The Role of ShcA Phosphotyrosine Signaling in the Myocardium

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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Abstract

Tyrosine kinases (TK) are important for cardiac function, but their downstream targets in the adult heart have yet to be established. The ShcA docking protein binds specific phosphotyrosine (pTyr) sites on activated TKs through its N-terminal PTB and C-terminal SH2 domains and stimulates downstream pathways through motifs such as pTyr sites in its central CH1 region. To explore the role of this TK scaffold in the adult heart, we generated a myocardial-specific knockout of murine ShcA (ShcA CKO). Such mice developed a dilated cardiomyopathy phenotype involving impaired systolic function with enhanced cardiomyocyte contractility. This uncoupling of global heart and intrinsic myocyte functions was associated with altered perimysial collagen and extracellular matrix compcance properties, suggesting disruption of mechanical coupling. In vivo dissection of ShcA signaling properties revealed that selective inactivation of the PTB domain in the myocardium had effects resembling those seen in ShcA CKO mice, while disruption of the SH2 domain caused a less severe cardiac phenotype. Downstream signaling through the CH1 pTyr sites was dispensable for baseline cardiac function, but necessary to prevent adverse remodeling after hemodynamic overload. Therefore, ShcA mediates pTyr signaling in the adult heart through multiple distinct signaling elements that control myocardial functions and response to stresses.
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I have not failed. I've just found 10,000 ways that won't work.

Thomas A. Edison
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**A** Alanine

**Ant wall** Anterior wall

**BSA** Bovine serum albumin

**Bpm** beats per minute

**CH1** Collagen homology region-1

**CH2** Collagen homology region 2

**CO₂** Carbon dioxide

**CML** Chronic myeloid leukemia

**Cre** Cre recombinase

**DNA** Deoxyribonucleic acid

**dP/dT** first derivative of pressure development

**dS/dT** first derivative of sarcomere shortening

**EGFR** Epidermal growth factor receptor

**ERK1/2** Extracellular-signal-regulated kinase ½

**F** Phenylalanine

**% FS** percent fractional shortening

**F-actin** Filamentous actin

**Grb2** growth factor receptor bound protein-2
G-actin  Globular actin
GSK-3  Glycogen synthases kinase-3
HF  Heart failure
HR  Heart rate
HW/BW  Heart weight-to-body weight ratio
K  Lysine
LungW/BW  Lung weight-to-body weight ratio
LVEDD  Left ventricle end diastolic dimension
LVESD  Left ventricle end systolic dimension
MMP  Matrix metalloproteinase
PI3K  Phosphoinositide 3-kinase
Post Wall  Posterior wall
PTB  Phosphotyrosine binding
pTyr  Phosphotyrosine
Q  Glutamate
R  Arginine
RTK  receptor tyrosine kinase
SDS PAGE  Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOS  Son of sevenless
SH2  Src homology 2
SH3  Src homology 3
TAC  Transverse aortic constriction
TR  Time to relaxation
Y  tyrosine
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Chapter 1
General Introduction
1 Introduction

1.1 Congestive Heart Failure and Hypertrophy

Heart failure (HF) is a leading cause of death in the western world and also contributes to considerable morbidity and health care costs. HF currently affects over half a million Canadians and sees over 50,000 new diagnoses per year (Liu et al. 2001). Despite a decline in acute cardiovascular prognoses, as with myocardial infarction, HF, the most common of all cardiovascular diseases for hospital admissions, has not seen improvement in its mortality rate (Ho et al. 1993; Ho et al. 1993; O’Connell et al. 1994). Indeed, the 5 year survival rate after diagnosis of HF is 45%, worse than most cancer prognosis (McMurray et al. 2000; Jessup et al. 2003). As 4% of all Canadians over the age of 70 live with HF, which incurs over 1 billion dollars for inpatient care alone, the aging population of Canada will further burden the public health care system in the coming years (Ross et al. 2006; Russell et al. 2008). In the pediatric population, HF results in multiple admissions, long term pharmacological management and, ultimately, cardiac transplantation (Boucek Jr et al. 2002). Understanding the pathogenic processes that contribute to HF is therefore paramount to impacting patient prognosis.

HF is operationally defined as the inability of the heart to meet the systemic metabolic needs of the body, and results in a clinical syndrome of fatigue, dyspnea, edema, and left ventricle dysfunction. HF can be caused by primary disease of the heart tissue (genetic cardiomyopathies) or secondary to complex polygenic disease such as hypertension, coronary artery disease or myocardial infarction (Figure 1.1). In the former case, cardiomyopathies can impact directly on the myocardium decreasing cardiac function at an early age of onset. In the latter case, various conditions, such as hypertension, can increase ventricular wall stress and hence initiate a pathological hypertrophic remodeling program. Under chronic conditions, these changes become maladaptive resulting in ventricle remodeling and/or dilation that leads to HF (Chien 1999; Hunter et al. 1999; Liew et al. 2004). Given the significant morbidity and mortality associated with HF, understanding the adaptive and maladaptive molecular mechanisms the heart employs to maintain cardiovascular function is clearly important.

Hypertrophy is the main protective response the heart employs to match pump function with enhanced systemic needs. Hypertrophy can be either physiological, as with pregnancy and
exercise, or pathological. The pathological hypertrophy response is the primary mechanism the heart employs to maintain pump function in the face of disease. This is a complex

![Diagram of cardiac remodeling pathways]

**Figure 1.1 Adaptive and maladaptive remodeling pathways in the heart.**

Physiological hypertrophy results in hypertrophy of the myocardium to accommodate increased blood volume resulting in myocytes lengthening (eccentric remodeling); however, no fibrosis or dysfunction is noted. Pathological hypertrophy is the disproportionate hypertrophy of the myocardium wall at the expense of the chamber volume, with dysfunction and fibrosis being
evident. Dilation of the ventricles occurs with eccentric remodelling whereby the cardiomyocytes lengthen in response to increased volume or mechanical instability. The syndrome of heart failure is the end consequence of chronic and maladaptive remodeling.
process that, at the level of the myocardium, involves neurohumoral activation and/or mechanoreceptors such as integrins to stimulate signaling pathways that ultimately effect gene transcription (MacLellan et al. 2000; Heineke et al. 2006). Hallmarks of the process are early expression of proto-oncogenes such as \textit{c-fos}, \textit{c-jun} and \textit{c-myc} (Mulvagh et al. 1987; Komuro et al. 1988; Izumo et al. 1998), coincident upregulation of fetal isoforms for various sarcomeric proteins such as β myosin and skeletal actin (Lompre et al. 1979; Schwartz et al. 1986; Schwartz et al. 1993), and expression of the biomarkers atrial naturetic peptide (ANP) and brain naturetic peptide (BNP) (Mercadier et al. 1989; de Bold et al. 2005) Ultimately, there is an increase in the number of contractile units of the myocyte by parallel addition of sarcomeres (concentric hypertrophy). Neurohumoral contributions such as adrenergic inputs, the renin –angiotensin system and cytokines also help to make the initial hypertrophy response compensatory, so that the heart is able to maintain cardiac homeostasis (Kan et al. 2001; Port et al. 2001). In chronic disease, where the initiating stress is persistent, the heart moves into a decompensated state, where fibrosis, cardiac enlargement and decreased systolic function evolve into symptomatic HF (Chien 1999). While current therapies attempt to relieve the symptoms of HF, understanding the complex signaling cascades that are involved in maintenance of normal cardiac function and the cascades involved in transitioning the heart into overt HF will potentially contribute to novel therapeutic strategies.

1.2 Signal Transduction: Cardiac Signaling in Hypertrophy and HF

From initiating signals at the cardiomyocyte membrane, a host of signal transduction events occur in a complex and integrated manner to evoke adaptive or maladaptive responses. Research has highlighted multiple signaling networks that receive converging inputs from various upstream molecules to impact on hypertrophy and the maintenance of cardiac function. While neither comprehensive nor inclusive, I will highlight signaling pathways important in hypertrophy and/or heart failure signaling, such as calcium kinetics, the mitogen-activated protein kinase (MAPK) pathways, the janus kinase/stat family, PKC signaling and the PI3K-GSK pathways. Important upstream membrane receptors that trigger these cascades are integrins
and sarcolemmal proteins, G-protein coupled receptors and tyrosine kinases (TK) (Section 1.3) (Figure 1.2).

**Figure 1.2. Signal transduction pathways in cardiac hypertrophy**

Proximal membrane inputs activate downstream signal transduction cascades that ultimately impact on hypertrophy. Important mediators of hypertrophy signaling are outlined in Section 1.2 and 1.3.
1.2.1 Calcium Kinetics

Calcium signaling is paramount to cardiomyocyte function as a rise in intracellular calcium can initiate both cardiomyocyte contraction and calcium triggered signal transduction pathways. With respect to cardiac contraction, calcium entry from the voltage gated L type calcium channel (Ca^{2+}_{L type}) triggers the ryanodine receptor (RyR) mediated sarcoplasmic reticulum calcium release. Relaxation of myocyte contraction involves reuptake of calcium by the sarcoplasmic reticulum ATPase pump (SERCA2a) and its negative regulatory protein phospholamban (PLN) (Bers 2002) (Chapter 4). Indeed, downregulation of SERCA2a gene expression is found in human and mouse models of hypertrophy and heart failure (Nagai et al. 1989; Anger et al. 1998; Frank et al. 2002), and mutations that prevent PKA mediated phosphorylation of PLN decrease SERCA2a mediated decay of the calcium transient, resulting in a dilated cardiomyopathy (Schmitt et al. 2003).

Within discrete microdomains, calcium can also act as a potent signal transduction molecule and can lead to modulation of key calcium activated pathways, termed excitation-transcription coupling (ETC); ETC is coordinated by the multifunction calcium sensor, calmodulin (CaM). CaM binds to the phosphatase, calcineurin, to activate the transcription factor NFAT and to Calmodulin kinase II (CaMKII) to impact on hypertrophy signaling. While both pathways can impact on excitation-contraction coupling (ECC), it is the modulation of transcription factors that impacts on hypertrophy signaling (Bers et al. 2005). Overexpression of CaM results in severe cardiac hypertrophy (Gruver et al. 1993), solidifying its role as a pivotal molecule in cardiac signaling. One arm of CaM signaling is through its interactions with calcineurin, a serine/threonine protein phosphatase that exists as a heterotrimer. Activation of calcineurin by sustained calcium levels (Dolmetsch et al. 1997) leads to dephosphorylation of NFAT, allowing coordination of hypertrophy genes in the nucleus with the transcription factor GATA4 (Frey et al. 2000). Indeed, overexpression of calcineurin and NFAT c4 leads to robust hypertrophy (Molkentin et al. 1998), while deficiency of calcineurin decreases the response to hypertrophy agonists (Wilkins et al. 2002). The CaM-CaMKII axis is activated by high calcium spikes (De Koninck et al. 1998) and can impact on hypertrophy through phosphorylation of CREB (cAMP binding response element) transcription factor (Sun et al. 1996; Deisseroth et al.
1.2.2 Mitogen Activated Protein Kinases

MAPKs are serine/threonine kinases that are primarily activated by tyrosine/threonine phosphorylation. Activated MAPKs phosphorylate downstream kinases and nuclear substrates such as c-myc, c-jun and ATF-2. The MAPK cascades include the stress activated protein kinases (SAPKs) and the 3 subfamilies include the extracellular regulated kinases (ERKs), the c-jun N-terminal kinases (JNKs) and the p38 MAPKs (Figure 1.3). These subfamilies are organized as hierarchal cascades with MAPK being the terminal kinase activated. The initiation of the kinase cascades is by activation of serine/threonine MAPK/ERK kinase kinases (MEKKs) which, through dual phosphorylation of a Ser-XXX-Ser/Thr motif (with x denoting an amino acid), activate MAPK/ERK kinases (MEK/MKK). These in turn activate MAPK by dual phosphorylation of Thr-X-Tyr motifs (Garrington et al. 1999).

In the heart, the 3 subfamilies are activated by varying stimuli, each contributing to different facets of hypertrophy signaling. The ERK pathway consists of 6 kinases, with ERK 1(44kDa) and ERK2 (42kDa) being the best characterized. The ERK cascade has the hierarchical order of: Raf kinase /MEKK1→MEK1 / MEK2→ERKs, with substrates including Elk-1, c-jun and 90 kDa S6 kinase (Garrington et al. 1999). In vivo data support a role for ERK signaling in hypertrophy, as overexpression of MEK1 in the myocardium of mice causes concentric hypertrophy that is not accompanied by fibrosis or premature death (Bueno et al. 2000). Overexpression of Ras, an upstream activator of Raf, also elicits robust hypertrophy, however due to pleiotropic effects, the mice die suddenly and have pathological remodeling (Hunter et al. 1995). Disruption of c-raf-1 in the myocardium leads to systolic dysfunction and chamber dilation, associated with an increased number of apoptotic cells (Yamaguchi et al. 2004). The clinical relevance of this pathway is reiterated as gain of function Raf mutations result in Noonan and LEOPARD syndromes with hypertrophic cardiomyopathies (Pandit et al. 2007). Therefore, proper levels of ERK signaling are important for maintaining normal cardiac function.
The JNK pathway is encoded by 3 genes that yield the 3 isoforms: JNK1/SAPKγ, JNK2/SAPKα and JNK 3/SAPK β. In the heart, the upstream kinases in the JNK pathway are ill defined, but appears to be MEKK1, MEKK2, MEKK3 and MEKK5, which activate MKK4 and MKK7 (Yan et al. 1994; Tournier et al. 1997; Wang 2007). JNKs activate transcription factors such as c-jun and ATF2 (Wang 2007). While the role of JNK signaling in hypertrophy is debated, loss of MEKK1 results in chamber dilation, systolic dysfunction and premature death after pressure overload (Sadoshima et al. 2002), while cardiac specific loss of MKK4 results in an exaggerated pathological hypertrophy response with apoptosis (Liu et al. 2009).

The p38 MAPK pathway is activated by MEKK5 which activates MKK3 and MKK6. These MKKs activated the isoforms p38 MAPK α, β, δ and γ leading to phosphorylation of MAPK-activated protein kinase 2 (MK2) which activates heat shock protein 25 and 27 and ATF 2 (Ichijo et al. 1997; Zechner et al. 1997; New et al. 1998; Kerkela et al. 2006). The main isoform expressed in the heart is p38α (Liao et al. 2002), and cardiac specific expression of upstream activators of p38, MKK3 and MKK6, show divergent signaling roles. Expression of activated MKK3 leads to chamber dilation with reduced wall thickness and fibrosis, while expression of activated MKK6 results in mild hypertrophy with preserved chamber dimensions (Liao et al. 2002). Myocardial expressed dominant negative p38α results in a hypertrophic response to pressure overload, with little fibrosis(Zhang et al. 2003). This suggests p38 is a critical player in ventricular remodeling.
Another family of signal transduction implicated in hypertrophy is the janus kinase / signal transducers and activator of transcription pathway (JAK/STAT). The JAK family are protein tyrosine kinases which consists of JAK 1, 2, and 3 and TYK2 (Ihle et al. 1995), while the STAT family are transcription factors activated by tyrosine phosphorylation and consist of STAT1, 2, 3, 4, 5a, 5b and 6 (Schindler et al. 1995; Horvath et al. 1997; Boengler et al. 2008). Initiation of the JAK pathway is through ligand binding to the cytokine receptor, such as cardiotrophin (CT-1) binding to the glycoprotein 130 receptor in the heart. This induces activation of the receptor-JAK complex and recruitment of STAT3 which migrate to the nucleus, regulating gene transcription (Horvath et al. 1997; Robledo et al. 1997). Indeed, loss of gp130 in the myocardium results in a dilated cardiomyopathy accompanied by apoptosis of myocytes (Hirota et al. 1999),
while constitutive expression of gp130 leads to cardiac hypertrophy (Hirota et al. 1995).
Clinically, cytokines have been shown to be involved in the transition into overt heart failure and are important biomarkers for disease prognosis (Wollert et al. 1997; Deswal et al. 2001; Fischer et al. 2007).

### 1.2.4 Protein Kinase C

Protein kinase C (PKC) is a serine/threonine kinase that has 3 subcategories for its isoenzymes: 1) classical PKC (α/β/γ) regulated by DAG, phosphatidylserine and calcium, 2) novel PKC (δ, ε, η, Ω, μ) not regulated by Ca+2 and 3) atypical PKC (ζ and λ) with ill defined regulation (Puceat et al. 1996; Newton 1997). PKC signaling can activate the ERK pathway through Raf, the IκB pathway, and modulated intracellular calcium all of which can initiate the hypertrophy response in cardiomyocytes (Kolch et al. 1993; Buchner 1995; Ho et al. 1998). In vivo, the role of PKC is difficult to decipher due to the overlapping function of multiple isoforms; however, with hemodynamic stress, PKCα has been shown to regulate hypertrophy and contractility, as the loss of PKCα in the myocardium leads to enhanced cardiomyocyte function and protection against heart failure (Braz et al. 2004).

### 1.2.5 PI3K/GSK 3 Signaling

The phosphoinositide-3’ kinase (PI3K) signaling pathway (Figure 1.4) involves the phosphorylation of phosphatidylinositols (PtdIns), with the phosphorylation pattern dictated by the class of PI3K. The two PI3K classes are delineated by their catalytic and accessory subunits, and in the heart, class 1, and its subclasses 1A and 1B, are distinguished by their effects on hypertrophy signaling (Oudit et al. 2009). Physiological hypertrophy is coupled to PI3K subclass 1A, 110 kDa lipid kinase (p110α) downstream of IGF-1 and other TKs (Lupu et al. 2001). Cardiac-specific constitutively active p110α does not cause transition to maladaptive hypertrophy (Shioi et al. 2000), while a dominant-negative mutant impairs physiological hypertrophy in response to exercise (McMullen et al. 2003). PI3K subgroup 1B, which utilizes the
catalytic p110γ subunit, is recruited to the membrane by the G-protein subunits Gβγ of Gq/11 receptor associated proteins, and is required for pressure overload induced hypertrophy (Crackower et al. 2002; Patrucco et al. 2004), while also regulating cAMP levels in cardiomyocytes (Crackower et al. 2002). These pathways converge on the protein kinase AKT, which is critical in trophic growth. AKT1 is the most important AKT gene in the heart, and germline deletion leads to reduction in organ size including the heart (Cho et al. 2001), while overexpression of AKT in the heart results in hypertrophy with depressed contractility (Matsui et al. 2002; Shioi et al. 2002). Other key molecules in this pathway are the negative regulator of PI3K, PTEN (phosphatase and tensin homolog on chromosome 10), and the upstream activator of AKT, phosphoinositide-dependant kinase-1 (PDK1). Cardiac specific loss of PTEN results in cardiac physiological hypertrophy (Schwartzbauer et al. 2001; Crackower et al. 2002); conversely, cardiac ablation of PDK-1 leads to reduced hypertrophy and subsequent cardiomyopathy (Mora et al. 2003).

The PI3K-AKT axis can activate mammalian target of rapamycin (mTOR) to effect protein synthesis, but can also negatively regulate the praline-directed serine/threonine protein kinase, GSK-3 (Fiol et al. 1987; Plyte et al. 1992). Of the 2 isoforms of this kinase, GSK-3β is the most studied in the heart. GSK-3β receives converging inputs from multiple protein kinases such as PKC and PKA and phosphorylation of Serine 9 of GSK-3β leads to release of negative inhibition to impact on hypertrophy (Hardt et al. 2002). The phospho-null mutant GSK-3βS9A, when overexpressed in the myocardium, inhibits hypertrophy after pressure overload and isoproterenol stimulation (Antos et al. 2002). While loss of GSK-3β downstream of the Gq activator, endothelin-1, results in hypertrophy in neonatal rat cardiomyocytes (Haq et al. 2000). As GSK-3β is a multifunctional protein, its ability to regulate hypertrophy can be through a variety of mechanisms. These could include regulation of important transcription factors involved in hypertrophy, such as GATA4 or through stabilization of β-catenin, the main consequence of Wnt signaling (Toyofuku et al. 2000; Morisco et al. 2001; Woodgett 2001). Recently, the GSK-3α isoform has been shown to regulate beta-adrenergic responsiveness and hypertrophy signaling in response to hemodynamic overload, therefore expanding our understanding of GSK signaling in the heart (Zhou et al.).
1.3 Membrane Signaling in Cardiac Maintenance and Hypertrophy

Proximal inputs implicated in the maintenance of the myocardium and the initiation of hypertrophy includes integrins and associated cytoskeletal proteins, G protein coupled receptors.
and tyrosine kinases. Engagement of these membrane proteins activates various signal transduction pathways mentioned above to impact on hypertrophy and cardiac function.

1.3.1 Integrins and Sarcolemmal Proteins

Maintenance of cardiac function and the initiation of the hypertrophy signal are sensed by integrins and/or sarcolemma proteins, particularly resulting from mechanical stimuli such as hypertension. Indeed, many of the genetic cardiomyopathies result from mutations in sarcomeric proteins such as cardiac α-actin, desmin, troponins, cardiac myosin heavy chain, dystrophin and sarcoglycan (Fatkin et al. 2002; Liew et al. 2004). Likewise, integrin signaling has been shown to initiate hypertrophy in cell culture in the absence of neurohumoral inputs, and the β1 integrin knockout mouse develops a dilated cardiomyopathy (Ross et al. 1998; Shai et al. 2002). Molecules downstream of integrins such as Melusin have also shown to be critical in hypertrophy signaling after hemodynamic overload (Brancaccio et al. 2003).

1.3.2 G-Protein Coupled Receptors

Currently, the main targets for therapeutics and the focus of much cardiac research are G protein-coupled receptors (GPCR) in cardiac tissue. GPCRs are ligand activated seven transmembrane receptors that are coupled to heterotrimeric guanine-nucleotide regulatory proteins. Over 200 GPCRs exist in the heart with the adrenergic, angiotensin, endothelin and muscarinic receptors being most notably implicated in heart failure (Salazar et al. 2007). In the treatment of heart failure, adrenergic and angiotensin GPCR therapeutics account for the majority of prescribed therapies (Tang et al. 2004).

Adrenergic signaling in the heart consists of α and β adrenergic receptors (AR), with β ARs being predominant: αARs are one tenth the number of βARs; within the βARs, there is an 80:20 expression ratio of β1ARs to β2ARs (Rockman et al. 2002). In general, the adrenergic receptors couple to either G stimulatory (Gs) or G inhibitory (Gi) proteins to modulate adenylyl cyclase and its second messengers cAMP to impact on PKA regulation. Clinically, in heart failure, when catecholamine levels are elevated for a sustained duration, desensitization of β
adrenergic receptors leads to loss of cardiac reserve (the inability to increase SNS mediated
effects on heart function) and compromised ventricular function (Tilley et al. 2006). Therefore,
βAR antagonists are used as a symptomatic therapeutic intervention. The β1ARs have a positive
ionotropic (enhanced contraction) and lustropic (enhanced relaxation) effect in that they
modulate calcium fluxes through phosphorylation of the L-type calcium channel, ryanodine
receptor, troponin I and phospholamban, the inhibitory regulator of SERCA2a, while β2ARs
are thought to exert their effect on vasodilation of the vasculature. Loss of β1AR leads to
dampened iontropic and chronotropic response to isoproterenol (Rohrer et al. 1996) and loss of
β2 ARs results in exercise-induced hypertension (Chruscinski et al. 1999). Conversely,
overexpression of β1AR results in hypertrophy and fibrosis which transitions into heart failure
(Engelhardt et al. 1999; Bisognano et al. 2000), while overexpression of β2ARs leads to
enhanced cardiac function without progression into heart failure (Milano et al. 1994; Liggett et
al. 2000). αARs are also important in mediating the hypertrophy response and the transition into
heart failure. Double knockouts of α1A/C and α1B fail to develop physiological hypertrophy, and
fail to initiate an adaptive hypertrophy response after pressure overload (O’Connell et al. 2003).

The Angiotensin receptors are another important class of GPCR implicated in
hypertrophy and heart failure and mainly signal through the peptide ligand Ang II which
activates Gq coupled AT1R (Lambert et al. 1995; Touyz et al. 2000). Overexpression of AT1aRs
result in exaggerated hypertrophy response and fibrosis (Paradis et al. 2000), while loss of
At1aRs diminishes these responses (Bridgman et al. 2005), thereby implicating Ang II signaling
in hypertrophy and extracellular matrix production.

1.3.3 Tyrosine Kinases

Individually, many tyrosine kinases have been shown to have an important role in cardiac
structure and function. Not only do they initiate discrete signaling pathways, but also serve as
nodes for other proximal signals. Indeed, integrins utilize FAK and Src, two non receptor TKs, to
impact on cardiac signaling (Plopper et al. 1995), while GPCRs, such as endothelin, use TKs to
activate growth pathways (Chung et al. 2007). Clinically, GPCRs have dominated the research
and treatment of heart failure, but tyrosine kinases have also been shown to be integral in cardiac
homeostasis. With research directed at elucidating the signaling networks from various tyrosine kinases perhaps novel and efficacious treatment strategies will evolve.

1.4 Tyrosine Kinase Signaling

Tyrosine phosphorylation, by protein TKs, is a key covalent modification that involves the transfer of the $\gamma$ phosphate of ATP to hydroxyl groups of tyrosines of target proteins thereby propagating signals within a cell (Hunter 1998). TKs exist as 2 main classes, the transmembrane receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (nRTK). Typically, RTKs have a common structure of a glycosylated extracellular domain which binds ligand, a transmembrane helix and the cytoplasmic tail containing a protein tyrosine kinase core and regulatory sites for covalent modifications (Hubbard et al. 2000; Schlessinger 2000). Signaling through RTKs involves ligand-induced oligimerization of the receptor to induce a conformational change with subsequent trans autophosphorylation in the activation loop of the cytoplasmic domain (Ullrich et al. 1990; Heldin 1995). nRTK can be activated by phosphorylation in the activation loop, either in trans or by a different nRTK, to increase tyrosine kinase activity (Superti-Furga 1995). Negative regulation of TK signaling can be through protein tyrosine phosphatases (PTP), autoinhibitory phosphorylation, receptor-mediated endocytosis, or ubiquitin-directed proteolysis (Hubbard et al. 2000; Schlessinger 2000). Tyrosine kinase signaling plays a critical role in development, differentiation, proliferation, and migration.

In the case of RTKs, tyrosine phosphorylation of consensus sites in the cytoplasmic domain creates docking sites to lend specificity and complexity to ligand induced activation. Autophosphorylation targets of PTKs are typically located outside the catalytic unit of the receptor and hence serve as binding sites for modular phosphotyrosine recognition proteins, such as those containing SH2 (Src homology 2) or PTB (phosphotyrosine binding) domains. Protein signaling/interaction domains are usually 3-120 amino acids in length and allow proteins to bind to covalently modified sites on target proteins (Pawson et al. 2000). Common examples of interaction domains include SH2 domains, which bind sequences pY-X-X-$\theta$ generally, PTB domains, which bind NP-X-pY motifs and SH3 domains which bind proline rich sequences defined by the P-X-X-P motifs (where $\theta$ is any hydrophobic amino acids, X is any amino acid).
The modular nature of proteins allows for the various domains to impact on signaling through diverse ways. First, domain binding to an activated kinases increases the probability of that protein being phosphorylated and hence propagation and amplification of the signal. Second, domains can be used as a means to localize signaling molecules to discrete microdomains, as seen with RTK mediated Ras activation, whereby recruitment of Grb2-SOS to the EGFR allows SOS to be in proximity to its substrate, Ras (Pawson et al. 1993). Finally, in the case of domain containing proteins with catalytic activity, binding to substrates can relieve intramolecular regulation allowing for subsequent activation, as in the case of Src (Superti-Furga et al. 1993).

1.4.1 Tyrosine Kinase Signaling in the Heart: Mouse Models

TK signaling has been shown to be critical in oncogenic transformation and in many developmental processes. Over the years, evidence has also demonstrated the importance of TK signaling in the heart, through use of mouse knockout models. Indeed, TKs have an under-appreciated role in both the maintenance of cardiac function and the complex signaling programs that are initiated with disease. As more conditional TK alleles emerge, the role of individual TKs within the myocardium will be elucidated. Additionally, the literature has demonstrated the importance of TK reciprocal signaling in the myocardium. Indeed, the paracrine induction of coronary vasculature by VEGF-A secreted from myocytes and NRG-1- Erbb2 interplay between the endothelium and the myocytes, suggests TK signaling is critical in maintaining tissue physiology. Therefore, I will highlight some of the heart specific findings for a variety of TK receptors (Figure 1.5).

1.4.1.1 Epidermal Growth Factor Receptor Family

The epidermal growth factor receptor family consists of 4 main receptors, the epidermal growth factor receptor (EGFR/ErbB1), ErbB2, ErbB3 and ErbB4. These RTKs exist in monomeric form and upon ligand binding, can form homo or heterodimers to initiate autophosphorylation events. EGF signaling ligands include EGF, transforming growth factor alpha, amphiregulin, epiregulin, neuregulins, heparin binding EGF-like growth factor and betacellulin. Complexity of signaling is
achieved by homo and heterodimer preferences, ligand binding preference and signaling recruitment by non ligand affiliated ErbB2 (Fuller et al. 2008). In the heart, EGF family members are required for heart development and for the maintenance of cardiac structure and function in the postnatal heart. Despite having no known ligand, ErbB2 facilitates dimerization and coordination of signaling networks and is critical in heart function (Horan et al. 1995; Graus-Porta et al. 1997). Mice null for ErbB2, ErbB4 and their endothelium-derived ligand NRG are embryonic lethal at E10.5 with poor trabeculation and peripheral nervous system defects (Gassmann et al. 1995; Lee et al. 1995; Meyer et al. 1995). Conditional loss of ErbB2 in the myocardium results in severely dilated ventricles, with potential defects in apoptosis (Crone et al. 2002; Ozcelik et al. 2002), while cardiomyocyte specific loss of ErbB4 results in dilated ventricles and electrical disturbances (Garcia-Rivello et al. 2005). ErbB3, though not expressed in cardiomyocytes, is a key player in valve morphogenesis as germline deletion results in defective cardiac cushion formation, thus resulting in valve abnormalities and lethality at E13.5 (Erickson et al. 1997; Riethmacher et al. 1997). HB-EGF KO mice have perinatal/postnatal lethality and demonstrate dilated cardiac chambers and cardiac valve enlargement (Iwamoto et al. 2003; Jackson et al. 2003). EGFR knockout mice have varying severity of phenotypes depending on the genetic background, and surviving KO mice have semilunar valve enlargement (Chen et al. 2000). As the NRG/EGF-ErbB2/ErbB4 axis is required for cardiac development and maintenance of cardiac function, understanding the signal transduction downstream of the autophosphorylation events is of interest. Phosphorylated consensus sites on the activated receptor allow many different adaptor and scaffold molecules to bind, thereby enhancing the complexity of receptor-ligand interactions; in the case of ErbB2, ShcA is the preferred adaptor, while Grb2 is preferred by ErbB4 (Schulze et al. 2005). Physiological processes regulated by NRG-ErbB2 signaling are apoptosis (Crone et al. 2002) and myocyte-myocyte/matrix interactions (Kuramochi et al. 2006); while the molecular mechanisms of NRG signaling are still emerging, the use of NRG-1 as a restorative agent for diseased myocardium, suggests it may be a clinically relevant therapy (Pentassuglia et al. 2009), especially as the NRG-1-ErbB4 axis has been shown to regenerate cardiomyocytes within the myocardium (Bersell et al. 2009).
Tyrosine kinase signaling has been shown to be critical in cardiovascular development and myocardial function. The structure of various TKs outlined in Section 1.4 are shown (adapted from (Hubbard et al. 2000)).
Figure 1.5. Domain structure of tyrosine kinases important in the cardiovascular system
1.4.1.2 Platelet-Derived Growth Factor Signaling

The platelet derived growth factor (PDGF) family consists of 4 ligands: PDGF-A, B, C and D, with the latter 2 being secreted latent factors. These ligands bind to PDGFRα and β and have a general mitogenic response on mesenchymal cells (Simm et al. 1998; Heldin et al. 1999). PDGF-B and PDFGR-β are important for development of vascular support cells, while cardiac overexpression of PDGF- C and D results in cardiac fibrosis and vascular defects (Edelberg et al. 2003; Ponten et al. 2003; Ponten et al. 2005). Conditional alleles, used for cardiac ablation studies, will allow evaluation of the importance of PDGF paracrine signaling in supporting vascular integrity within the myocardium.

1.4.1.3 Vascular Endothelial Growth Factor Family

The vascular endothelial growth factor (VEGF) family of receptors consists of VEGF receptors 1, 2, and 3 which can bind the ligands VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PIGF) (Tammela et al. 2005). While VEGF -C and D bind VEGFR3 to stimulate lymphangiogenesis, VEGF-A is the primary ligand for hypoxia induced angiogenesis (Tammela et al. 2005). VEGF-A can form homo or heterodimers with other VEGF ligands and binds VEGFR1 and 2 in the vascular endothelium to stimulate the formation of new blood vessels. Germline ablation of VEGF-A results in early embryonic lethality resulting from defects in endothelial cell development, blood island formation and vascular formation (Ferrara 1999). Loss of specific VEGF-A isoforms in mice results in impaired myocardial angiogenesis and ischemic cardiomyopathy (Carmeliet et al. 1999). In the heart, paracrine signaling is paramount for maintaining vascular-myocyte function. Conditional loss of VEGF-A in the myocardium showed that the paracrine release of VEGF from myocytes is required for heart function, as loss of Vegf-A resulted in thin, dilated ventricles due to coronary hypovascularization (Giordano et al. 2001). The clear role for VEGF signaling in hypoxic induced angiogenesis has lead to
therapies designed to increase collateral vessel formation in ischemic heart disease (Carmeliet et al. 2000; Freedman et al. 2002; Yla-Herttuala 2003).

1.4.1.4 Integrins/FAK/Src Signaling

Integrins are membrane associated receptors that are comprised of heterodimers of α and β subunits. As 8 β and 18 α subunits consisting of a large extracellular domain connected to a shorter cytoplasmic domain can dimerize, signal diversity is generated by combinations of heterodimers and ligand preference. Extracellular matrix proteins such as fibronectin, laminin and collagen are examples of common ligands (Barczyk et al.; Hynes 2002). In the adult heart, laminin is bound predominately by α7β1D heterodimer, and signaling is directed by the consensus motifs in the cytoplasmic domain (Brancaccio et al. 1998; Ross et al. 2001). The β1D integrin isoform is necessary for cardiac structure and function as loss of β1D in the postnatal period results in a dilated cardiomyopathy with enhanced fibrosis deposition (Ross et al. 1998). Signaling downstream from integrins involves many cytoskeletal proteins such as talin and α actinin, but of particular importance are the cytoplasmic tyrosine kinase FAK and Src (Manso et al. 2009). FAK is a FERM domain containing tyrosine kinase, that has a C-terminal Focal adhesion targeting domain to localize it to costomer (focal adhesion complexes) in cardiomyocytes (Mitra et al. 2005). Recruitment to clustered integrins at the costomer leads to autophosphorylation of Tyr-397 to allow for the SH2 domain docking of ShcA, Src or p85 subunit of PI3K (Mitra et al. 2005). FAK has been implicated in the hypertrophic response of cardiomyocytes, particularly upon mechanical stretch (Franchini et al. 2000; Domingos et al. 2002; Torsoni et al. 2003), while selective loss of FAK in the myocardium results in heart failure after pressure overload (DiMichele et al. 2006; Peng et al. 2006), as well as eccentric remodeling associated with age (Peng et al. 2006). Downstream effectors of FAK signaling include ERK 1/2 and AKT, thereby impacting both on hypertrophy effectors and apoptosis (Heidkamp et al. 2002; Brancaccio et al. 2006).

1.4.1.5 Ephrin Family
The largest subfamily of tyrosine kinases is Eph receptors, and, with their surface-associated ligands, ephrins, they specialize in cell-cell communication. Eph receptors initiate forward signaling in the receptor bearing cell, while reverse signaling occurs in the ephrin expressing cell (Holland et al. 1996; Kullander et al. 2002). Eph receptors are broken into 2 subclasses where generally EphA (A1-A10) receptors bind ephrin A (A1-A6) ligands and EphB (B1-B6) receptors bind ephrinB (B1-B3) ligands. Eph receptors are critical in blood vessel formation as ephrin A1 is expressed in developing vasculature (McBride et al. 1998) and EphB2, EphB3 and EphB4 along with their ligands ephrin B2 and B1 are important in directing circulatory system development (Wang et al. 1998; Gerety et al. 1999; Foo et al. 2006; Himanen et al. 2007). Eph signaling has also been shown to be important in heart morphogenesis and cardiac function. EphA3 null mice have defects in atrial septa and atrioventricular endocardial cushions, possibly from impaired epithelial to mesenchymal transformation (Stephen et al. 2007). Cardiac trabeculation during ventricular chamber morphogenesis is critical for embryonic viability, and recent studies have demonstrated that Notch controls the expression of ephrin B2 to regulate cardiomyocyte differentiation (Grego-Bessa et al. 2007). To date, no conditional deletion of any ephrin or Eph receptors has been reported in the myocardium; however, given their importance in development and general cell-cell communication, they are likely to play a role in maintaining the adult myocardium.

1.4.1.6 Trk Family

Signaling by neurotrophins (NT1-5) is mediated by their binding to one of 3 Trk receptors (TrkA, B, and C), and is well known for its effects on axon guidance and neuronal development (Huang et al. 2003). The NT3-TrkC axis has been shown to have a role in embryonic heart formation, as loss of either NT3 or TrkC results cardiac abnormalities consistent with defects in cardiac neural crest migration, such as atrial and ventricular septal defects and valvular abnormalities (Donovan et al. 1996; Tessarollo et al. 1997). While no known role in the adult myocardium has been reported, the observation that TrkC is expressed in adult cardiomyocytes suggests it could play a role in a post mitotic myocardium (Kawaguchi-Manabe et al. 2007).
1.4.1.7 Insulin Growth Factor Family

The insulin family consists of multiple peptides, such as insulin and insulin growth factors 1 and 2 (IGF1 and IGF2). The peptide ligands bind 3 main receptors, the insulin receptor, IGF-1 receptor (IGF1R) and the non tyrosine kinase IGF2-receptor (IGF2R), that exist on the cell surface as homodimers or heterodimers (Nakae et al. 2001). While loss of insulin signaling results in the metabolic syndrome of diabetes and obesity (Accili et al. 2001), IGF-1 signaling is implicated in physiological cardiac growth upstream of AKT (McMullen et al. 2004). Indeed, loss of the IGF-1R in cardiomyocytes resulted in a blunted hypertrophy response to exercise (Kim et al. 2008).

1.4.1.8 Fibroblast Growth Factor Family

The fibroblast growth factor (FGF) family consists of 23 related polypeptide growth factors that bind to receptors FGF receptor 1 and 2 (FGFR1 and FGFR2) (Turner et al.). Fibroblast growth factor 2 (FGF2) is the most well known in the myocardium and is expressed in multiple cell types and binds to mainly to FGF receptor 1 (FGFR-1) (Detillieux et al. 2003). FGF-2 has a protective role in ischemia/reperfusion as mice overexpression FGF-2 show limited injury following an ischemic event (184). Solidifying its role in hypertrophy signaling, mice deficient in FGF-2 have a reduced hypertrophy response in a pressure overload model (Schultz et al. 1999) and a dilated cardiomyopathy phenotype with Angiotensin II stimulus (Pellieux et al. 2001). The ability of FGF-2 to act in many different cell types gives rise to pleiotropic effects on fibrosis and inflammation in addition to ischemic injury and hypertrophy signaling (Detillieux et al. 2003).

1.4.1.9 Discoidin Domain Receptor Family

Discoidin Domain Receptors (DDR) 1 and 2 bind collagen and are critical in cell-matrix interactions (Vogel et al. 2006). While no cardiomyocyte specific knock out exists, the germline
deletion of DDR1 results in altered remodeling at atherosclerotic lesions (188) while preliminary studies suggest a role in ventricular remodeling (Unpublished Results, Vanderlaan, RD; Vogel, WF; Backx, PH).

1.4.1.10 Ror Family

Ror family receptor (Ror) TKs consist of Ror1 and Ror2 receptors, and in the mouse, Ror1 null mice display no obvious phenotype, while Ror2 displays skeletal and cardiac defects in embryonic development (189-192). Mutations in human Ror genes result in Robinow syndrome, comprised of bone abnormalities with some cardiac findings, and brachydactyly type B, a syndrome affecting skeletogenesis. While little is known regarding the signal transduction cascades involved, the Ror family of receptors is important in the pathogenesis of specific hand-heart syndromes.

1.4.1.11 Abl Kinase

Recently, the nonRTK, c-Abl has been implicated in cardiac function due to the cardiotoxicity seen with Gleevec in oncology patients (Section 1.4.2) (193). In mice, c-Abl was found to be important in heart development in a strain-dependant manner. Loss of c-Abl results in perinatal lethality with dilation of both ventricles and atria that is also accompanied with increased proliferation of cardiomyocytes and abnormal mitochondria (Qiu et al.). This study provides insight into the mechanism by which TK inhibitors impact on cardiac function.

1.4.2 Tyrosine Kinase Signaling: Clinical Evidence

Research has shown the importance of tyrosine kinases in oncogenic processes. Mutations in tyrosine kinases such as the BCR-Abl fusion protein in chronic myeloid leukemia (CML) (Burke et al.) and over-amplification of the ErbB2 receptor in some breast cancers (Tagliabue et
al.) are examples of the pivotal role that TKs can play in the initiation and progression of oncogenic events. As targeted tyrosine kinase inhibitors (TKI) have been developed, either as humanized monoclonal antibodies or small molecule inhibitors, the efficacy of these treatments have been proven; however, a side effect of cardiac disease has been reported in a subset of patients treated with TKIs. In particular, two TKIs known to cause cardiac side effects are Herceptin/Trastuzumab and Gleevec/Imatinib, highlighting the central role of TKs in the maintenance in cardiac function.

1.4.2.1 Herceptin Mediated Cardiotoxicity

TKI cardiac effects were first reported for Herceptin/Trastuzumab, a humanized monoclonal antibody against the ErbB2 receptor. Clinical trials demonstrated left ventricle dysfunction with an incidence of 4-7% when Herceptin is used alone. When Herceptin is used in conjunction with chemotherapy, such as anthracyclines, the incidence of ventricular dysfunction increased to 27% *. Herceptin binds ErbB2-ErbB3 heterodimers (De Keulenaer et al. 2010), and while the direct mechanism by which Herceptin mediates the cardiotoxicity is unknown, clinical trials with Pertuzumab, a humanized monoclonal antibody against the dimerization domain of ErbB2, and Lapatinib, a small molecular inhibitor against the kinase activity of ErbB1 and ErbB2, will help to understand ErbB2 signaling in the myocardium *. Basic science research focused on understanding ErbB2 signaling networks in the heart will perhaps lead to selective targeting of ErbB2 signaling to minimize cardiotoxicity.

1.4.2.2 Gleevec Mediated Cardiotoxicity

Recently, the cardiotoxicity seen in Gleevec/Imatinib patients, has further focused the importance of TKs in cardiac maintenance. Imatinib is an Abl kinase ATP competitive small molecule inhibitor used in the treatment of CML. Other tyrosine kinases inhibited are Abl-related
gene (ARG), the PDGF receptors α and β and KIT (Cheng et al.). While the overall incidence of cardiotoxicity reported for Imatinib is low (1%) (Force et al. 2007), many feel this is an underestimation due to short nature of monitoring in clinical trials, the lack of specific cardiac end points and the sample bias of patients entering clinical trials (Force et al. 2007). Although the exact mechanism by which Imatinib mediates cardiotoxicity is poorly understood, one study has shown that cultured myocytes treated with Imatinib have significant mitochondrial dysfunction. In mice receiving Imatinib, mitochondrial dysfunction can be recapitulated, and clinically, heart biopsies taken from 2 patients administered Imatinib demonstrated non-specific abnormalities in mitochondria while taking Imatinib (Kerkela et al. 2006). While the incidence of HF remains low in current TKI clinical trials, the potential for retrospective reports of cardiotoxicity is high, especially if multikinase TKIs are utilized (Force et al. 2007). Therefore, understanding downstream signaling from TKs in the myocardium will help to design therapeutics that minimizes side effects and potential cardiotoxicities.

1.5 ShcA, an Adaptor for Tyrosine Kinases

Adaptor proteins are critical signaling molecules downstream of TKs, as their recruitment localizes and coordinates signal transduction networks. Adaptor proteins are non-enzymatic signaling molecules that are modular in nature, in that they contain multiple protein domains and regulatory sites for binding other proteins and phospholipids (Pawson et al. 1997). Examples of adaptor proteins include Grb2, Shc, Nck and Crk. Of particular interest in this thesis is the broadly expressed adaptor/scaffold molecule ShcA, Src homology 2 containing transforming protein 1(Shc1), and how its two phospho-tyrosine recognition domains mediate downstream signaling from TKs in the myocardium.

1.5.1 ShcA Isoforms and Signaling Domain Architecture

ShcA is a member of the Shc family that contains ShcB, ShcC and ShcD. While ShcB, C and D are expressed in neuronal tissue, with ShcD also expressed in the neural crest lineage, ShcA is found in most tissues (Ravichandran 2001). ShcA has 3 isoforms: p66, p52 and p46. The p52/46
isoforms result from translation initiation sites within the same transcript, while the p66 isoform arises from induction at a distant promoter site (Migliaccio et al. 1997) (Figure 1.6). All 3 isoforms contain an N terminal phospho-tyrosine binding (PTB) domain (truncated in p46) and C terminal Src homology 2 (SH2) domain with an intervening collagen homology region (CH1); the p66 isoform is unique in that it has an additional N terminal collagen homology 2 region (CH2) (Figure 4). Given the modular architecture of ShcA, many different signaling partners and downstream pathways can be utilized, in a context-dependant manner. In the myocardium, the in vivo role of ShcA remains largely unexplored; therefore, to begin to understand the biological significance of ShcA in the myocardium, one must first consider its domain architecture.

1.5.1.1 The PTB domain of ShcA

PTB domains are 100-170 amino acid sequences that typically bind phosphopeptides with the NP-X-pY motif (X is any amino acid). Additional specificity is conferred by hydrophobic residues 5 amino acids C terminal to the pTyr (Ravichandran 2001). The first structure of the PTB domain of ShcA was completed in 1995 in complex with the TrkA phosphopeptide (Zhou et al. 1995). This enabled the tertiary structure of a β sandwich comprised of 2 nearly orthogonanol β sheets and 3 α helices to be described. The phosphotyrosine (pTyr) binding site is formed by a cleft of β5 and the C terminal of α3. Zhou and colleagues showed that the phosphopeptide forms a β turn to fit precisely into the binding pocket, establishing interactions with Arg 67, Ser 151, Lys 169 and Arg 175 (Zhou et al. 1995). Of these residues, Arg 175 was found to be required for pTyr binding as site directed mutagenesis of Arg 175 to glycine or lysine resulted in loss of phosphopeptide binding. This is recapitulated in Drosophila studies where a mutation in the site analogous to R175 inactivates ShcA function (Ravichandran 2001).

Despite sharing little sequence similarity, the topology of the PTB domain closely resembles that of a pleckstrin homology domain, which binds acidic phospholipids (Ravichandran 2001). Phospholipid-binding by the ShcA PTB domain could allow membrane-protein interactions, perhaps in the absence of phosphopeptide binding, and could explain the localization of ShcA at membranes in unstimulated cells (Ravichandran 2001). While initial observations for ShcA binding was shown for TrkA and middle T antigen, ShcA was also shown
to bind the EGFR, ErbB3, cytokine receptors for GM CSF and IL2, ErbB2 and ErbB4, amongst others (Ravichandran 2001) (See Table 1.1 for complete list of interactions).
Figure 1.6 ShcA locus and domain architecture.

(A) ShcA locus and exon arrangement in ShcA transcripts. P66ShcA transcript is formed by exons 2-13, while p52/p46 transcripts are formed by exons 1-2a and 3-13 through alternative splicing. ShcA isoforms have a common domain architecture containing a PTB and SH2 domain separated by an intervening CH1 region. P66ShcA has an additional CH2 domain, while p46 has a truncated PTB domain. (B) ShcA pTyr signaling. The phosphotyrosine recognition motifs bind to consensus motifs on activated TKs, while subsequent phosphorylation leads to activation of downstream signaling pathways.
Figure 1.6. ShcA locus and domain architecture.
1.5.1.2 The SH2 domain of ShcA

SH2 domains are ~100 amino acid elements that bind to phosphotyrosine motifs and are divided into 2 broad groups: group I have consensus sites pY-φ-θ-φ (φ denoting hydrophobic residues and θ as hydrophilic residues) and group II SH2 domains which prefer pY-φ-x-φ (Marengere et al. 1994). SH2 domains typically have a central antiparallel β sheet flanked by 2 α helicities, and SH2 domains bind the pTyr containing sidechain into their pTyr binding pocket by coordinating the negatively charged phosphate with the positive charge on the sidechain of Arg βB5 (Marengere et al. 1994), which is part of the signature FLVR sequence found in most SH2 domains (Blaikie et al. 1994). Loss of this critical anchor through point mutation prevents SH2 recognition of phosphoproteins (Mayer et al. 1993). The SH2 domain of ShcA is located at the C-terminus, and preferentially binds pY -X- X- Ile/Leu (Songyang et al. 1993; Songyang et al. 1994). While sharing much homology with other SH2 domains, the SH2 domain of ShcA has a leucine at βD5, where βD5 residues influences phosphopeptide binding specificity (Zhou et al. 1995). The SH2 domain of ShcA has relatively low binding affinity for phosphopeptides, and may use its N terminal phosphotyrosine binding domain to help augment recruitment of ShcA to signaling networks (Ravichandran 2001). Initially, the SH2 domain of ShcA was shown to bind EGF, PDGF and the ζ chain of the T-cell receptor, but subsequently, many other binding partners have been demonstrated (Ravichandran 2001) (See Table 1.2 for a complete list). Of interest, the VEGF family, EGF family, and PDGF family of receptors have the potential to influence cardiac structure and function.

1.5.1.3 The CH1 Region of ShcA

The collagen homology region is a 52-167 amino acid region that like its namesake collagen, is highly enriched in prolines and glycines. Within the CH1 region of ShcA, there are critical tyrosine residues for mediating downstream phosphotryosine signaling, primarily through recruitment of Grb2, a SH3-SH2-SH3 adaptor protein. These tyrosine residues, Y239/Y240 and Y313 (denoting mouse nomenclature), may represent a mechanism that has evolved in multi-cellular organisms to increase the complexity of signaling (Luzi et al. 2000). While all three
tyrosines are present in higher mammals, Y239/240 are present in Drosophila but absent in nematodes, and Y313 is generally absent in “lower” metazoans (Luzi et al. 2000). Given the requirement for CH1 phosphotyrosine signaling in only some morphogenic processes (Hardy et al. 2007), the evolutionary advantage conferred by pTyr sites in the CH1 region could be through the introduction of specificity to signal transduction downstream of activated TKs.

Y239/240 and Y313 are phosphorylated by a variety of receptor kinases, and mutagenesis studies suggest that these are essential for ShcA mitogenic signaling leading to Ras activation. Upon receptor tyrosine kinase activation, phosphorylation of tyrosines in their cytoplasmic tail creates docking sites for either the PTB or SH2 domain of ShcA. ShcA is subsequently phosphorylated in its CH1 region to create consensus motifs (pY-X-Asn) for Grb2-SH2 domain binding. The Ras guanine nucleotide exchange factor SOS (Son of Sevenless) is recruited by the SH3 domain of Grb2, and localized to the plasma membrane (Ravichandran 2001). The TK-ShcA-Grb2-SOS complex thus allows for Ras activation by conversion of GDP bound Ras to the active GTP bound state. ShcA has also been shown to activate PI3K signaling via Grb2-Gab associations, whereby phosphorylation of the pTyr sites in the CH1 region can potentially lead to the recruitment of Grb2-Gab complexes to activate AKT signaling (Ravichandran 2001).

While Grb2-MAPK activation is the predominant signaling pathway from the CH1 region, other studies have shown that pY239/240 can lead to c-Myc activation (Gotoh et al. 1997). A diverse array of phosphopeptides can also bind to the pTyr sites in the CH1 region of ShcA, however the functional relevance of these is still unknown (van der Geer et al. 1996). In addition to SH2 binding motifs, the CH1 region also has several P-X-X-P motifs for SH3 binding (Weng et al. 1994) and a documented adaptin binding site (Okabayashi et al. 1996). Recent studies suggest that IQGAP links ShcA to the actin cytoskeleton through a non-canonical interaction with the PTB domain (Smith et al.) (see Table 1.3). Therefore, ShcA is able to coordinate multiple different signal transduction networks when recruited to upstream TKs. The importance of these, in the context of the myocardium, is largely unexplored.
<table>
<thead>
<tr>
<th>PTB domain interaction partner</th>
<th>Reference</th>
<th>Cardiovascular phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>APP (amyloid β (A4) precursor protein)</td>
<td>(Zambrano et al. 1997; Tarr et al. 2002)</td>
<td></td>
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<tr>
<td>Tetraspanin CD81</td>
<td>(Carloni et al. 2004)</td>
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<tr>
<td>CSF2RB (β subunit of IL-3 R)</td>
<td>(Bone et al. 2000)</td>
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<tr>
<td>DDR1 (Discoidin domain receptor)</td>
<td>(Vogel et al. 1997; Foehr et al. 2000)</td>
<td>Section 1.4.1.9</td>
</tr>
<tr>
<td>EGFR</td>
<td>(O'Bryan et al. 1996; Sakaguchi et al. 1998)</td>
<td>Section 1.4.1.1</td>
</tr>
<tr>
<td>EphA2</td>
<td>(Pratt et al. 2002)</td>
<td></td>
</tr>
<tr>
<td>ERBB2</td>
<td>(Olayioye et al. 1998)</td>
<td>Section 1.4.1.1</td>
</tr>
<tr>
<td>ERBB3</td>
<td>(Vijapurkar et al. 1998)</td>
<td>Section 1.4.1.1</td>
</tr>
<tr>
<td>ESR1 (ERα)</td>
<td>(Song et al. 2002)</td>
<td>Associated with Coronary artery disease(Kunnas et al.)</td>
</tr>
<tr>
<td>IGF-I receptor</td>
<td>(Dey et al. 1996)</td>
<td>Section 1.4.1.7</td>
</tr>
<tr>
<td>IL-2R β chain</td>
<td>(Ravichandran et al. 1996)</td>
<td></td>
</tr>
<tr>
<td>IL-4Rα</td>
<td>(Ikizawa et al. 2000)</td>
<td></td>
</tr>
<tr>
<td>Insulin Receptor</td>
<td>(Wolf et al. 1995; Sasaoka et al. 2000)</td>
<td>Section 1.4.1.7</td>
</tr>
<tr>
<td>β 3 integrin</td>
<td>(Cowan et al. 2000)</td>
<td>Section 1.4.1.4 and (Ren et al. 2007)</td>
</tr>
<tr>
<td>β 4 integrin</td>
<td>(Dans et al. 2001)</td>
<td></td>
</tr>
<tr>
<td>SHIP (SH2 containing inositol phosphatase)</td>
<td>(Damen et al. 1996; Lamkin et al. 1997)</td>
<td></td>
</tr>
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LDL receptor-related protein 1  (Barnes et al. 2003)
Mpl (myeloproliferative leukemia virus oncogene)  (Drachman et al. 1997)
TrkA  (Dikic et al. 1995; Obermeier et al. 1996)  Section 1.4.1.6
TrkC  (Guiton et al. 1995)  Section 1.4.1.6
PP2A  (Ugi et al. 2002)
PTP-PEST (PTPN12)  (Habib et al. 1994; Faisal et al. 2002)
T-cell protein tyrosine phosphatase (PTPN2)  (Tiganis et al. 1998)
ZAP-70 (Lck, Syk)  (Pacini et al. 1998; Walk et al. 1998)

Table 1.2. ShcA SH2 domain interaction partners

<table>
<thead>
<tr>
<th>SH2 domain interaction partner</th>
<th>Reference</th>
<th>Cardiovascular Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEACAM1 (carcinoembryonic antigen-related cell adhesion molecule 1)</td>
<td>(Poy et al. 2002)</td>
<td></td>
</tr>
<tr>
<td>AP2A1/2 (Adaptin)</td>
<td>(Okabayashi et al. 1996)</td>
<td></td>
</tr>
<tr>
<td>AXL tyrosine kinase</td>
<td>(O'Bryan et al. 1996)</td>
<td></td>
</tr>
<tr>
<td>Cbl</td>
<td>(Fukazawa et al. 1996; Park et al. 1999)</td>
<td>Noonan-like syndrome (Martinelli et al.)</td>
</tr>
<tr>
<td>CD22</td>
<td>(Poe et al. 2000)</td>
<td></td>
</tr>
<tr>
<td>CD3E (CD3ε)</td>
<td>(Guirado et al. 2002)</td>
<td></td>
</tr>
</tbody>
</table>
CD3Z (CD3ζ) (Labadia et al. 1996)

CDH5 (cadherin 5, type 2) (Zanetti et al. 2002)

CSF1R (colony stimulating factor 1 receptor) (Lioubin et al. 1994)

CSF2Rβ (β subunit of IL-3 R) (Bone et al. 2000)

CSF3R (G-CSF receptor) (Ward et al. 1998)

DAG1 (β-Dystroglycan; dystroglycan 1) (Sotgia et al. 2001) Cardiomyopathy/Muscular dystrophy (Lapidos et al. 2004)

DDR2 (discoidin domain receptor) (Ikeda et al. 2002) Section 1.4.1.9

EphA2 (Pratt et al. 2002)

Erythropoetin receptor (Damen et al. 1993)

ESR1 (ERα) (Song et al. 2002)

Fc γRIIb (Koncz et al. 1999)

FcγRIII (CD16) (Galandrini et al. 1997)

Flt1 (Warner et al. 2000) Section 1.4.1.3

Flt4 (Fournier et al. 1995) Section 1.4.1.3

Gab1 (Ingham et al. 1998)

Growth hormone receptor (VanderKuur et al. 1995; Moutoussamy et al. 1998)

β4 integrin (Dans et al. 2001) Section 1.4.1.4

Jak2 (VanderKuur et al. 1995) Section 1.2.3

Lyn (Ptasznik et al. 1995)
<table>
<thead>
<tr>
<th>Interaction partner</th>
<th>Interaction Region/motif</th>
<th>References</th>
<th>Cardiovascular phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGF receptor</td>
<td></td>
<td>(Pelicci et al. 1995)</td>
<td></td>
</tr>
<tr>
<td>STK/RON tyrosine kinase</td>
<td></td>
<td>(Iwama et al. 1996)</td>
<td></td>
</tr>
<tr>
<td>PDGF β-receptor</td>
<td>(Yokote et al. 1994)</td>
<td>Section 1.4.1.2</td>
<td></td>
</tr>
<tr>
<td>Fak</td>
<td>(Hecker et al. 2002)</td>
<td>Section 1.4.1.4</td>
<td></td>
</tr>
<tr>
<td>Pyk2</td>
<td>(Lev et al. 1995)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ret</td>
<td>(Borrello et al. 1994)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tie2</td>
<td>(Audero et al. 2004)</td>
<td>Section 1.4.1.3</td>
<td></td>
</tr>
<tr>
<td>PAL (Protein expressed in Activated Lymphocytes)</td>
<td>(Schmandt et al. 1999)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLP-76</td>
<td>(Chu et al. 1998)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.3. ShcA interactions by conserved signaling motifs

<table>
<thead>
<tr>
<th>Interaction partner</th>
<th>Interaction Region/motif</th>
<th>References</th>
<th>Cardiovascular phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crk</td>
<td>CH1 pTyr sites for SH2 domain binding</td>
<td>(Matsuda et al. 1994; Gesbert et al. 1998)</td>
<td>Embryonic cardiac dilation and thin ventricles (Park et al. 2006)</td>
</tr>
<tr>
<td>CrkL</td>
<td>CH1 pTyr sites for SH2 domain binding</td>
<td>(Chin et al. 1997)</td>
<td></td>
</tr>
<tr>
<td>Source, lyn, fyn</td>
<td>PXXP motif for SH3 domain binding; (Weng et al. 1994; Sato et al. 2002)</td>
<td>Section 1.4.1.4</td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>---------------------------------------------------------------------</td>
<td>-----------------</td>
<td></td>
</tr>
<tr>
<td>Grb7</td>
<td>CH1 pTyr sites for SH2 domain binding (Yokote et al. 1996)</td>
<td>Inhibitory function on cardiac K channels (Ureche et al. 2009)</td>
<td></td>
</tr>
<tr>
<td>Grb2</td>
<td>CH1 pTyr sites for SH2 domain binding (Nicholson et al. 2001)</td>
<td>Section 1.2.2</td>
<td></td>
</tr>
<tr>
<td>Shp-2 (PTPN11)</td>
<td>Binds pTyr sites on ShcA with its SH2 domains (Eck et al. 1996)</td>
<td>Noonan and LEOPARD syndromes (Neel et al. 2003)</td>
<td></td>
</tr>
<tr>
<td>Grap</td>
<td>Binds pTyr sites on ShcA with its SH2 domains (Trub et al. 1997)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gads</td>
<td>Binds pTyr sites on ShcA with its SH2 domains (Liu et al. 1998)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAPKAP kinase 2, (MK2)</td>
<td>Outside of pTyr binding motif on the PTB domain (Yannoni et al. 2004)</td>
<td>Section 1.2.2 (Streicher et al.)</td>
<td></td>
</tr>
<tr>
<td>IQGAP1</td>
<td>Outside of pTyr binding motif on the PTB domain (Smith et al.)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 1.5.1.4 The CH2 region of ShcA

The second collagen homology region is found only in p66 ShcA at its N terminus and also in the long forms of ShcB and C (205). It is thought to be recently acquired in vertebrates, as it is lacking in Drosophila Shc protein (Luzi et al. 2000). The CH2 region is linked to the oxidative stress pathway, as selective loss of the p66 isoform results in mice that are long lived with reduced levels of intracellular free radicals (Migliaccio et al. 1999; Pinton et al. 2008). It has been demonstrated that p66ShcA is phosphorylated at serine 36 in response to exposure of $\text{H}_2\text{O}_2$, PMA or UV irradiation and is implicated in apoptosis signaling through p53 mediated
increase in intracellular reactive oxygen species (ROS) (Trinei et al. 2002; Pacini et al. 2004). P66ShcA also participates in the oxidative stress response by inhibiting FOXO transcription factors (Nemoto et al. 2002) and localizing to the mitochondria membrane to aid in depolarization (Orsini et al. 2004; Giorgio et al. 2005). Overall, the loss of p66 ShcA confers protection from conditions related to oxidative stress damage (Purdom et al. 2003; Cosentino et al. 2008).

1.5.2 Biological Role of ShcA Signaling

The first biological role for ShcA was shown by Lai and Pawson in 2000 (Lai et al. 2000). Mice homozygous for the ShcA null allele are embryonic lethal at E12.5 resulting from defects in the cardiovascular system. ShcA null mice demonstrated dilated ventricles and poorly developed vasculature; the developing myocardium displayed decreased trabeculation in the ventricles at E10.5 and hypoplastic endocardial cushions resulting in poor contractility and cardiac congestion. Whole mount RNA in situ hybridization demonstrated ShcA is highly expressed in the developing cardiovascular system, specifically in the endothelium and mesenchymal cells. The vasculature in these mice was found to have decreased complexity and aberrant cell-cell contacts, owing to defects in late angiogenic remodeling. As ShcA is important for MAPK activation, Lai and Pawson demonstrated decreased MAPK activation in the developing cardiovascular system, while in embryonic fibroblasts, ShcA sensitized cells to growth factor stimulation.

To circumvent the embryonic lethality and to address more specific roles for ShcA in vivo, a ShcA allele series was generated that allowed for dissection of pTyr requirements of ShcA through mutant Knock-in (KI) models, as well as conditional excision of ShcA by use of loxP/Cre recombinase technology (Hardy et al. 2007). This allows tissue and temporal specificity of ShcA signaling to be addressed.

The ShcA KI models contained individual point mutations that precluded 1) pTyr binding of the PTB domain to upstream TKs by mutating arginine 175 to glutamate (δPTB), 2) pTyr binding of the SH2 domain by mutation of arginine 397 to lysine (δSH2) and 3) downstream signaling from the tyrosines in the CH1 region by mutating tyrosines 239/240/313 to
phenylalanine (1F (Y313F), 2F (Y239/240F) and 3F (Y239/240/313F). Mice homozygous for the PTB mutation phenocopied the ShcA null mouse, demonstrating a requirement for TK signaling in heart development, which is myocardial-specific (Hardy et al. 2007). Surprisingly, mice homozygous for the 3F allele showed normal heart development, suggesting an alternative downstream signaling mechanism is required for heart development.

Mice homozygous for the ShcA 3F and SH2 alleles were viable, but postnatal phenotyping revealed severe coordination defects. ShcA 3F/3F mice failed to develop normal muscle spindles, while ShcA SH2/SH2 mice had defects in spindle morphogenesis. Both genotypes had altered synaptic conductivity of group Ia sensory neurons. Using the conditional allele (ShcA $^{flx/flx}$) in conjunction with the muscle specific Mlc1f Cre, Hardy et al demonstrated that ShcA functions in the stretch reflex circuit and requires signaling from both the PTB domain and protein-protein interactions downstream of the pTyr sites in the CH1 region using a novel genetic strategy (Hardy et al. 2007). These studies not only highlight the diversity of ShcA signaling requirements for various morphogenic processes in different tissues, but also the potential for a ShcA signaling independent of Grb2.

The emerging theme that CH1 signaling is context dependent was solidified by experiments demonstrating differential signaling from the CH1 region in an ErbB2 breast cancer model (Ursini-Siegel et al. 2008). By using the phospho-deficient 3F ShcA alleles, Ursini-Siegel et al showed differential pTyr signaling requirements in early stages of mammary tumour progression: Y313 is critical for tumor cell survival, while Y239/240 augments tumour vascularization. In vitro studies also have demonstrated differential signaling from the pTyr residues based on receptor type (Gotoh et al. 1997; Pratt et al. 1999). These studies demonstrate that ShcA is critical in coordinating morphogenic processes in multiple tissues. They also highlight the complexity of signaling as ShcA can recruit downstream effectors either through pTyr sites in the CH1 region or other signaling motifs.

These studies also demonstrated the importance of reciprocal signaling in tissue physiology. Hardy and colleagues demonstrated that the loss of SH2 signaling in the muscle compartment acts to limit paracrine signaling required in proper spindle development. Ursini-Siegel and colleagues also showed that loss of Y313 signaling in the stroma can limit tumour progression by disrupting cellular communication between the endothelial and myoepithelial
compartments. ShcA is therefore an important mediator of TK signaling that maintains cell autonomous and non-cell autonomous effects.

1.5.3 The Role of ShcA in the Myocardium

The importance of ShcA signaling in the developing cardiovascular system and the strong requirement for TK signaling in the myocardium suggests that ShcA could play a role in the postnatal myocardium. While no in vivo data have been reported for the conditional loss of ShcA in the functioning myocardium, there have been several reported roles for ShcA in the cardiovascular system. One of the most explored roles for ShcA is that of p66ShcA in cardiovascular diseases associated with free radical damage. The discovery that serine36 phosphorylation of p66 ShcA leads to its translocation into the mitochondria to induce ROS generation and apoptosis (Pinton et al. 2007) has offered mechanistic insight into the protection incurred by germline ablated p66 ShcA mice in conditions of ROS damage, such as diabetic cardiomyopathies (Rota et al. 2006), endothelial dysfunction (Camici et al. 2007), atherosclerosis (Napoli et al. 2003), ischemia-reperfusion (Zaccagnini et al. 2004) and AngII-mediated myocardial remodeling (Graiani et al. 2005). In vitro data have placed ShcA downstream of thrombin activation of the GPCR PAR-1 (Obreztchikova et al. 2006), while a role for ShcA in integrin/FAK signaling has also been described in feline pressure overload studies (Barberis et al. 2000; Laser et al. 2000). These studies point to an important role for ShcA in the myocardium, however, little is known regarding the signaling mechanisms by which ShcA mediates its effects or the physiological implications within the myocardium. Therefore, to address this, a conditional gene targeting strategy must be employed to define tissue specificity and circumvent embryonic lethality.

1.5.4 Strategies for Condition Gene Targeting in the Myocardium

As germline deletion of key signaling molecules can result in embryonic lethality or phenotypic ambiguity due to cellular heterogeneity within the tissue of interest, conditional gene targeting approaches are preferred. In the heart, choice of Cre transgenic mouse models allow for spatial
regulation and the use of ligand inducible systems refine excision, allowing for cardiomyocyte specific excision by Cre/loxP recombination. Techniques for inducing point mutations are currently evolving (Bayascas et al. 2006) and new recombination methods await rigorous in vivo testing (Feil 2007).

The main system to achieve conditional allele activation is through LoxP/Cre recombinase technology. Cre is a bacteriophage P1 gene that recognizes a 34 bp site designated LoxP and catalyzes the reciprocal DNA recombination between 2 loxP sites (Sauer 1998). The directionality and lack of requirement for host factors to mediate efficient recombination make it suitable for eukaryotic cells (Sauer 1998). LoxP/Cre recombination can be used to ‘turn on’ genes by excision of a STOP cassette between a promoter and its transgene or ‘turn off’ genes by excision of targeted alleles. Conditional targeting of genes of interest in the myocardium has allowed for regulation, as the choice of the promoter used to drive Cre expression determines the developmental window for the phenotype and specific cell lineage. To circumvent any developmental deficiencies, one can use a ligand inducible Cre system such that Cre expression is acutely turned on by drug administration (Table 4).

Table 1.4. Cardiomyocyte specific Cre transgenic mouse models

<table>
<thead>
<tr>
<th>Heart Specific Cre Promoters</th>
<th>Temporal Expression Pattern</th>
<th>Tissue Expression Pattern</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isl1</td>
<td>Early Embryonic</td>
<td>1st and 2nd heart field</td>
<td>(Ma et al. 2008)</td>
</tr>
<tr>
<td>Nkx2.5</td>
<td>E9.5-adulthood</td>
<td>Cardiomyocyte</td>
<td>(Moses et al. 2001)</td>
</tr>
<tr>
<td>β-MHC</td>
<td>E8.5-birth/perinatal</td>
<td>Cardiomyocyte</td>
<td>(Lyons et al. 1990; Parsons et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>Reactivated in disease states</td>
<td>Fetal: V&gt;A</td>
<td></td>
</tr>
<tr>
<td>α-MHC</td>
<td>E8.5 to midgestation</td>
<td>Cardiomyocyte</td>
<td>(Agah et al. 1997; Wang et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>Reactivated at birth</td>
<td>Fetal: A&gt;V</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Downregulated with disease</td>
<td>Adult: V and A</td>
<td></td>
</tr>
<tr>
<td>MLC2v</td>
<td>Fetal (E10-E15) to adulthood</td>
<td>Cardiomyocyte</td>
<td>(Chen et al.</td>
</tr>
</tbody>
</table>
The germline deletion of ShcA demonstrated a requirement for ShcA in the developing cardiovascular system, but due to the embryonic lethality, the defined roles of ShcA in the postnatal myocardium have remained elusive. Therefore, conditional ablation of ShcA in the myocardium is required to fully understand the role of ShcA in cardiac structure and function. Genetic analysis of embryonic heart development demonstrated the CH1 pTyr sites of ShcA are not required for heart morphogenesis, therefore, generation of cardiac-specific mutant ShcA KI mice using the ShcA allelic series would allow for evaluation of the PTB and SH2 domains of ShcA in coupling to upstream TKs and the importance of the pTyr sites in the CH1 region in cardiac structure and function. As ShcA has the potential to signal downstream of multiple TKs in the myocardium, and can activate Erk- MAPK and PI3K pathways, these studies can further our understanding of pTyr signaling networks in the myocardium. Of clinical importance, the
ability of ShcA to be recruited to activated ErbB2 suggests it could be relevant to Herceptin-mediated cardiotoxicity, thereby furthering our understanding of the effects of TKIs in the myocardium. Therefore, the objectives of this thesis are: 1) to understand the role of ShcA in the postnatal myocardium by evaluating its effects on cardiac structure and function, 2) to explore the molecular mechanism by which ShcA effects cardiac structure and function, and lastly 3) to understand how ShcA coordinates its effects on cardiac structure and function through its various signaling domains and motifs. To this end, ShcA was conditionally excised in ventricular cardiomyocytes and the ShcA allelic series (Hardy et al. 2007), which is comprised of ShcA KI alleles each containing discrete point mutations that inactivate specific domains or motifs, were restricted to ventricular cardiomyocytes only and evaluated for effects on cardiac structure and function.

Our studies demonstrate that the loss of ShcA in ventricular cardiomyocytes leads to a dilated cardiomyopathy characterized by elevated cardiomyocyte contractility and impaired myocyte-matrix interactions. In vivo dissection of ShcA signaling properties revealed that selective inactivation of the PTB domain in the myocardium had effects resembling those seen in ShcA CKO mice, while disruption of the SH2 domain caused a less severe cardiac phenotype. Downstream signaling through the CH1 pTyr sites was dispensable for baseline cardiac function, but necessary to prevent adverse remodeling after hemodynamic overload. Therefore, ShcA mediates pTyr signaling in the adult heart through multiple distinct signaling elements that control myocardial functions and response to stresses.
Chapter 2

Phenotypic Analysis of the ShcA Allele Series
2 Phenotypic Analysis of ShcA Allele Series

This section will highlight all the methods used to do the experiments in the subsequent data chapters.

2.1 Mouse Models

All mice were housed in a pathogen-free facility (Department of Comparative Medicine, University of Toronto) and handled using standard protocols in accordance with animal welfare regulations. All study protocols were approved by the Animal Care Committee at the University of Toronto.

The ShcA allele series was generated by W.R Hardy in the Pawson lab and targeting was described elsewhere (Hardy et al. 2007). The ShcA allele series consists of the ShcA floxed allele and mutant domain KI alleles of ShcA (Hardy et al. 2007). The Mlc2v Cre KI mouse (Chen et al. 1998) and α MHC- MerCreMer transgenic mouse (Sohal et al. 2001) were used to conditionally excise ShcA in the myocardium through Cre/loxP strategies. Targeting and recombination strategies were previously described (Hardy et al. 2007) and outlined in results section. All mouse lines were bred in a mixed background and used appropriate littermate controls.

Mouse tails were used for PCR amplification of the various genotypes. In short, mouse tails were digested in SDS tail buffer (50Mm Tris (pH 8.5), 0.5mM EDTA, 0.1% SDS, 0.1M NaCl with 100ug/ml Proteinase K) diluted and deactivated at 100°C for 15 minutes. Genotyping primers distinguishing the KI and wild type alleles and the various Cre recombinase lines were as previously described (Chen et al. 1998; Sohal et al. 2001; Hardy et al. 2007). DNA sequencing from tail samples confirmed the individual mutations as previously reported (Hardy et al. 2007). The excision of the ShcA floxed allele was confirmed using genomic DNA as previously described (Hardy et al. 2007).

2.2 Cardiac Phenotyping
Cardiac function is determined by various levels of regulation. Depending on the context, cardiac contractility can have many layers of compensation built in to maintain cardiac homeostasis. Systemic regulation, external to the heart itself, involves autonomic nervous system modulation and volume regulation at the level of the kidneys, while effective contraction at the tissue level requires proper electrical and mechanical coupling. Electrical propagation ensures synchronous and progressive waves of depolarization and repolarization with each heart beat, while effective mechanical force transmission requires intact extracellular matrix that is continuous along the myocytes. At the most fundamental level, cardiomyocyte contractility, governed by calcium handling and myofilament interactions, determines the ability of the individual myocyte to contract. Therefore, to understand heart function in various mouse models, one must look at each level of regulation to understand the various contributions, which ultimately lead to the phenotype.

2.2.1 Global Cardiac Function Methods

At the organ level, the heart must function to meet the systemic needs of the body, while maintaining beat to beat regulation. As an organ, global cardiac function is determined by the heterogeneous cellular composition and is also subject to regulation extrinsic to the myocardium itself. For example, in the face of disease, systemic regulation (sympathetic stimulation) can augment poor cardiomyocyte function to maintain cardiac homeostasis. To ascertain the global cardiac function in the mouse, echocardiography, cardiac catheterization and transverse aortic constriction were employed. These methods look at characterization of left ventricle function, the main systolic pumping chamber, to infer global heart mechanics.

2.2.1.1 Echocardiography of the Mouse Heart

Transthoracic echocardiography allows for in vivo assessment of cardiac function under physiological conditions and also the serial evaluation of disease states. Echocardiography visualizes the left ventricle dimensions during the cardiac cycle, thereby assessing dimensions
during the ejection phase (systole) and during the relaxation phase (diastole). Trans thoracic echocardiography was done as previously described (Crackower et al. 2002). In brief, mice were anaesthetized with 2% isoflurane, shaved in the pericardial region and then brought to a semi-conscious state with 0.6% isoflurane. Mice were maintained at homeostatic conditions with the use of a heating pad and low isoflurane with a target heart rate between 450 to 650 beats per minute (bpm). Imaging was recorded on the Sequoia C256 ultrasound machine (15L8 transducer, Acuson). For visualization and assessment of left ventricular systolic function, as measured by the degree of reduction of chamber dimension, mice were placed in a lateral dicubitus position and the transducer was placed to first visualize wall motion in the M-mode view at the mid papillary level and then positioned to read the aortic velocities by Doppler in the 2 dimensional plane using the parasternal short axis view. 3 independent views were obtained for each position. Analyses of 3 consecutive cardiac contractions were averaged using the Sequoia platform and standard parameters of cardiac structure and function were assessed. Parameters included left ventricular end diastolic and systolic dimensions (LVEDD and LVESD, respectively), anterior and posterior wall thickness and % fractional shortening (LVEDD-LVESD)/LVEDD*100), heart rate (beats per minute) and velocity of circumferential shortening (VCF = %FS/AoV). Doppler imaging evaluates the aortic velocities (AoV) and time to peak ejection amplitude (ET) for subsequent estimations of ejection performance. Parameters recorded on tables are: LVEDD, left ventricular end diastolic dimension; LVESD, left end systolic dimension; %FS, % fractional shortening; VCF, velocity of circumferential shortening; Ant, anterior; post, posterior.

2.2.1.2 Cardiac Catheterization

Cardiac Catheterization allows for detection of pressure development within the aortic and left ventricular compartments. A pressure transducer is introduced by a wire catheter to measure the hydrostatic pressure responsible for blood ejection within the left ventricle. These surgical procedures were done by Dr. M.G. Kabir at the Heart and Stroke Richard Lewar Centre of Excellence at the University of Toronto as described previously (Crackower et al. 2002). In brief, mice for the various time points were anesthetized with inhaled isoflurane (3% induction, 1% maintenance) placed in a supine position and a small midline incision from the peristernal notch toward the mandible was made to expose the right common carotid artery and a 1.4 French
pressure transducer catheter (Millar Instruments) was inserted in a 1mm incision. Retrograde advancement of the catheter into the ascending aorta and subsequently through the aortic valve into the left ventricle allowed for 3 independent recordings of aortic and left ventricle pressures to be collected. Measurements were recorded at a stable internal temperature of 37ºC with a target heart rate above 350 bpm. Both aortic and left ventricle parameters were recorded. Dobutamine challenge (3.0mg/kg body weight) was injected in the peritoneal cavity and measurements were recorded at baseline and 20 minutes after injection. Parameters recorded on tables are: LVESP, Left ventricular end systolic pressure; LVEDP, Left venticle end diastolic pressure; dP/dT\text{max}, first derivative of maximal pressure development in left ventricle; dP/dT\text{min}, first derivative of minimal pressure in left ventricle.

2.2.1.3 Transverse Aortic Constriction Model

To analyze the effect of biomechanical stress on the remodeling processes of the left ventricle in the various genetic models, a minimally invasive transverse aortic constriction procedure was employed. These surgical procedures were done by Dr. M.G. Kabir at the Heart and Stroke Richard Lewar Centre of Excellence at the University of Toronto as described previously (Crackower et al. 2002). Briefly, 25-30g male mice were anesthetized (3% isoflurane) and intubated. Once placed on a respirator and held at a stable internal temperature of 37ºC, a small incision above the suprasternal notch was made to expose the aortic arch. A 6.0 mm silk suture was positioned under the aorta with a blunted 27 gauge needle to set the diameter of the ligation. Sham animals underwent every aspect of the surgery with the exception of the ligation procedure. The animal was then sutured closed and allowed to recover in a heated environment.

2.2.2 Tissue and Cellular Physiology Methods

To isolate various physiological parameters of cardiac muscle, tissue physiology and isolated myocyte function were investigated. This allowed for tissue mechanics and cardiomyocyte contractility to be evaluated independent of integrated systemic compensation responses.
2.2.2.1 Isolated Ventricular Muscle Preparation Methods

The heart possesses structures that have myocardium arranged into linear strips. One example is the papillary muscle which assists in holding the valves in place during cardiac ejection. Trabeculae are small ultrathin myocardial strips that prevent inversion of the mitral and tricuspid valve during systole. The force length relationship was investigated in papillary and trabeculae muscles to evaluate the contribution of extracellular matrix to the passive tension. The force length relationship in tissue preparations, allows for isolation of the mechanical components of the Frank Starling principle, which states an increase in end diastolic volume will necessitate an increase in pressure generation. At the tissue level, this translates to increased force as sarcomere length is increased.

Adult mice (10-12wks) were killed by cervical dislocation. The hearts were rapidly excised and cannulated and perfused with a cardioplegic Krebs solution (112.5mM NaCl, 4.7mM KCl, 1.2mM MgCl₂, 1.2mM KH₂PO₄, 25mM NaHCO₃, 12mM Glucose, containing1mM CaCl₂ and 15mM KCl, pH 7.4). The right ventricle was opened from the pulmonary artery to the apex and the papillary muscle (septal side) or trabeculae (right ventricle free wall) was dissected by cutting a portion of the valve leaflet above and a small portion of the ventricular wall at the base of the muscle. Only muscles preparations free from the wall and cylindrical in shape were selected, while muscle preparations with spontaneous electrical activity were discarded. Muscles were measured manually using a stereoscopic microscope for their thickness and width to allow for calculation of cross sectional area to normalize the force measurements.

The muscle was then placed in the bath and mounted on the force transducer (Aurora Scientific Canada, 5mN) (Figure 2.1). Muscles preparations were mounted horizontally with the ventricular wall piece mounted on a small wire basket connected to the force transducer by a glass tube while the valve leaflet is connected to a hook on the motor server arm allowing for incremental length changes. The perfusion set up allowed for constant perfusion rate and thermostatic conditions at 27°C. The preparation was field-stimulated by platinum electrodes allowing for pulse durations of 2ms, at 1 Hz with voltages a minimum of 20% over threshold. The preparation was gradually stretched to 90% of its maximal value and allowed to equilibrate for 30 minutes. The standard Krebs salt solution (112.5mM NaCl, 4.7mM KCl, 1.2mM MgCl₂,
1.2mM KH$_2$PO$_4$, 25mM NaHCO$_3$, 12mM Glucose, (1.5mM CaCl$_2$ (papillary) 1mM CaCl$_2$ (trabeculae)) was used and gassed with 95% O$_2$ and 5% CO$_2$ to a pH of 7.4.

Figure 2.1 Papillary muscle force-length apparatus.

(A) Isolated papillary muscles are mounted in a perfusion chamber and stimulated with platinum electrodes. (B) The papillary muscle undergoes incremental length changes by the motor server arm while the changes in force generation are acquired through a force transducer. Sarcomere length is recorded as outlined in Section 2.2.2.1.
When muscle contracts, the contractile proteins undergo structural changes resulting in shortening of the myocyte, leading to pressure generation. Shortening can be monitored by measuring the sarcomere length. After the equilibration period of 30 min, the force length relation was determined. Muscle preparations were put to slack length whereby no force was detected and the baseline for the force transducer was set. Sarcomere length was measured throughout the protocol using a high speed video length (HSVL) detection system from Aurora Scientific (papillary muscles) and laser diffraction for trabeculae. Micromanipulators were used to increase the length of the muscle over the protocol. Small changes in length were made and muscle was allowed to equilibrate for 1 min between length changes. Length was decreased from 2.25um to slack and then increased from slack to Lmax (2.3um).

Force and sarcomere length raw data were analyzed and force was normalized to the cross sectional area (mN/mm2) and plotted against the diastolic sarcomere length. Passive tension data was fitted to the exponential equation \( y = e^{(x-x_0)/t} + a \), whereby \( y \) is the passive tension, \( x_0 \) is the average resting passive tension, \( x \) is the measured sarcomere length, \( a \) is the measurement offset and \( t \) is the compliance constant of the myocardium. Statistical significance was determined by Adrian Pasculucae at the Samuel Lunenfeld Research Institute using F statistics of the experimental and control data normalized linear model. Developed tension comparisons were compared by generalized additive model to generate splines.

### Isolated Cardiomyocyte Methods

Isolated cardiomyocyte experiments enable one to investigate function independent of systemic compensatory mechanisms and local paracine signaling from surrounding tissue. To investigate excitation contraction coupling (Chapter 4), single myocyte experiments were performed, while myofilament mechanics were investigated by force-calcium experiments.

#### Dissociation of Murine Cardiomyocytes

Adult mice ages 8-12 wks were used for the studies. Mice were euthanized by cervical dislocation and the heart was rapidly removed and placed in chilled calcium free Tyrodes
solution (137mM NaCl, 5.4mM KCl, 10mm HEPES, 0.5mM NaH₂PO₄7H₂O, 1.0mM MgCl₂6H₂O and 10mM Glucose, pH 7.4(NaOH)). The aorta was then cannulated with a blunted 18 gauge needle and perfused for 3-4 minutes with calcium free Tyrodes solution at 37°C. Collagenase containing Tyrodes solution was then perfused (1 mg/ml, CLS2 Worthington’s) for 10-12 minutes at 37°C. The ventricular free wall was dissected free and myocytes were mechanically dispersed in high K + containing KB solution (100mM K-glutamate, 10mM K-aspartate, 2.5mM KCl, 20mM Glucose, 10mM KH₂PO₄, 2mM MgSO₄7H₂O, 20mM Taurine, 5mM Creatine, 0.5mM EGTA, 5mM HEPES and 0.1% albumin, pH 7.2 (KOH)). Myocytes were used within 6 hours of being isolated.

2.2.2.2.1.1 Single Myocyte Morphometry

Using the 5x objective microscope, fields of plated cardiomyocytes were photographed, and length and width measurements were recorded, after calibration with micrometer, using GNU image manipulation program.

2.2.2.2.1.2 Single Myocyte Contractility

Freshly isolated myocytes were plated on glass perfusion plate and allowed to settle for 2 minutes. Calcium containing Tyrodes (1 mM CaCl) heated to 32°C was perfused for 20 minutes to ensure proper equilibration of calcium and temperature. Cells were then whole field stimulated by platinum electrodes (5-7volts, 1 Hz, 5 ms duration). Cells were allowed to equilibrate (~5 minutes) to ensure stable contraction and then myocyte sarcomere length measurements were captured using the HSVL program (Aurora Scientific, Canada) at 0, 3 and 5 minutes for 10 second intervals. Only stable cells with no spontaneous contractions were selected for analysis (Figure 2.2).

2.2.2.2.1.3 Single Myocyte Calcium Transients
Freshly isolated myocytes were incubated with Indo AM calcium indicator dye (Molecular Probes, final concentration 1 uM with 0.45% Pluronic Acid) for 5 minutes at room temperature. Cells were then plated on a glass perfusion dish and perfused with calcium containing Tyrodes solution (1mM CaCl, 32°C) for at least 20 minutes. Cells were whole field stimulated with platinum electrodes (5-7 volts, 1 Hz, 5ms duration). The selected cell was allowed to equilibrate, and calcium transients were recorded at 0, 3 and 5 min of the protocol for 10 second intervals and captured with the Felix software program. At 5 minutes, single myocyte contractility was also recorded using the HSVL program. Only cells with stable contraction with no spontaneous contraction were selected for analysis. Background subtraction to control for autofluorescence and varied background was evaluated by 1) recording of fluorescence in the absence of a cell 2)
Figure 2.2 Configuration of single cardiomyocyte apparatus

(A) Single myocyte contractility apparatus. Single myocytes are plated on the perfusion chamber and stimulated with platinum electrodes. Sarcomere Shortening is captured by the high speed video sarcomere length program by Aurora Scientific. Specific detail is outlined in Section 2.2.2.2

(B) Calcium Transient apparatus. Single myocytes are plated on a perfusion chamber and stimulated with platinum electrode. Fluroscence signals were captured and analzed as outlined in Section 2.2.2.
unloaded cells and 3) manganese perfusion. Amplitude of the calcium transient was expressed as a ratio of the emissions (405/485nm) corrected for background.

### 2.2.2.2 Force-Calcium Relationship in Skinned Myocytes

I did these experiments in the laboratory of Dr. Pieter deTombe at the University of Illinois at Chicago as previously described (Belin et al. 2007). Briefly, mice were euthanized by cervical dislocation and the heart was rapidly excised and placed in ice cold PBS solution. After excess blood was removed, ~10 mg pieces of left ventricle free wall were dissected and quickly placed in liquid nitrogen. The frozen biopsies were then kept at –80°C until they were used for mechanical isolation of single myocytes. To isolate the single myocytes, the sample was placed in relaxing solution (5.55 mM Na$_2$ATP, 7.11mM MgCl$_2$, 2.0mM EGTA, 108.01mM KCl, 8.91 mM KOH and 10.0mM Imidazol, pH 7.0 and Ionic strength 149.17) with the addition of protease inhibitor cocktail (Sigma), 10mM DTT and 0.3% Triton. On ice, the sample was homogenized for 35 seconds at 1500rpm with a polytron and then incubated on ice for 10-15 minutes. After centrifugation at 560g for 1 min at 4°C, the supernatant was decanted and the pellet resuspended in 2ml of relaxing solution. Centrifugation and resuspension of the pellet was carried out 3 times with a final resuspension in 500ul of relaxing solution on ice.

Once the myocytes were isolated, a small aliquot was placed on a microscope mounting cover slip, and myocytes were selected based on size (100-150um long and ~30um thick) and striation uniformity. Selected myocytes were attached to a 5mN force transducer arm (Aurora Scientific, Canada) and a motor server arm by silicon adhesive (Dow corning, ML USA). Once the adhesive had cured, the myocyte was moved to an adjacent well containing relaxing solution (5.95mM Na$_2$ATP, 6.41mM MgCl$_2$, 10.0mM EGTA, 100.0 mM BES, 10.0mM creatine phosphate, 50.25mM potassium prop, protease inhibitor cocktail (Sigma) and 10mM DTT, pH 7.0), and the sarcomere length of the myocyte preparation was set to 2.25um using a spatial Fourier transform, whereby the peak power spectrum corresponded to a mean sarcomere length.
The myocyte then alternated between relaxing solution wells and activation solution (5.95mM Na$_2$ATP, 6.20mM MgCl$_2$, 10.0mM Ca$^{+2}$EGTA, 100.0mM BES, 10.0mM creatine phosphate and 29.98mM potassium prop, protease inhibitor cocktail (Sigma) and 10mM DTT, pH 7.0) wells with varying calcium concentrations at 15$^\circ$C. For each calcium concentration, the preparation was released to slack after peak isometric tension development to obtain a baseline before switching to the relaxing solution. The difference in peak tension and the baseline tension is the total force developed for the given calcium concentration. As force generation in the heart is highly cooperative, whereby force generation is highly dependant on the amount of intracellular calcium available, the data was fitted using the equation:

$$P=\frac{[Ca^{+2}]nH}{(KnH+[Ca^{+2}]KnH)}$$

Whereby $P =$ tension, $K =$ calcium concentration required for half-maximal activation, and $nH$ is the Hill coefficient for cooperatively of the myofilaments. Data was analyzed as mean ± SEM.

2.3 Cellular Biology and Signal Transduction Methods

To understand the molecular changes that underpin the physiological responses seen at the whole heart level, the tissue level and at the cellular level, a variety of molecular and biochemical techniques were employed.

2.3.1 Histological Analysis and Microscopy

Excised hearts were washed with PBS and fixed in 10% buffered formalin and paraffin embedded. 8μm sections were stained with hemotoxylin and eosin, masson trichrome stain or picrosirius red by the histology core at the Centre for Phenogenomics (Toronto, Canada) and slides were scanned at 20x lens using the Mirax microdigital slide scanner (Carl Zieiss MicroImaging). Picrosirius red with 10% PMA was used for visualization of perimysial fiber
content using the Optigrid structured illumination microscopy (Qioptiq LINOS) as previously described (Fedak et al. 2003). Using the 20x objective lens multiple areas of the myocardium were captured and analyzed for percent area of collagen (Image J software) to infer collagen content. Only sections absent of interstitial fibrosis and perivascular fibrosis were analyzed.

2.3.2 Electron Microscopy

Electron Microscopy was done by Doug Holmyard at the electron microscopy facility at Mount Sinai Hospital, Toronto, Canada. Briefly, left ventricle free wall specimens were rapidly dissected and cut into 2mm$^3$ pieces and placed in fixative solution containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.15M sodium phosphate buffer, pH7.4. The specimens were post fixed for 1 hr in sodium phosphate buffer containing 1% osmium tetroxide and 1.25% potassium ferrocyanide then dehydrated in a series of alcohol steps and embedded in Epon Araldite. Thin sections were obtained and stained with uranyl acetate and lead citrate. Random fields were scanned at various magnifications and photographed for subsequent analysis.

2.3.3 Western Blotting and Immunoprecipitation

Hearts used for biochemical analysis were excised, rinsed in cold PBS and immediately frozen in liquid nitrogen for subsequent analysis. Whole hearts or isolated cardiomyocytes were homogenized in chilled radioimmunoprecipitation buffer containing 50mM Tris pH7.4, 150 NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton, 1% NP –40, 100mM sodium fluoride, 1mM sodium orthovandadate, 1x protease cocktail containing leupeptin, aprotinin and PMSF. Lysates were clarified by centrifugation at 14 000x g for 15 minutes at 4C. Protein Quantification was carried out following standard protocol for the BCA kit (Pierce) and proteins (40ug) were boiled in 2x sample buffer, resolved by SDS PAGE and transferred to nitrocellulose. For Immunoprecipitations, Flag M2 antibody immobilized on agarose beads (Sigma) was used to enrich for ShcA containing fraction, and beads were washed 3 times and boiled in sample buffer, resolved by SDS PAGE and transferred to nitrocellulose. Membranes were blocked in 5% BSA
and incubated with the appropriate primary antibody overnight, washed and incubated with the appropriate HRP conjugated secondary antibody for 1 hr. Blots were visualized after incubation with Chemiluminescence reagents. Additional antibodies used were: p44/42 and phospho-p44/42 Erk (Cell Signaling Technologies), GAPDH (Biosciences).

2.3.4 Zymography

Left Ventricle free wall was rapidly dissected from hearts, washed in PBS and placed in liquid nitrogen. Heart specimens were then homogenized in RIPA buffer and clarified 2x by centrifugation at 14,000g for 20 minutes at 4°C. 50 ug of protein was loaded onto an 8% Tris glycine gel containing 0.5% gelatin A substrate (Sigma). Zymography sample buffer contained: 0.625M Tris pH 6.8, 10% glycerol, 2% SDS and 2% bromophenol blue. The gel was then washed 3 times for 20 minutes each at room temperature with 2.5% Triton wash. The gel was then incubated at 37°C for the desired time in incubation buffer containing 50mm Tris pH 7.4, 150mM NaCl and 5mM CaCl₂. The gel was then stained with Coomassie and destained to resolve the digested bands.

2.3.5 RNA Isolation and Real Time RT PCR

Hearts were excised and rinsed in DEPC-treated PBS and quickly frozen in liquid nitrogen. Ventricle tissue was homogenized in Trizol reagent (Invitrogen) to isolate RNA and cDNA synthesis was carried out following the Superscript II cDNA synthesis kit (Invitrogen). Real time RT PCR template detection was carried out by SYBRgreen (Applied Biosystems) and read using the Applied Biosystems 7900HT machine. Data was normalized to GAPDH internal control and expressed as fold increase or decrease from control samples (Schmittgen et al. 2008). Primers for real time RT PCR were described previously (Schoenfeld et al. 1998).

2.3.6 Preparation of Tamoxifen
Injected tamoxifen was prepared as previously described (Sohal et al. 2001). Briefly, Tamoxifen citrate (Sigma) was sonicated in peanut oil (Sigma) at a concentration of 5mg/ml. 20mg/kg/day was injected in the peritoneal cavity for 5 days. Mice were studied 7 days post injection.
Chapter 3

ShcA is required to maintain cardiac structure and function

A version of this appeared in:

The ShcA phosphotyrosine docking protein uses distinct mechanisms to regulate myocyte and global heart function (manuscript submitted)

Attributions:

Cardiac Catheterization studies were done in collaboration with the Surgical Technician at the Heart and Stroke/Richard Lewar Centre of Excellence, Dr.M.Golum Kabir

Histology and Electron Microscopy were done in collaboration with the facilities at the Samuel Lunenfeld Research Institute
3 ShcA is required for Cardiac Structure and Function

3.1 Introduction

Despite heart failure (HF) being a leading cause of death and morbidity in the western world, current treatment strategies have not significantly impacted on long-term survival (Ho et al. 1993; O'Connell et al. 1994; Russell et al. 2008). Recent studies have established tyrosine kinase (TK) signaling to be integral to cardiac function, as disruption of various TKs in animal models leads to a dilated cardiomyopathy phenotype. In particular, loss of myocyte-specific signals downstream of the receptor TK ErbB2 results in a dilated cardiomyopathy (Crone et al. 2002; Ozcelik et al. 2002), while myocyte-specific deletion of FAK, a non-receptor TK, results in eccentric remodeling in response to hemodynamic overload (DiMichele et al. 2006) and aging (Peng et al. 2006). The clinical relevance of TK signaling is supported by clinical trials in which tyrosine kinase inhibitors such as Herceptin, which targets ErbB2, and Gleevec, an inhibitor of kinases such as Abl and the platelet-derived growth factor receptor, have shown cardiac side effects in a subset of oncology patients (De Keulenaer et al.; Slamon et al. 2001; Sparano 2001; Chu et al. 2007; Force et al. 2007). Therefore, through dissection of signaling networks downstream of TKs, we expand our understanding of cardiac function and potentially contribute to novel therapeutic strategies (Chien 1999; MacLellan et al. 2000; Katz 2002).

An important molecular mechanism for adding complexity and specificity to TK signaling utilizes the docking protein ShcA. This broadly expressed protein is one of 4 Shc family members and is expressed in the forms of 66, 52 and 46 kDa polypeptides capable of binding to phosphotyrosine (pTyr)–containing motifs on activated TKs both through an N-terminal pTyr binding (PTB) domain and a C-terminal Src Homology 2 (SH2) domain (Pelicci et al. 1996; Migliaccio et al. 1997; Ravichandran 2001; Jones et al. 2007). Once recruited to activated TKs through such pTyr recognition domains, ShcA can itself undergo tyrosine phosphorylation in the central CH1 (collagen homology 1) region, thereby stimulating the activation of specific cytoplasmic signaling pathways. Notably, phosphorylation of the tyrosine residues 239/240 and 313 in the CH1 region creates two consensus pY-X-N motifs that bind the
SH2 domain of the Grb2 adaptor, leading to stimulation of the Erk-MAP Kinase (MAPK) and phosphatidylinositol 3’kinase (PI3K) pathways (McGlade et al. 1992; van der Geer et al. 1996; Walk et al. 1998; Ravichandran 2001). Protein-protein interactions mediated by phosphorylation of the CH1 tyrosines of ShcA has been shown to be important in the development of a functional monosynaptic stretch reflex circuit (Hardy et al. 2007) and ErbB2-induced breast cancer in the mouse (Ursini-Siegel et al. 2008).

The first biological role for ShcA was demonstrated by Lai and Pawson in 2000. Surprisingly, mice homozygous for the ShcA null allele are embryonic lethal at E12.5, and displayed decreased trabeculation, hypoplastic endocardial cushions and poorly developed vasculature. Subsequent studies demonstrated that ShcA is required specifically in the developing myocardium and that embryonic cardiovascular development requires ShcA PTB domain recruitment to activated TKs; however, downstream signaling through the CH1 pTyr sites were not essential (Hardy et al. 2007). While p66 ShcA has been shown to be important in mediating protective cardiovascular effects (Cosentino et al. 2008), it is unknown how p52 and p46 ShcA impact on cardiac structure and function in the postnatal myocardium.

The observation that ShcA is pivotal in the developing heart led us to investigate the role of ShcA in the postnatal myocardium. To this end, we conditionally excised ShcA in ventricular cardiomyocytes, whereby the loss of ShcA in the myocardium results in a dilated cardiomyopathy phenotype that evolves over the course of one year. The remodeling process that results in eccentric remodeling, suggests ShcA signaling is uniquely required to maintain cardiac structure and function in the post-mitotic myocardium, in the absence of ultrastructural changes or exaggerated fibrosis.

3.2 Results

3.2.1 Generation of ventricular cardiomyocyte - specific ShcA null mice

To generate ventricular cardiomyocyte-specific ShcA null mice we employed a Cre/loxP strategy by intercrossing mice with a ShcA floxed (ShcA^{flx}) allele (Hardy et al. 2007) with mice possessing the myosin light chain 2v (Mlc2v) Cre KI (Mlc2v^{KI/wt}) allele (Chen et al. 1998). The
ShcA floxed allele has a triple flag-tagged ShcA cDNA (ShcA minigene) fused with exon 3 of the endogenous ShcA gene. LoxP sites 5’ of the first coding exon and 3’ of the polyadenylation site (pA) of the ShcA minigene were used for conditional excision by the Cre recombinase (Figure 3.1A). Upon Cre mediated

**Figure 3.1 Ventricular cardiomyocyte-specific deletion of ShcA.**

(A) Schematics of ShcA floxed and deleted (CKO) alleles. (B) PCR of genomic DNA showing recombination of the floxed allele in the heart of ShcA CKO mice only (C) Timeline of ShcA excision in the heart as seen by western blot analysis of protein levels at the timepoints indicated. Heart lysates collected at 12 weeks of age showed significant reduction in the levels of ShcA protein suggesting excision of the ShcA gene by Cre recombinases (D) Lysates from partially purified cardiomyocytes demonstrated effective recombination as ShcA protein levels were
significantly reduced. (E) Mlc2v Cre is specific to cardiomyocytes as lysates prepared from spleen and lung showed no reduction in ShcA protein levels in the presence or absence of Mlc2v Cre. PND, postnatal day; WK, week
recombination, the first coding exon, the intervening ShcA cDNA and exon 3 are excised leaving a Neo\(^r\) cassette in-frame, thus ensuring a functionally inactive allele (Figure 3.1A and B). Mating of ShcA\(^{flx/flx}\) Mlc2v\(_{wt/wt}\) and ShcA\(^{flx/wt}\) Mlc2v\(_{Kl/wt}\) mice yielded the expected 1:1:1:1 Mendelian ratio. As expected, ShcA\(^{flx/flx}\) Mlc2v\(_{Kl/wt}\) mice (designated ShcA CKO) showed selective deletion of ShcA in ventricular cardiomyocytes (Chen et al. 1998). ShcA\(^{flx/flx}\) Mlc2v\(_{wt/wt}\) mice were employed as littermate controls (Table 3.1) and were indistinguishable from ShcA\(^{flx/wt}\) Mlc2v\(_{Kl/wt}\) mice (LVEDD: 4.00± 0.11 mm and % FS: 44.89± 2.38; n=4 ) at 6 months of age, as previously shown (Chen et al. 1998; Peng et al. 2006).

Mlc2v Cre recombinase-mediated excision of ShcA, as inferred by the level of ShcA protein, was detected at a minimal level at 2 weeks of age as previously reported (Chen et al. 1998; Peng et al. 2006), while excision markedly increased by 12 weeks of age (Figure 3.1C), consistent with previous studies (Peng et al. 2006). ShcA CKO ventricular lysates at 12 weeks of age contained some residual ShcA protein, likely from non- myocyte sources (fibroblasts, smooth muscle cells and endothelial cells), since robust loss of ShcA protein levels was observed in partially purified left ventricle cardiomyocytes at similar timepoints (Figure 3.1D). Lysates from spleen and lung confirmed the specificity of excision, as ShcA levels were comparable to control lysates in these tissues (Figure 3.1E).

3.2.2 ShcA is required for the maintenance of cardiac structure and function

Homozygous ShcA\(^{-/-}\) mice die at embryonic day (E) 11.5 (Lai et al. 2000), while mice with germline ablation of the p66 ShcA isoform are long lived (Migliaccio et al. 1999). In contrast, ShcA CKO mice survive embryonic development, but demonstrate significant cardiac dysfunction with age. At 6 weeks of age, echocardiography data showed no significant difference in cardiac dimensions (LVEDD (n>6): 3.79± 0.05 mm for ShcA Con vs. 3.92 ± 0.05 mm for ShcA CKO), or cardiac contractility as measured by fractional shortening of the left ventricle (%FS (n=6): 48.30 ± 1.17 for ShcA Con vs. 45.49 ± 1.02 for ShcA CKO). In addition, the heart weight-to-body weight (HW/BW) ratio for ShcA Con mice (5.30±0.25) was not different from ShcA CKO animals (5.00±0.15) (n>7). However, at 12 weeks, ShcA CKO mice developed decreased fractional shortening and distended chamber morphology without evidence
of concentric hypertrophy (Figure 3.2A and B, Table 3.1). This cardiac dysfunction combined with the absence of antecedent cardiac

**Figure 3.2 ShcA is required for the maintenance of cardiac function.**

(A) Echocardiography time course of cardiac contractility as measured by % fractional shortening showing a decline in function of ShcA CKO hearts over the course of 1 year compared to controls. (B) Echocardiography time course of chamber morphology as measured by left ventricular end diastolic dimensions (mm) showing a progressive increase in chamber dimension over the course of 1 year in ShcA CKO hearts compared to controls. See Supplemental Table I for complete data. (C) Representative histology of transverse heart sections stained with hemotoxylin and eosin at 1 year of age; ShcA CKO hearts show enlarged chamber dimensions compared to littermate controls. 6.25 x magnification (D) Isolated cardiomyocyte dimensions of ShcA CKO and control mice. The mean ± SEM of 3 independent experiments are shown. (E) Quantitative real time RT PCR of heart failure markers showing an increase in mRNA expression of atrial naturetic factor (ANF) and Skeletal Actin (Sk Actin) in ShcA CKO mice compared to controls at 3 months of age (n=4). Data was normalized to GAPDH internal control and expressed as a fold change (arbitrary units, AU) relative to control mRNA expression. No difference was detected in mRNA expression levels of Sarcoplasmic reticulum ATPase pump 2A (SERCA), brain naturetic peptide (BNP) and beta –myosin heavy chain (B-MHC). (F) Hemodynamic measurements of ShcA CKO (n=5) and control mice (n=5) at 20minutes after 3.0 mg/kg body weight IP injection of dobutamine at 3 months of age. Values expressed as mean ±SEM of the percent change from baseline measurements. dP/dT max, maximal first derivative of LV pressure development; dP/dT min, minial first derivative of LV pressure development. HR, heart rate; bpm, beats per minute. *P<0.05 compared to littermate control values for the given timepoint.
Figure 3.2. ShcA is required for the maintenance of cardiac function
**Table 3.1. Echocardiography timecourse data.**

<table>
<thead>
<tr>
<th></th>
<th>6 weeks Con</th>
<th>6 weeks ShcA CKO</th>
<th>12 weeks Con</th>
<th>12 weeks ShcA CKO</th>
<th>6 months Con</th>
<th>6 months ShcA CKO</th>
<th>1 Year Con</th>
<th>1 Year ShcA CKO</th>
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<tr>
<td>HR (bpm)</td>
<td>473.2±4.02</td>
<td>484.2±11.8</td>
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<td>509.4±8.4</td>
<td>571.9±19.9</td>
<td>526.2±9.7</td>
<td>530.5±10.04</td>
<td>506.6±13.8</td>
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<td>LVEDD (mm)</td>
<td>3.78±0.05</td>
<td>3.93±0.06</td>
<td>3.91±0.06</td>
<td>4.25±0.05*</td>
<td>3.97±0.07</td>
<td>4.41±0.09*</td>
<td>4.02±0.1</td>
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<td>LVESD (mm)</td>
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<td>2.17±0.06</td>
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<td>2.78±0.09*</td>
<td>2.17±0.14</td>
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<td>% FS</td>
<td>49.16±1.44</td>
<td>45.52±1.4</td>
<td>44.65±0.87</td>
<td>39.23±0.89*</td>
<td>47.12±2.6</td>
<td>36.4±1.33*</td>
<td>46.7±2.3</td>
<td>27.8±1.7*</td>
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<td>VCF</td>
<td>0.98±0.022</td>
<td>0.913±0.056</td>
<td>0.96±0.03</td>
<td>0.84±0.0*</td>
<td>1.105±0.07</td>
<td>0.814±0.04*</td>
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<td>0.619±0.047*</td>
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<td>Ant wall (mm)</td>
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<td>Post wall (mm)</td>
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</table>

LVEDD, left ventricle end diastolic dimension; LVESD, left ventricle end systolic dimension; %FS, %fractional shortening; VCF, velocity of circumferential shortening. * p<0.05 from littermate control for given timepoint.
hypertrophy at 6-8 weeks of age, suggests that ShcA CKO mice enter a dilated cardiomyopathy phenotype in parallel with the kinetics of maximal ShcA excision in adult cardiomyocytes. Indeed, isolated cardiomyocytes from ShcA CKO mice at 12 weeks of age had increased cell lengths indicating eccentric remodeling (Figure 3.2D), while ShcA CKO hearts had elevations in atrial natriuretic factor (ANF) and skeletal actin transcripts (Figure 3.2E), two genetic markers of cardiac pathology. Nevertheless, responsiveness to the adrenergic agonist dobutamine was unaltered in ShcA CKO mice compared to controls (Figure 3.2F). While ShcA has been reported to be critical in MAPK activation, no significant changes in phospho-Erk, indicative of Erk activation, were noted at 6 months (Figure 3.3A). This could be due to the heterogeneous cell population within the myocardium and the multiple converging signals leading to MAPK activation (Bueno et al. 2002).

By one year of age, ShcA CKO mice had a further decline in cardiac function with coincident enlargement of the left ventricle chamber dimensions (Figure 3.2A, B and C, Table 3.1) in conjunction with an increase in HW/BW ratios (Figure 3.3B). Despite severe ventricle dilation accompanied by a trend of increasing lung weight-to-body weight ratios (Figure 3.3B), electron microscopy revealed no evidence of myofibrillar disarray or aberrant intercalated disc structure or mitochondrial abnormalities (swollen or disrupted cisternae) (Figure 3.3C). Masson trichrome staining revealed only minimal myocardial interstitial fibrosis in ShcA CKO mice at 1 year of age (Figure 3.3D). As interstitial fibrosis and myocardial ultrastructure changes are typically present in advanced remodeling states in association with myocyte loss (Sabbah et al. 2000), these studies suggest that the loss of ShcA impacts on factors leading to cardiac dilation without promoting myocyte loss or interstitial fibrosis.
Figure 3.3 ShcA CKO mice undergo eccentric remodeling with preserved cyto-ultrastructure and minimal fibrosis.

(A) Representative western blot detection of pErk in heart lysates at 6 months of age. pErk levels were similar between ShcA CKO and control hearts. (B) Gravimetric data showing increased heart weight (HW) to body weight (BW) in ShcA CKO mice (n=5) compared to controls (n=5). Lung weight (LungW) to body weight ratios showed an increasing trend in ShcA CKO mice compared to controls. (C) Transmission electron microscope showing preserved ultrastructure and normal mitochondrial morphology at 1 year of age in ShcA CKO and littermate controls. Scale=2 μm. (D) Histological analysis of masson trichome stained transverse sections showing minimal interstitial fibrosis with peri-venous fibrosis in She CKO hearts compared to littermate controls at 1 year of age. Magnification for upper panel 6.25x, lower panel 20x. *P< 0.05 from littermate controls.
Figure 3.3. ShcA CKO mice undergo eccentric remodeling with preserved cyto-ultrastructure and minimal fibrosis.
3.3 Discussion

Our study demonstrates that the loss of ShcA in the ventricular cardiomyocytes leads to a dilated cardiomyopathy that evolves over the course of one year. The dilated cardiomyopathy is characterized by progressive chamber dilation mediated by eccentric remodeling without excessive deposition of interstitial fibrosis or ultrastructural changes. This study extends our understanding of the biological requirement for ShcA, which was previously established for embryonic heart development, neuromuscular development and oncogenic signaling (Lai et al. 2000; Hardy et al. 2007; Ursini-Siegel et al. 2008), and demonstrates that ShcA is essential in the postnatal myocardium.

This study also highlights the important and predominant role of the p52 ShcA and p46 ShcA isoforms in maintaining cardiac structure and function. Germline ablation of the p66 ShcA isoform results on a small cardiac chamber size at baseline and results in protection against angiotensin II- induced ventricular remodeling (Graiani et al. 2005); however, these studies demonstrate that the loss of all 3 isoforms results in a dilated heart. Therefore, from these data, one can infer that p52 and p46 ShcA are critical in the maintenance of cardiac structure and function, independent of the protective properties resulting from a loss of p66 ShcA.

While in vitro data has shown ShcA to be central to Erk-MAPK activation in response to mitogenic stimuli, we did not see a loss of Erk activation. Other signal transduction pathways important in cardiac maintenance and hypertrophy were also interrogated, but did not show a significant effects attributed to the loss of ShcA. This could be due to the heterogenous cell type composition within the myocardium as well as multiple converging inputs regulating the dynamic nature of cardiac function. Critical to advancing the role of ShcA in the myocardium requires identification of downstream signaling targets important in modulating cardiac structure and function.

These studies highlight the essential role of ShcA signaling within the postnatal myocardium, but also set the foundation to investigate the mechanism by which the loss of ShcA leads to a dilated cardiomyopathy, and how the loss of ShcA in cardiomyocytes affects their ability to contract. Lastly, as ShcA is a modular protein, it will be of great interest to understand
how the individual domains and signaling motifs contribute to the dilated cardiomyopathy phenotype seen in the ShcA CKO mouse.
Chapter 4

The loss of ShcA in the myocardium uncouples single myocyte function and global heart function

A version of this appeared in:

The ShcA phosphotyrosine docking protein uses distinct mechanisms to regulate myocyte and global heart function. (Submitted manuscript)

Attributions:

Cardiac Surgical Operations were done in collaboration with Dr.M. Golum Kabir at the Heart and Stroke/Richard Lewar Centre of Excellence.
The loss of ShcA in the myocardium uncouples single myocyte function and global heart function

4.1 Introduction

The loss of ShcA in the myocardium leads to a dilated cardiomyopathy phenotype; however, the mechanism by which the loss of ShcA precipitates the phenotype is unclear. Dilated cardiomyopathies are associated with early functional changes that encompass systolic dysfunction, diastolic dysfunction, cytoskeletal deficiencies and aberrant electromechanical coupling (Backx et al. 1995; Giordano et al. 2001; Houser et al. 2003; Ji et al. 2004). The loss of ShcA in the cardiomyocyte population obligates investigations of cellular and tissue function to gain insight into the complex nature of the dilated cardiomyopathy phenotype.

Given the chamber dilation and depressed systolic function seen at the whole heart level, we investigated the effect of ShcA deficiency on baseline cardiomyocyte contractility with respects to calcium handling and myofilament interactions. Cardiomyocyte contractility is regulated by excitation contraction coupling (ECC) (Figure 4.1), whereby depolarization of the cardiomyocyte triggers a series of ion movements that culminate in a rise in intracellular calcium, allowing for cross bridge formation and subsequent force generation. When an action potential causes depolarization of the myocyte membrane, voltage gated calcium channels (dihydropyridine receptor, \( \text{Ca}^{2+} \)) have an increased probability of opening, allowing for a local rise in calcium (\( \sim 10-20 \mu\text{M} \)). This rise of calcium triggers a large efflux of calcium from the juxtaposed sarcoplasmic reticulum (SR) via the ryanodine receptor (RyR), in a process termed calcium induced calcium release (CICR) (Bers 2002). The rapid release of SR calcium (\( \sim 300 \text{mM} \)) removes the inhibition imposed by the troponin-tropomyosin complex to initiate myofilament crossbridge formation and the subsequent generation of force (Figure 4.1A and B). Relaxation of force requires the calcium to be pumped back into the SR by the SR ATPase pump, Serca2a, whose function is gated by phosphorylation of phospholamban (PLN) (Bers 2008).
Figure 4.1 Excitation Contraction Coupling in Cardiomyocytes.

(A) Calcium entry into the cardiomyocyte initiates CICR (Section 4.1). (B) With increased calcium in systole, calcium is available to bind to TnC and induce a conformation change within the Tm-Tn complex and allows for myosin binding to actin and subsequent force generation. (C) Temporal relation of calcium and force development and the relationship of force and calcium in the cardiomyocyte.
Myofilaments are the end effectors of excitation contraction coupling. The large rise in intracellular calcium initiated by the AP, allows for calcium to bind to various components of the myofilament and initiate contraction. The myofilaments within the cardiac sarcomere are composed of 2 main filaments, myosin (thick filaments) and actin and its associated proteins, tropomysin (Tm) and hetero-trimeric troponin (Tn) (thin filaments).

The thin filaments are the main site for Ca$^{2+}$ regulation of contraction. Actin is the backbone thin filament and is comprised of monomeric G (globular)-actin that polymerizes to form F( filamentous)-actin, two of which associate to form a double standed structure. Actin contains sites for myosin head binding that initiate contractile force (Gordon et al. 2000). Actomyosin binding is regulated in part by Tm and the Tn complex. Tm is coiled coil molecule that spans 7 monomeric actin molecules in F-actin and overlaps with its neigbouring Tm in a head to tail configuration (Gordon et al. 2000). Tm is expressed from 2 genes, α and β, and cardiac Tm is predominantly α,α (tobacman, ls, 1996). The hetero-trimeric Tn complex triangulates with Tm, actin and Tn subunits to regulate contraction. Tn subunits consist of Tn-C, which binds Ca$^{2+}$, Tn-I, which binds actin to inhibit crossbridge formation, and Tn-T, which links Tn to Tm (Salaro 1995; Gordon et al. 2000). Cardiac Tn-C contains 4 calcium binding sites, 2 high affinity (for which Ca$^+$ is always bound) and 1 low affinity sites (Gordon et al. 2000).

Myosin is composed of two heavy chains and 4 light chains. The two heavy chains associate as a coiled coil structure for most of their length (rod section), and after the hinge region (S2 fragment), there is a projecting N-terminal globular head termed subfragment 1 (S1 fragment) (Gordon et al. 2000). The S1 fragment contains the actin binding domain and the ATP hydrolysis site and also associates with a pair of light chain molecules. The rod section of myosin associates with C protein, whereby altered phosphorylation of C protein can modify thick filament structure and ultimately contraction (Gordon et al. 2000). Titin, the giant elastic protein, interacts with myosin , C protein and actin and is thought to generate passive tension in the sarcomere and dictate interfilament lattice spacing (Granzier et al. 2005).

In diastole, intracellular calcium is low and Tn-I exerts an inhibitory effect such that Tn-T is associated with Tm and prevents actomyosin crossbridges. In systole, when Ca$^{2+}$ binds the low affinity site on Tn-C, it induces a conformational change, such that Tn-C and Tn-I associate
more closely, thus weakening Tn-I-Tn-T interactions. This removes the constraint on Tm that causes it to shift, no longer blocking the actomyosin crossbridge sites. This increases the probability of the actin sites to be occupied by myosin heads in a three state model of thin filament activation (Solaro 1995; Gordon et al. 2000) (Figure 4.1B). The degree by which myofilaments generate force at a given calcium concentration is the calcium sensitivity, and this is represented by the concentration of calcium required to generate half maximal force generation when interrogating the force-calcium relationship (Figure 4.1C). Alteration in calcium sensitivity is associated with myofilament isoform shifts and altered phosphorylation of key residues of various myofilaments and is also implicated in cardiomyopathies (Willott et al.; Solaro 2008).

While factors intrinsic to the cardiomyocyte, such as ECC, can affect global cardiac function. There are also properties of the myocardium, extrinsic to the myocyte that also influence cardiac performance. The main properties are electrical synchronisity of AP,propogation and the mechanical properties of the tissue, such as the properties contributed by the extracellular matrix. Action potential depolarization initiates in the sino-atrial node and propogates through the atria to the atrio-ventricular node. From here, AP depolarization spreads through the Bundle of His, the purkinje fibres and then through the ventricles in a stereotypic pattern to ensure the heart functions as a syncytium. Abberent propagation of depolarization or repolarization result in cardiac dysfunction, as effective contraction is compromised (Lehnart et al. 2007).

Another mechanism by which the heart is coupled is through the extracellular matrix. The extracellular matrix is a heterogenous microenvironment that contains structural molecules, such as collagen, matricellular molecules, such as osteoponin and proteoglycans, and also serves as a reservoir for signaling molecules, such as HB-EGF (Spinale 2007). Deficiencies in many aspect of the ECM can potentially contribute to cardiac demise (see Table 4.1). For example, collagen, a major ECM structural molecule, when disrupted can rapidly induce cardiac dilation and dysfunction (Spinale 2007). Collagen fibrils encase myocytes (endomysial fibres) and perimysial fibres run along myocytes, typically spanning 4-5 myocytes (Streeter et al. 1969). It is thought that the uncoiling of the perimysial collagen fibres contributes to the stiffness or mechanical diastolic properties of the myocardium (MacKenna et al. 1996; MacKenna et al. 1997; Matsubara et al. 2000), and it is the loss of these fibres that results in cardiac dilation and
increased distensibility seen in heart failure (Caulfield et al. 1992). Alterations in collagen mechanical properties can also be affected by changes in collagen crosslinking (Avendano et al. 1999; Herrmann et al. 2003). Indeed, turnover of collagen by matrix metalloproteinases (MMPs), tissue zinc-dependant proteases, have been implicated in various disease models (Spinale 2007). While the mechanical contributions of other components of the ECM are still emerging (Fomovsky et al.), the literature has demonstrated an important and dynamic role for the ECM in regulating cardiac structure and function.

This chapter addresses both the cell autonomous and non cell autonomous effects of ShcA deficiency on cardiomyocytes and myocardial mechanical tissue properties, respectively, to provide insight into the molecular defects contributing to the dilated cardiomyopathy phenotype seen in the ShcA CKO mouse. Surprisingly, cardiomyocyte function was enhanced in ShcA CKO mice compared to controls, suggesting global systolic dysfunction is not attributed to poor cardiomyocyte function. Rather, ShcA CKO mice have deficiencies in myocyte/matrix interactions that lead to mechanical uncoupling, that precipitate the dilated cardiomyopathy phenotype.
Table 4.1. ECM components that contribute to mechanical integrity of the myocardium
<table>
<thead>
<tr>
<th>Gene Deletion</th>
<th>Enzymatic substrate or endproduct (Spinale 2007)</th>
<th>Phenotype in animal model</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP 2</td>
<td>denatured fibrillar collagen, basement membrane proteins, proteoglycans</td>
<td>Reduced dilation post MI and pressure overload (Matsumura et al. 2005) Over expression: DCM with age (Bergman et al. 2007)</td>
</tr>
<tr>
<td>MMP 3</td>
<td>All basement membrane proteins, elastin and proteoglycans</td>
<td>Impaired MI scar maturation (Morita et al. 2006)</td>
</tr>
<tr>
<td>MMP 7</td>
<td>Fibrillar collagen (I and III)</td>
<td>Increased post MI survival (Lindsey et al. 2006)</td>
</tr>
<tr>
<td>MMP 9</td>
<td>denatured fibrillar collagen, basement membrane proteins, proteoglycans</td>
<td>Attenuates contractile dysfunction in HF (Moshal et al. 2008) Reduced LV enlargement post MI (Ducharme et al. 2000; Romanic et al. 2002)</td>
</tr>
<tr>
<td>MMP13</td>
<td>Fibrillar collagen and ECM proteins</td>
<td>No ablation study to date</td>
</tr>
<tr>
<td>MMP 14 (MT1)</td>
<td>Diverse array of collagens and proteins</td>
<td>Non cardiac phenotype</td>
</tr>
<tr>
<td>Tissue inhibitor of metalloproteinase 1</td>
<td>Increased LV dilation and dysfunction post MI (Ikonomidis et al. 2005)</td>
<td></td>
</tr>
<tr>
<td>Tissue inhibitor of metalloproteinase 3</td>
<td>LV dilation with age (Fedak et al. 2004)</td>
<td></td>
</tr>
<tr>
<td>Osteopontin</td>
<td></td>
<td>LV dilation and dysfunction post MI (Trueblood et al. 2001)</td>
</tr>
<tr>
<td>Thrombospondin</td>
<td></td>
<td>Cardiac rupture after MI and increased load (Schroen et al. 2004)</td>
</tr>
<tr>
<td>Hyaluronic acid synthase 2</td>
<td>Synthesis of hyaluronic acid</td>
<td>Failure to form endocardial cushions in embryonic heart development (Camenisch et al. 2000; Camenisch et al. 2002)</td>
</tr>
</tbody>
</table>
4.2 Results

4.2.1 The loss of ShcA signaling results in enhanced single myocyte contractility

To address whether the dilated cardiomyopathy phenotype resulting from ShcA deficiency is associated with changes in baseline cardiomyocyte function, we measured isolated single myocyte sarcomere length shortening in 12 week old mice. Surprisingly, despite a global reduction of systolic function, ShcA CKO cardiomyocytes had enhanced contractility compared to controls (8.85±0.51% vs. 6.78±1.16% respectively; p<0.05; Table 4.2). Calcium transient experiments demonstrated no significant change in amplitude between ShcA CKO and littermate controls (Table 4.2), suggesting altered calcium sensitivity of the myofilament (Kobayashi et al. 2005; Solaro 2008). Indeed, the calcium sensitivity of ShcA CKO myocytes was enhanced, but was not obviously associated with a decrease in serine 23/24 phosphorylation of Troponin I (Table 4.2, data not shown). These results demonstrate that the enhanced contractility seen at baseline in ShcA CKO mice is due to changes in myofilament properties that are regulated by ShcA signaling.

As the elevation in contractility could be a compensatory mechanism resulting from developmental defects mediated by the variable excision of ShcA in the postnatal period by the Mlc2v Cre recombinase KI mouse (Chen et al. 1998; Peng et al. 2006), we utilized the tamoxifen inducible Cre transgenic mouse (MerCreMer) driven by the alpha MHC promoter to acutely excise ShcA (Sohal et al. 2001; Andersson et al. 2009). ShcA MCKO (ShcA^{flx/flx} MerCreMer^{+/wt}) and their littermate controls, ShcA MCON (ShcA^{flx/flx} MerCreMer^{wt/wt} and ShcA^{flx/wt} MerCreMer^{+/wt}), were injected with tamoxifen for 5 days. ShcA MCKO mice showed no evidence of chamber dilation or depressed systolic dysfunction 7 days post injection (Table 4.4); however, 7 days after the tamoxifen protocol, ShcA MCKO cardiomyocytes showed elevated baseline contractility compared to controls (6.42±0.34% vs. 7.76±0.36%, respectively; n=5 hearts with >25 cells, p=0.016). The single myocyte data suggest that the defect in isolated...
myocyte function is a cell autonomous effect due to the loss of ShcA that can be uncoupled from whole heart function.
### Table 4.2. Single myocyte assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>Parameter</th>
<th>Control</th>
<th>ShcA CKO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Contractility</strong></td>
<td>% <em>Sarcomere shortening</em></td>
<td>6.90±0.58</td>
<td>8.85±0.30*</td>
</tr>
<tr>
<td></td>
<td><em>dSL/dT max (µm/sec)</em></td>
<td>3.57±0.16</td>
<td>4.93±0.34*</td>
</tr>
<tr>
<td></td>
<td><em>dSL/dT min (µm/sec)</em></td>
<td>2.65±0.22</td>
<td>3.73±0.31*</td>
</tr>
<tr>
<td></td>
<td><em>TR&lt;sub&gt;50&lt;/sub&gt; (sec)</em></td>
<td>0.032±0.005</td>
<td>0.033±0.002</td>
</tr>
<tr>
<td></td>
<td><em>TR&lt;sub&gt;80&lt;/sub&gt; (sec)</em></td>
<td>0.052±0.006</td>
<td>0.064±0.005</td>
</tr>
<tr>
<td><strong>Ca&lt;sup&gt;2+&lt;/sup&gt; Transients</strong></td>
<td><em>Amplitude (F405/485nm)</em></td>
<td>0.323±0.018</td>
<td>0.335±0.019</td>
</tr>
<tr>
<td><strong>Force Ca&lt;sup&gt;2+&lt;/sup&gt; Curve</strong></td>
<td><em>F&lt;sub&gt;max&lt;/sub&gt; (mN/mm&lt;sup&gt;2&lt;/sup&gt;)</em></td>
<td>18.04±0.55</td>
<td>19.81±1.015</td>
</tr>
<tr>
<td></td>
<td><em>EC50 (µmoles/L)</em></td>
<td>2.46±0.08</td>
<td>2.18±0.13*</td>
</tr>
<tr>
<td></td>
<td><em>Hill Coefficient</em></td>
<td>3.57±0.34</td>
<td>3.74±0.64</td>
</tr>
</tbody>
</table>

Contractility and transient assays have a minimum of 3 hearts per group with a minimum of 15 cells. Force-Ca<sup>2+</sup> experiments have a minimum of 6 hearts per group. dSL/dT max, maximum first derivative of sarcomere shortening; dSL/dTmin, minimum first derivative of sarcomere shortening; TR<sub>50</sub>, time to 50% relaxation; TR<sub>80</sub>, time to 80% relaxation. F, Fluorescence of calcium transient; F<sub>max</sub>, maximal force; EC50, Ca<sup>2+</sup> concentration at 50% maximal force. * p<0.05 from littermate controls at given time point.
4.2.2 The loss of ShcA leads to deregulation of the ECM components in the heart

The presence of elevated single myocyte contractility despite decreased global systolic function suggests a mechanical uncoupling within the myocardium; therefore, we investigated whether the chamber dilation in ShcA CKO mice results from impaired ECM-myocyte interactions. To this end, we examined extracellular matrix (ECM) properties by measuring the force-sarcomere length relationship in isolated ventricular papillary preparations and by examining perimysial connective tissue surrounding individual myocytes at early time points of disease. In response to sarcomere length stretch, ShcA CKO papillary muscles had reductions in passive tension due to higher compliance (p<0.001) compared to controls (compliance parameter (t) = 0.42 vs. 0.20, respectively; Figure 4.2A), consistent with a disrupted ECM (Granzier et al. 1995; MacKenna et al. 1996; Fedak et al. 2003; Spinale 2007). As papillary muscles can be heterogeneous in shape leading to a lack of uniformity of sarcomere length, a small cohort of ultrathin trabeculae muscle preparations were also examined and gave similar results (data not shown). Consistent with the mechanical results, perimysial collagen fibres stained with picrosirius red showed decreased complexity and structural collagen content, at 12 weeks of age in ShcA CKO mice (Figure 4.2B and C). In further support of mechanical uncoupling, ShcA CKO papillary muscles also showed reduced developed tension (p<0.001), suggesting that poor force transmission could potentially contribute to the systolic dysfunction seen in the whole heart. While the cause of the altered ECM structure and function in ShcA CKO myocardium is unclear, MMP activity in six month old ShcA CKO hearts was elevated compared to controls (Figure 4.2D), suggesting activation of remodeling signaling pathways. Overall, our data supports the conclusion that, despite causing enhanced cardiomyocyte contractility, ShcA excision leads to a progressive dilated cardiomyopathy phenotype as a result of impaired maintenance of the ECM.
Figure 4.2 The loss of ShcA leads to deregulation of the ECM components in the heart.

(A) Papillary passive tension, expressed as mN/mm² (miliNewtons normalized to cross sectional area), across increasing sarcomere lengths (µm) as described in Methods. All data for individual papillary muscles (≥5 independent experiments per group) were plotted and fitted using a exponential equation \(y=e(x-xo)/t +a\), where \(x=\text{sarcomere length (µm)}\), \(xo = \text{average resting tension, a= measurement offset, t= compliance constant}\). A significant reduction in ShcA CKO compliance (\(t=0.42\)) was demonstrated compared to control (\(t=0.20\); \(p<0.001\)). (B) Visualization of perimysial collagen fibres stained with Picrosirius Red using Optigrid structured illumination microscopy. ShcA CKO hearts demonstrated a decreased complexity of collagen staining compared to controls (63x Magnification). (C) Collagen area was quantified from multiple images from each heart and demonstrated a decrease in % collagen area in the ShcA CKO hearts compared to controls (\(n=3\)). (D) Zymography of hearts from ShcA CKO (\(n=3\)) and their littermate controls (\(n=3\)) at 6 months demonstrate ShcA CKO mice have increased MMP activity as the ventricle chambers dilate. Increased activity was noted for proMMP9 and an unidentified MMP species at ~200kDa. The positive control sample was supernatant from mouse embryonic fibroblasts stimulated with concanavalin A for 24hrs. Values are means ± SEM with *\(p<0.05\) compared to littermate controls for given time point.
Figure 4.2. The loss of ShcA leads to deregulation of the ECM components in the heart.
4.2.3 ShcA CKO mice undergo an eccentric remodeling response with transverse aortic constriction

To test the hypothesis that ShcA is critical for the maintenance of mechanical integrity of the heart through ECM-myocyte interactions, 8 week old ShcA CKO mice and their littermate controls were subjected to biomechanical stress by transverse aortic constriction (TAC). The introduction of acute mechanical stress to the hearts of ShcA CKO mice, prior to overt dilation, would be predicted to induce a rapid transition into HF. After 4 weeks of TAC, control mice mounted the expected concentric hypertrophy response (Figure 4.3A, Table 4.3), accompanied by multifocal interstitial fibrosis (Figure 4.3B) and preserved overall heart function (Figure 4.3A), Table 4.3). ShcA CKO mice, in contrast, showed a rapid decline in cardiac function that quickly transitioned into congestive HF after the 4 weeks of TAC, characterized by severe chamber enlargement, depressed fractional shortening, minimal ventricular wall thickening, and elevated lung weights in ShcA CKO mice (Figure 4.3A and B, Table 4.3 and 4.5). Despite marked chamber enlargement and impaired cardiac function, ShcA CKO mice had a similar level of interstitial fibrosis compared to controls after TAC (Figure 4.3B). These changes in ShcA CKO hearts were accompanied by elevation of matrix-metalloproteinase 2 (MMP2) activity at 1 week TAC and subtle depressions in pErk signaling (Figure 4.3C and D).

As ShcA excision with the Mlc2v Cre recombinase KI mouse occurs slowly in postnatal development (Chen et al. 1998; Peng et al. 2006), we acutely excised ShcA using the tamoxifen inducible Cre transgenic mouse driven by the alpha MHC promoter (Sohal et al. 2001). ShcA MCKO and their littermate controls, ShcA MCON, were injected with tamoxifen for 5 days, and 7 days post injection both groups underwent the TAC procedure (n≥5). Although acute ligand mediated excision of ShcA can show some inherent variability in excision (Figure 4.3E)(Sohal et al. 2001), ShcA MCKO showed dilated chamber dimensions upon echocardiography with decreased systolic function compared to ShcA MCON mice after 4 weeks of TAC (Figure 4.3F, Table 4.4), while hypertrophy, indicated by anterior wall thickness (Table 4.4), and HW/BW ratios (5.86±0.35 vs. 4.55±0.14; n=5) were not different. These results suggest that ShcA is not initially required for mounting a hypertrophic response, but is necessary for preventing ventricular dilation.
Figure 4.3 ShcA CKO mice undergo an eccentric remodeling response with TAC.

(A) Echocardiography data shows a decline in ShcA CKO cardiac function with TAC, as demonstrated by % fractional shortening of ShcA CKO and control mice after a 4 week TAC protocol; see Table 4.3 for complete echocardiography analysis. (B) Histological analysis of transverse heart sections stained with masson trichrome showing dilation of ShcA CKO hearts compared to controls after TAC. (C) Zymography of ShcA CKO and control hearts after a 4 week TAC protocol showing enhanced MMP2 activation in ShcA CKO mice after TAC. The positive control (+) sample was supernatant from mouse embryonic fibroblasts stimulated by concanavalin A for 24hrs. (D) Western blot analysis of ShcA CKO and control hearts for pERK activation after TAC. ShcA CKO hearts after 4 weeks of TAC show subtle depressions in pERK activation compared to controls. Data shows 3 independent hearts per group. (E) Western blot analysis showing loss of ShcA protein in heart and isolated cardiomyocytes in the presence of MerCreMer transgene driven by the αMHC promoter compared to littermate controls, 7 days after tamoxifen injection protocol as explained in Methods. (F) Histological analysis of transverse sections stained with masson trichrome showing chamber dilation in ShcA MCKO hearts compared to controls after 4 weeks of TAC. See Table 4.4 for complete echocardiography study. Values are means ±SEM with ^p<0.05 compared to same genotype sham, * p<0.05 compared to littermate control values for the given time point. WK, week
Figure 4.3. ShcA CKO mice undergo an eccentric remodeling response with TAC
Table 4.3. Echocardiography and cardiac catheterization data for TAC studies

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham</th>
<th>1 wk TAC</th>
<th>4 wk TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con</td>
<td>ShcA CKO</td>
<td>Con</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>500.1±1.24</td>
<td>517.2±1.46</td>
<td>501.62±1.05</td>
</tr>
<tr>
<td>LVEDD(mm)</td>
<td>3.94±0.01</td>
<td>4.34±0.014*</td>
<td>3.85±0.01</td>
</tr>
<tr>
<td>LVESD(mm)</td>
<td>2.16±0.01</td>
<td>2.64±0.016*</td>
<td>2.07±0.016</td>
</tr>
<tr>
<td>% FS</td>
<td>45.6±0.180</td>
<td>39.5±0.24*</td>
<td>47.04±0.306</td>
</tr>
<tr>
<td>VCF (circ/sec)</td>
<td>0.94±0.004</td>
<td>0.817±0.0057*</td>
<td>0.97±0.0067</td>
</tr>
<tr>
<td>Ant wall (mm)</td>
<td>0.70±0.03</td>
<td>0.67±0.04</td>
<td>0.99±0.05^</td>
</tr>
<tr>
<td>Post wall (mm)</td>
<td>0.67±0.03</td>
<td>0.65±0.03</td>
<td>1.13±0.04^</td>
</tr>
<tr>
<td>N</td>
<td>13</td>
<td>9</td>
<td>12</td>
</tr>
</tbody>
</table>

| HR (bpm)       | 433.88±6.27 | 444.3±4.56 | 415.1±10.4 | 398.6±9.5 | 460.5±10.2 | 386.2±24.8 |
| LVESP          | 101.4±1.04  | 96.5±1.16  | 148.9±1.42^ | 147.1±1.7^* | 136.7±5.8^ | 91.5±3.0^  |
| LVEDP          | 10.88±0.73  | 8.26±0.57  | 13.6±2.16^ | 17.5±2.3^* | 21.2±1.1^  | 21.1±0.89^ |
| Dp/dt max      | 7479.3±153  | 6200.2±88.6* | 6763.6±139 | 6943.6±388 | 6664.9±354 | 3371.8±250^* |
| Dp/dt min      | -6582.9±16  | -5978.1±118* | -5695.1±228 | -5792.8±397 | -5977.7±35 | -3041±191^* |
| N              | 10          | 10         | 5         | 5        | 8        | 7        |

* p<0.05 compared to littermate control value for the given timepoint.
Table 4.4. Echocardiography data of TAC study for acute model of ShcA excision

<table>
<thead>
<tr>
<th>Parameter</th>
<th>7 days P.I. #</th>
<th>Sham</th>
<th>4 wk TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>ShcA MCON 446.7±14.56</td>
<td>ShcA MCKO 473.8±11.09</td>
<td>Sham 467.8±7.07</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>4.06±0.09</td>
<td>0.38±0.005</td>
<td>4.16±0.011</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.29±0.09</td>
<td>0.21±0.008</td>
<td>2.67±0.012</td>
</tr>
<tr>
<td>% FS</td>
<td>43.75±1.53</td>
<td>45.33±1.67</td>
<td>36.07±1.67</td>
</tr>
<tr>
<td>VCF (circ/sec)</td>
<td>0.809±0.103</td>
<td>0.819±0.039</td>
<td>0.731±0.028</td>
</tr>
<tr>
<td>Ant wall (mm)</td>
<td>0.69±0.02</td>
<td>0.73±0.02</td>
<td>0.69±0.02</td>
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<tr>
<td>Post wall (mm)</td>
<td>0.63±0.03</td>
<td>0.61±0.02</td>
<td>0.69±0.03</td>
</tr>
</tbody>
</table>

P.I., post injection; # No significant difference was noted at 7 days post injection between ShcA<sup>wt/flx</sup> MerCre/ Mer<sup>+/wt</sup> and ShcA<sup>flx/flx</sup> MerCreMer<sup>wt/wt</sup>. * <sup>p</sup> < 0.05 compared to same genotype sham; * <sup>p</sup> < 0.05 compared to littermate control value for the given timepoint.
Table 4.5. Gravimetric data for ShcA CKO and control mice after TAC

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham</th>
<th>4 wk TAC</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Con</td>
<td>ShcA CKO</td>
</tr>
<tr>
<td>BW (g)</td>
<td>30.69±0.06</td>
<td>29.80±0.79</td>
</tr>
<tr>
<td>Left Ventricle (mg)</td>
<td>100.0±4.1</td>
<td>103.2±10.1</td>
</tr>
<tr>
<td>LVW/TL (mg/mm)</td>
<td>0.611±0.025</td>
<td>0.633±0.056</td>
</tr>
<tr>
<td>LungW/TL (mg/mm)</td>
<td>1.14±0.032</td>
<td>1.09±0.077</td>
</tr>
</tbody>
</table>

BW, body weight; LVW/TL, left ventricle weight/tibial length; LungW/TL, lung weight/tibial length. ^ p<0.5 compared to same genotype sham, * p<0.05 compared to littermate control for given timepoint.

4.3 Discussion

The enhanced cardiomyocyte contractility found in ShcA deficient cardiomyocytes was surprising in the context of global cardiac dysfunction. The absence of changes to calcium handling but alterations in the calcium sensitivity suggests ShcA impacts on homeostatic myocyte contractility at the level of the myofilaments. Regulation of the force-calcium relationship occurs through phosphorylation and isoform shifts of the major myofilaments. In particular, calcium sensitivity is regulated by kinase and phosphatases, such as beta adrenergic
modulation of serine 23/24 of troponin I and other residues of troponin C and tropomyosin (de Tombe et al. 2000; Solaro et al. 2002; Solaro 2008). While little data is available regarding the role of TK signaling and contractility, genistein, a broad spectrum TK inhibitor has been shown to increase calcium sensitivity at the level of the myofilaments (Min et al. 2002). The ability for ShcA to interact with multiple phosphatases (Table 1.1 and 1.2) and influence various signal transduction pathways, suggest ShcA signaling, potentially through upstream TKs, is important in single myocyte contractile function. The acute excision of ShcA using the α MHC-MerCerMer mouse also resulted in hypercontractile cardiomyocytes suggesting that this is a cell autonomous effect of the loss of ShcA in cardiomyocytes.

The ShcA CKO mice have enhanced cardiomyocyte contractility despite whole heart cardiac dysfunction, suggesting an uncoupling of global regulators of cardiac function from myocyte function. When cardiomyocyte function is compromised global cardiac function is expected to be reduced (Backx et al. 1995; Houser et al. 2003); however, just as importantly, blood flow change as a result of altered vascular function (Giordano et al. 2001), disruption of normal propagation of electrical signals (Ji et al. 2004) and uncoupling of force transmission from the myocyte along collagen struts (Spinale 2007) can all contribute to impaired cardiac function, thereby precipitating heart failure. The absence of necrosis with fibrosis, arrhythmias or sudden death in the ShcA CKO mice suggests that cardiomyopathy in these mice did not result from myocyte loss and stimulation of fibrosis. While the activation of the MMP2 and MMP9 in ShcA-deficient mice may result from generic stimulation of a final common pathway associated with adverse cardiac remodeling (Heymans et al. 1999; Ducharme et al. 2000; Spinale 2007), the early detection of mechanical changes in the passive tension in parallel with the excision of ShcA suggests disruption of pathways involved in myocyte/myocyte or myocyte/matrix coupling. Indeed, several matrix-associated TKs have docking sites for the PTB and SH2 domains of ShcA (Mainiero et al. 1995; Vogel et al. 1997; Ravichandran 2001), so that ShcA, through reciprocal signaling between cellular compartments (Hardy et al. 2007; Ursini-Siegel et al. 2008) could potentially affect mechanical coupling through adhesion interactions, secretion of matricellular proteins or direct remodeling of the matrix (Bowers et al.; Spinale 2007). It will be of great interest to explore more deeply the underlying mechanism through which ShcA impacts on global heart structure and function. The presence of enhanced cardiomyocyte function in the context of a dilated cardiomyopathy reiterates that HF is a mosaic syndrome and requires
stratification that is based on the precipitating etiology to effectively impact on prognosis (Liew et al. 2004).

Overall, these data suggest single myocyte contractility and global cardiac function can be uncoupled, and highlight the signaling complexity underlying cardiac physiology. These data also highlight a central role for ShcA in mediating TK signaling in the myocardium. As ShcA has a modular domain structure, it will be of interest to understand the role of ShcA PTB and SH2 domains coupling to upstream TKs and of the CH1 pTyr sites in activation downstream pathways in regulating cardiac structure and function.
Chapter 5

ShcA uses distinct signaling mechanisms to regulate myocyte contractility and global heart function

A version of this appeared in:

The ShcA phosphotyrosine docking protein uses distinct mechanisms to regulate myocyte and global heart function. (Submitted manuscript)

Attributions:

Cardiac Catheterization and Transverse Aortic Constriction studies were done in collaboration with the Surgical Technician at the Heart and Stroke/Richard Lewar Centre of Excellence, Dr. M. Golum Kabir

Histology was done in collaboration with the facilities at the Samuel Lunenfeld Research Institute
5 ShcA uses distinct signaling mechanisms to regulate myocyte contractility and global heart function

5.1 Introduction

Adaptor or docking proteins are molecules containing multiple signaling domains and motifs that generally lack kinase activity (Pawson et al. 1997). The presence of multiple potential signaling mechanisms allows for the introduction of complexity to protein-protein interactions. ShcA has 2 pTyr containing recognition domains, the N terminal PTB domain and the C terminal SH2 domain (Introduction Section 3.2). Other SH2 domain containing adaptors are Grb2, Crk and Nck, while other PTB containing adaptors are the Dok and IRS family of proteins. In the myocardium, ShcA then has the potential to interact with a multitude of TKs to regulate cardiac structure and function (Table 1.1-3)

ShcA also have various signaling motifs that contribute to downstream signal propagation. In the CH2 domain, Serine 36 has been identified as critical in mediating oxidative stress signaling (Trinei et al. 2009), while the Y239/240 and Y313 of the CH1 region, when phosphorylated, generate two consensus pY-X-N motifs that bind the SH2 domain of the Grb2 adaptor, leading to stimulation of the Erk-MAP Kinase (MAPK) and phosphatidylinositol 3’kinase (PI3K) pathways (Introduction Section 4.2). Alternative ShcA signaling mechanisms include adaptin binding in the CH1 region, SH3 domain containing proteins recruited to the proline rich CH1 region, or serine/threonine phosphorylation motifs within the ShcA protein (Pelicci et al. 1992; Weng et al. 1994; Bonfini et al. 1996; Okabayashi et al. 1996). In addition, recent data suggests that IQGAP links ShcA to the actin cytoskeleton through a non-canonical interaction with the PTB domain (Smith et al.). Therefore, ShcA has several alternative downstream signaling mechanisms that could be utilized to impact on cardiac function.

To understand how ShcA mediates it effects in the myocardium, we used the ShcA mutant allele series (Hardy et al. 2007), which is comprised of ShcA knock-in (KI) alleles each containing discrete point mutations that allowed us to evaluate the functional roles of ShcA PTB and SH2 domains in coupling to upstream TKs in the postnatal heart and of the CH1 pTyr sites in activating downstream pathways. Analysis of specific KI mutants indicate that PTB domain
inactivation produced similar cardiac dysfunction found in ShcA CKO mice, indicating that
PTB-pTyr interactions are essential for ShcA function in the myocardium, while the SH2 domain
has a more modest role in hypertrophy signaling in response to biomechanical stress and aging.
In contrast, the CH1 pTyr sites are dispensable in maintaining normal cardiac structure and
function, but are required for preventing cardiac dysfunction induced by biomechanical stress.
Our results therefore indicate that ShcA is a critical hub protein that transmits pTyr-dependant
signals to control adult myocardial function and response to stress through a variety of distinct
molecular mechanisms.

5.2 Results

5.2.1 Generation of mice with ventricular specific ShcA point mutations

Given its modular domain architecture, ShcA can potentially signal downstream of multiple
tyrosine kinases important in both cardiac maintenance and the disease model of pressure
overload (Ravichandran 2001). Therefore, to evaluate the individual contributions of the various
domains and signaling motifs of ShcA, we utilized a ShcA mutant allelic series (Hardy et al.
2007) to generate mice in which the KI mutations (δKI) are restricted to cardiomyocytes. To test
the importance of the PTB or SH2 domains of ShcA in recognizing pTyr motifs of activated TKs
in the postnatal myocardium, mutant PTB and SH2 alleles were evaluated with respect to cardiac
structure and function. To understand signaling mechanism downstream of ShcA in the post
mitotic myocardium, we evaluated CH1 pTyr signals in cardiac structure and function using the
phospho-null 3F allele (see below).

Mice with ShcA KI alleles containing individual point mutations were intercrossed with
ShcA^{flx/flx} Mlc2v^{KI/wt} mice to give ShcA^{δKI/flx} Mlc2v^{KI/wt}. In non-cardiac tissue, the KI mutant
allele yields no phenotype owing to expression of the fully functional floxed allele. However,
Cre recombinase excision of the floxed allele in ventricular cardiomyocytes unmasksthe
properties of the mutated allele (cardiac specific KI (CKI))(Figure 13A)(Hardy et al. 2007). The
ShcA KI alleles have a single flag tagged ShcA cDNA fused with exon 3, each with discrete
point mutations (δKI). The individual point mutations preclude 1) pTyr binding of the PTB
domain to
Figure 5.1 Schematic of ShcA allele strategy to generate ventricle cardiomyocyte specific ShcA KI mutants.

(A) Breeding strategy to generate mice containing one mutant ShcA δKI allele and one ShcA floxed allele. In the presence of Mlc2v Cre, the floxed allele becomes functionally null, unmasking the individual mutant domain ShcA δKI allele. (B) Individual ShcA δKI alleles are outlined denoting the point mutations. (C) Representative western blot analysis showing reduction in protein levels of the ShcA floxed allele (3x flag) and expression of the individual mutant ShcA δKI allele (1x flag) only in the presence of Mlc2v Cre in SH2 CKI hearts compared to littermate controls.
Figure 5.1. Schematic of ShcA allele strategy to generate ventricle cardiomyocyte specific ShcA KI mutants.
upstream TKs by mutating arginine 175 to glutamate (δPTB CKI; ShcA^{δPTB/flx Mlc2v Cre^{KI/wt}}),
2) pTyr binding of the SH2 domain by mutation of arginine 397 to lysine (δSH2 CKI;
ShcA^{δSH2/flx Mlc2v Cre^{KI/wt}}) and 3) downstream signaling from the tyrosines in the CH1 region
by mutating tyrosines 239/240/313 to phenylalanine (3F CKI; ShcA^{3F/flx Mlc2v Cre^{KI/wt}}) (Figure
13B and C). Mice were viable and recovered at the expected 1:4 Mendelian frequency. Controls
were littermates with the genotypes ShcA^{δKI/flx Mlc2v Cre^{wt/wt}}, ShcA^{wt/flx Mlc2v Cre^{wt/wt}} or
ShcA^{wt/flx Mlc2v Cre^{KI/wt}}.

5.2.2 The PTB domain of ShcA couples to upstream TKs to maintain cardiac structure
and function

Like the ShcA CKO mice, δPTB CKI mice demonstrated a dilated cardiomyopathy phenotype at
an early age. Echocardiography and histological analysis revealed enlarged left ventricle
chamber dimensions and reduced fractional shortening (Figure 5.2A and B, Table 5.1). Over the
course of 1 year, ventricle dilation progressed, while cardiac function declined. A trend in the
elevation of HW/BW ratios in δPTB CKI mice at 1 year of age, suggests a remodeling heart
(Figure 5.3A). Masson trichrome staining of hearts at 1 year revealed minimal areas of interstitial
fibrosis, as in ShcA CKO hearts (data not shown). As the δPTB CKI mice displayed phenotypic
defects at 8 weeks of age (LVEDD: 4.01± 0.06 mm for δPTB Con vs. 4.51± 0.10 mm for δPTB
CKI and %FS: 50.7 ± 1.31 for δPTB Con vs. 39.6 ± 1.68 for δPTB CKI; n>7), TAC studies were
not carried out. The early presentation of the dilated cardiomyopathy in the δPTB CKI mice
could result from subtle gene dosage effects originating from the presence of one functionally
null allele that precludes PTB coupling to upstream TKs during development. Thus, cardiac
chamber dimensions and cardiac function require ShcA coupling to upstream TKs through the
pTyr binding activity of its PTB domain.

δSH2 CKI mice were followed for 1 year to see if ShcA SH2 domain signaling is
required to maintain cardiac structure and function. By 6 months of age, δSH2 CKI mice
demonstrated subtle cardiac dysfunction, and by 1 year of age, systolic function was reduced and
chamber dimensions were slightly enlarged (Figure 5.2A and B, Table 5.1). At one year of age,
no significant difference in HW/BW ratio was noted (Figure 5.3A). As the cardiac function of
Figure 5.2 TK-ShcA signaling is required to maintain cardiac structure and function, while downstream signaling from the CH1 pTyr sites is dispensable.

(A) Echocardiography data showing % fractional shortening of the mutant ShcA allele series. δPTB CKI mice have reduced cardiac function as early as 3 months, while 3F CKI mutants have normal cardiac function. Over the course of 1 year, δSH2 CKI mice have a progressive decline in cardiac function. (B) δPTB CKI mice have enlarged cardiac chambers (left ventricle end diastolic dimensions) that progress over the course of 1 year. In contrast, 3F CKI mice maintain chamber dimensions similar to controls. δSH2 CKI have a small increase in chamber dimensions over the course of 1 year compared to controls. (C) Histological cross sections of hearts from the various mutant ShcA allele mice. 6.25 magnification. (D) Heart Weight to Body weight ratios of the various mutant ShcA allele mice. ^ p < 0.05 compared to same genotype control shams, * p<0.05 compared to littermate controls at given time points.
Figure 5.2 TK-ShcA signaling is required to maintain cardiac structure and function, while downstream signaling from the CH1 pTyr sites is dispensable.
δSH2 CKI mice was unchanged at 2-3 months of age, (Figure 5.2A, Table 5.1), the mice were subjected to the TAC protocol. δSH2 CKI mice had similar remodeling of the left ventricle chamber dimensions compared to controls, but showed reduced cardiac function and blunted hypertrophy response compared to controls after 4 weeks of TAC (Figure 5.2 C and D, Table 5.3). These results demonstrate that SH2 domain-mediated interactions play a role in hypertrophy signaling in response to biomechanical stress and aging.

5.2.3 ShcA phosphotyrosine derived signaling is required in hemodynamic overload

While in vitro and in vivo studies have shown the CH1 pTyr sites are important in downstream signaling, genetic analysis has indicated that phosphorylation of tyrosines 239/240 and 313 is dispensable for embryonic heart development (Hardy et al. 2007). Consistent with this, phospho-null 3F CKI mice did not develop significant cardiac dilation and cardiac dysfunction (Figure 5.2A-D., Table 5.1) over a 1 year period. However, 3F CKI mice subjected to TAC at 2-3 months rapidly advanced into HF, as demonstrating by dilated ventricle chambers coupled with reduced cardiac function (Figure 5.3 A and B, Table 5.2). These data suggest that the tyrosine phosphorylation sites in the CH1 region of ShcA are dispensable in maintaining normal heart function. However, in response to biomechanical stress, ShcA signaling requires phosphorylation of CH1 tyrosine sites associated with recruitment of SH2 domain proteins such as the Grb2 adaptor (Walk et al. 1998; Velazquez et al. 2000).

These data suggest that the tyrosine phosphorylation sites in the CH1 region of ShcA are dispensable in maintaining normal heart function, but become important in the context of a mechanical stress, such as hemodynamic overload. Therefore, ShcA maintains cardiac function through coupling to upstream TKs and, in the context of hemodynamic overload, requires recruitment of downstream SH2 domain proteins such as Grb2 to CH1 pTyr sites. Of interest, the lack of requirement for CH1 pTyr signaling in the maintenance of normal cardiac function
Figure 5.3 TK-ShcA signaling is required to maintain cardiac structure and function, while downstream signaling from the CH1 pTyr sites is necessary after hemodynamic overload.

(A) % Fractional shortening demonstrates that 3F CKI mice have reduced cardiac function after 4 weeks of TAC compared to controls, while δSH2 CKI have a slight reduction in cardiac function. (B) Left ventricle end diastolic dimensions are increased in 3F CKI mice compared to controls after 4 weeks of TAC, while δSH2 CKI have preserved chamber morphology. Individual KI controls were not significantly different and pooled for graphing purposes, individual echocardiography data is found in Table 5.3. (D) Single myocyte contractility demonstrates hypercontractility in δPTB CKI and 3F CKI isolated single myocytes, while δSH2 CKI isolated single myocytes have contractility similar to controls. Single myocyte contractility consists of >3 independent experiments preformed with >15 cells. Individual KI controls were pooled (n=9, >45 cells) (See Table 5.4). ^ p<0.05 compared to same genotype control shams, * p<0.05 compared to littermate controls at given timepoints.
Figure 5.3 TK-ShcA signaling is required to maintain cardiac structure and function, while downstream signaling from the CH1 pTyr sites is necessary after hemodynamic overload.
### Table 5.1. Echocardiography data for 1 year timecourse

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Heart Rate (bpm)</th>
<th>LVEDD (mm)</th>
<th>LVESD (mm)</th>
<th>% FS</th>
<th>VCF (circ/sec)</th>
<th>Ant Wall (mm)</th>
<th>Post Wall (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 week</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>δPTB CON</td>
<td>6</td>
<td>500.2±14.8</td>
<td>3.85±0.07</td>
<td>2.07±0.06</td>
<td>46.4±0.98</td>
<td>0.98±0.03</td>
<td>0.72±0.02</td>
<td>0.61±0.04</td>
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<td>10</td>
<td>513.5±6.21</td>
<td>4.50±0.10*</td>
<td>2.90±0.11*</td>
<td>34.6±1.22*</td>
<td>0.76±0.03*</td>
<td>0.64±0.02*</td>
<td>0.65±0.03</td>
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<tr>
<td>3F CON</td>
<td>12</td>
<td>517.0±11.0</td>
<td>4.00±0.06</td>
<td>2.12±0.05</td>
<td>46.8±0.99</td>
<td>1.04±0.038</td>
<td>0.79±0.02</td>
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<td>501.1±10.9</td>
<td>3.87±0.06</td>
<td>2.19±0.05</td>
<td>44.0±1.0</td>
<td>0.93±0.022</td>
<td>0.79±0.02</td>
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<td>δSH2 CON</td>
<td>8</td>
<td>512.1±9.59</td>
<td>3.94±0.07</td>
<td>2.12±0.07</td>
<td>46.3±1.24</td>
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<td>δSH2 CKI</td>
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<tr>
<td>6 Months</td>
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<tr>
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<td>2.31±0.10</td>
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<td>0.93±0.03*</td>
<td>0.77±0.01</td>
<td>0.70±0.02</td>
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Continued
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<th>LVESD (mm)</th>
<th>% FS</th>
<th>VCF (circ/sec)</th>
<th>Ant Wall (mm)</th>
<th>Post Wall (mm)</th>
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<td>δPTB CON</td>
<td>6</td>
<td>545.2±14.78</td>
<td>4.05±0.09</td>
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<td>δPTB CKI</td>
<td>9</td>
<td>518.4±12.8</td>
<td>5.00±0.17*</td>
<td>3.26±0.22*</td>
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<td>0.78±0.035*</td>
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<tr>
<td>3F CON</td>
<td>8</td>
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^ p<0.05 from same genotype sham; * p<0.05 from littermate control for the given time point
## Table 5.2. Echocardiography and cardiac catheterization for TAC study

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<th>Heart Rate (bpm)</th>
<th>LVEDD (mm)</th>
<th>% FS</th>
<th>Ant Wall (mm)</th>
<th>Heart Rate (bpm)</th>
<th>LVESP (mmHg)</th>
<th>LVEDP (mmHg)</th>
<th>dP/dT max (mmHg/s)</th>
<th>dP/dt min (mmHg/s)</th>
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<tr>
<td><strong>12 wk Sham</strong></td>
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<tr>
<td>3F CON</td>
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<td>6795.7±425*</td>
<td>-6690.3±387*</td>
</tr>
<tr>
<td>δSH2 CON</td>
<td>512.1±9.59</td>
<td>3.94±0.07</td>
<td>46.3±1.24</td>
<td>0.74±0.02</td>
<td>500.1±18.5</td>
<td>109.3±6.39</td>
<td>16.4±2.83</td>
<td>8387.1±464</td>
<td>-7609.0±618</td>
</tr>
<tr>
<td>δSH2 CKI</td>
<td>502.2±11.7</td>
<td>3.78±0.04</td>
<td>45.01±2.14</td>
<td>0.74±0.04</td>
<td>453.8±26.7</td>
<td>106.9±4.58</td>
<td>11.74±2.44</td>
<td>8594.2±989</td>
<td>-7827.3±753</td>
</tr>
<tr>
<td><strong>4 wk TAC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3F CON</td>
<td>502.9±10.3</td>
<td>4.37±0.1^</td>
<td>35.9±1.62^</td>
<td>0.92±0.02^</td>
<td>448.5±18.9</td>
<td>150.2±11.9^</td>
<td>16.7±4.65</td>
<td>6561.9±791^</td>
<td>-6875.9±908^</td>
</tr>
<tr>
<td>3F CKI</td>
<td>498.7±11.2</td>
<td>5.05±0.11^*</td>
<td>18.1±1.71^*</td>
<td>0.73±0.02*</td>
<td>466.1±29.7</td>
<td>129.8±11.5^</td>
<td>23.3±3.66</td>
<td>5545.8±806^</td>
<td>-5124.3±562^</td>
</tr>
<tr>
<td>δSH2 CON</td>
<td>530.4±11.7</td>
<td>4.35±0.11^</td>
<td>38.3±1.61^</td>
<td>0.94±0.03^</td>
<td>499.8±11.3</td>
<td>178.2±11.16^</td>
<td>20.20±3.44</td>
<td>8807.1±1091</td>
<td>-8614.1±896</td>
</tr>
<tr>
<td>δSH2 CKI</td>
<td>487.3±9.22</td>
<td>4.41±0.11^</td>
<td>29.5±1.49^</td>
<td>0.85±0.02^</td>
<td>545.6±9.48</td>
<td>135.9±11.77^</td>
<td>16.79±2.41</td>
<td>7294.7±533^*</td>
<td>-7194.7±768*</td>
</tr>
</tbody>
</table>

^ p<0.05 from same genotype sham; * p<0.05 from littermate control for the given time point
suggests that an alternative signaling mechanism is the predominant mode by which ShcA directs TK signaling in the absence of stress.

5.2.4 Myocyte contractility requires ShcA phosphotyrosine-based signaling

As the loss of ShcA in cardiomyocytes enhanced myocyte contractility, the three ShcA mutant allele CKI mouse lines were subjected to single myocyte contractility assays and analysis of global calcium transients. Both the δPTB CKI and 3F CKI myocytes were hypercontractile compared to their littermate controls independent of changes in calcium handling, while δSH2 CKI myocytes were not different from control myocytes (Figure 5.3C, Table 5.5). This suggests that ShcA requires PTB domain coupling to upstream TKs and subsequent signaling through the CH1 pTyr sites to maintain homeostatic contractility at the myofilament level. This cell autonomous function of ShcA therefore utilizes the well established signaling through pTyr residues in the CH1 region, in contrast to the spontaneous dilated cardiomyopathy phenotype, in which pTyr signaling from the CH1 region is dispensable. Together, these data suggest that in the myocardium, ShcA signals downstream of TKs to differentially impact on cardiomyocyte contractility and global heart morphology.

5.3 Discussion

The ability of ShcA to impact on different aspects of cardiovascular function highlights the modular nature of the ShcA protein and its ability to direct signaling by recruitment to specific TKs through the PTB and SH2 domains. Indeed, loss of signals derived from PTB domain docking to upstream tyrosine kinases phenocopies the loss of ShcA in the myocardium, while loss of signals involving the SH2 domain are required to augment the response to TAC and hypertrophy associated with age.
Table 5.3. Contractility and calcium transient assays for ShcA mutant domain KI mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pooled Controls</th>
<th>PTB CKI</th>
<th>3F CKI</th>
<th>SH2 CKI</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Sarcomere shortening</td>
<td>7.17±0.24</td>
<td>9.04±0.41*</td>
<td>9.44±0.55*</td>
<td>6.72±0.26</td>
</tr>
<tr>
<td>dSl/dT max</td>
<td>2.85±0.18</td>
<td>4.26±0.25*</td>
<td>3.65±0.27*</td>
<td>2.93±0.25</td>
</tr>
<tr>
<td>dSl/dT min</td>
<td>3.89±0.20</td>
<td>5.53±0.34*</td>
<td>4.96±0.40*</td>
<td>3.92±0.22</td>
</tr>
<tr>
<td>TR&lt;sub&gt;50&lt;/sub&gt;</td>
<td>0.039±0.002</td>
<td>0.035±0.002</td>
<td>0.038±0.002</td>
<td>0.034±0.003</td>
</tr>
<tr>
<td>TR&lt;sub&gt;80&lt;/sub&gt;</td>
<td>0.063±0.003</td>
<td>0.056±0.003</td>
<td>0.061±0.003</td>
<td>0.055±0.004</td>
</tr>
<tr>
<td>Amplitude (F405/485nm)</td>
<td>0.316±0.011</td>
<td>0.329±0.02</td>
<td>0.281±0.014</td>
<td>0.307±0.02</td>
</tr>
</tbody>
</table>

For each group n=3, minimum 15 cells. Pooled KI controls (n=9, minimum 45 cells). dSl/dT<sub>max</sub>, maximum first derivative of sarcomere shortening; dSl/dT<sub>min</sub>, minimum first derivative of sarcomere shortening; TR<sub>50</sub>, time to 50% relaxation; TR<sub>80</sub>, time to 80% relaxation. F, Fluorescence of calcium transient. * p<0.05 to littermate control for given time point.

The PTB domain of ShcA has been shown to interact with multiple receptor TKs critical in cardiac function, such as VEGFR3, ErbB2 and ErbB4 (Segatto et al. 1993; Wolf et al. 1995; Fournier et al. 1996); in particular, loss of ErbB2 in cardiomyocytes produces a similar phenotype to ShcA in cardiac ablation studies (Crone et al. 2002; Ozcelik et al. 2002). The loss of ShcA in other tissues also phenocopies conditional ErbB2 loss (Dankort et al. 2001; Andrechek et al. 2002; Hardy et al. 2007) and supports the idea that ShcA is a preferential scaffold for ErbB2 signaling, especially as it contains multiple ShcA consensus binding motifs (Segatto et al. 1993; Schulze et al. 2005). Of clinical relevance, Herceptin, an effective tyrosine kinase inhibitor of ErbB2-ErbB3 heterodimers, causes HF in a subset of patients (De Keulenaer et al.); it is suggestive then that ShcA could function in a pathway that is regulated by Herceptin, perhaps influencing matrix/myocyte interactions. The clinical utility of Neuregulin (Jiang et al.), the ligand of heterodimers containing ErbB2 (Shi et al. 2009), in improving cardiac function in diseased myocardium suggests the ErbB2-ShcA signaling network could be of great interest therapeutically.
The slow evolution of a HF phenotype in the SH2 CKI mice, that does not follow the timecourse of the loss of ShcA in the myocardium, suggests that SH2 coupling to various upstream TKs has a supportive role in cardiac structure and function, distinct from PTB domain-mediated signaling. Indeed, the SH2 domain has been shown to bind to the platelet derived growth factor receptor β (Yokote et al. 1994), FAK (Hecker et al. 2002) and the epidermal growth factor receptor (Pelicci et al. 1992), unique role for ShcA SH2-mediated signaling in response to biomechanical stress and aging.

Downstream signaling induced by phosphorylation of tyrosine residues in the CH1 region of ShcA appears to have evolved as a mechanism to increase signal complexity in multicellular organisms (Luzi et al. 2000). These tyrosines are covalently modified by activated tyrosine kinases when ShcA is recruited by either its PTB or SH2 domain and have been shown to be critical in Ras mediated MAPK activation and PI3K activation through ShcA-Grb2 interactions (Ravichandran 2001). To understand the requirement for downstream signaling from the pTyr residues in the CH1 region of ShcA in the adult myocardium, we evaluated the phospho-null mutant 3F CKI mouse for effects on cardiac structure and function. Of interest, the pTyr sites required for Grb2 recruitment were not required for normal cardiac function, but become functionally important in situations of mechanical stress, such as hemodynamic overload. A small percentage of 3F CKI mice (<20%) did spontaneously dilate with age, but this seems to be an isolated phenomenon and could be attributed to some unknown stress. Alternative ShcA signaling mechanisms include adaptin binding in the CH1 region, SH3 domain containing proteins recruited to the proline rich CH1 region, or serine/threonine phosphorylation motifs within the ShcA protein (Pelicci et al. 1992; Weng et al. 1994; Bonfini et al. 1996; Okabayashi et al. 1996). In addition, recent data suggests that IQGAP links ShcA to the actin cytoskeleton through a non-canonical interaction with the PTB domain (Smith et al.). Therefore, ShcA has several alternative downstream signaling mechanisms that could be utilized to impact on cardiac function.

Thus, in the postnatal myocardium, ShcA is able to use distinct signaling pathways to impact on isolated cardiomyocyte and global heart function. For example, the PTB domain is required to maintain cardiac function essential for suppressing the dilated cardiomyopathy phenotype; however, this is independent of downstream signaling from the CH1 pTyr sites. Nonetheless, the CH1 pTyr sites become important in regulating cardiomyocyte contractility and
coordinating signals in response to hemodynamic stress. These data show that ShcA is a key
docking protein for TK signaling in the postnatal myocardium that coordinates signaling
networks underlying cardiac physiology.
Chapter 6

Summary and Future Directions
6 Summary and Future Directions

6.1 Summary

ShcA is a key protein that acts immediately downstream of receptor tyrosine kinases, especially members of the ErbB family, to organize cytoplasmic signaling pathways. It has a modular architecture, containing an N-terminal phosphotyrosine-binding (PTB) domain, a C-terminal SH2 domain, and a central region with phosphotyrosine sites that bind to the adaptor protein Grb2 and thus regulate the Ras-MAP kinase pathway. Using Cre/LoxP recombination, removal of murine ShcA from the myocardium leads to a dilated cardiomyopathy, accompanied by eccentric remodeling, indicating that it is a critical molecule in controlling cytoplasmic signaling pathways in the heart. Of interest, individual ShcA-deficient cardiomyocytes show an enhanced contractility, in contrast to the defect at the level of the whole heart. Loss of ShcA in the myocardium apparently precipitates a dilated cardiomyopathy phenotype through defects in signaling pathways governing interactions of cardiomyocytes with the extracellular matrix. To understand more fully the molecular mode of action of ShcA, we examined the effects of an allelic series of ShcA point mutants on cardiac function, which either eliminate the phosphotyrosine-recognition domains through which ShcA interacts with activated receptors, or inhibit its ability to couple to selective downstream pathways. The resulting data indicate that the ability of ShcA to bind activated receptors through its PTB domain is essential for physiological cardiac function, whereas the integrity of the SH2 domain is required to augment cardiac function. The phosphotyrosine sites that couple to Grb2 are more important for stress-induced responses and cardiomyocyte contractility. Our results identify ShcA as an important cytoplasmic protein for phosphotyrosine signaling in the heart, demonstrate that it has distinct cell autonomous and non-cell autonomous roles, and show that different domains and motifs in this modular protein are of distinct significance in regulating normal and stress responses. These findings significantly extend our understanding of phosphotyrosine-based signaling in the control of heart function, and are of both basic and clinical interest.
Figure 6.1 Model of ShcA signaling in the myocardium

(A) ShcA uses the PTB domain to dock to activated TKs critical in maintaining cardiac structure and function, while the SH2 domain is important in hypertrophy signaling in response to hemodynamic overload and aging. ShcA uses an alternative mode of signaling to maintain cardiac function, as mutation of the CH1 pTyr sites did not elicit a dilated cardiomyopathy phenotype. (B) ShcA pTyr signaling downstream of the CH1 region is required for appropriating signals after hemodynamic overload and signaling that regulates myocyte contractility.
6.2 Future Directions

These studies demonstrated a role for ShcA in the postnatal myocardium and highlighted the complex regulation and signaling underlying cardiac physiology. However, many unanswered questions remain. Some areas for potential study are outlined below.

6.2.1 What are the specific TK interactions of the PTB and SH2 domains of ShcA within the myocardium?

Ventricular specific ShcA KI mice with mutations in the various domains and motifs demonstrated a requirement for the binding of the PTB domain of ShcA to upstream TKs to maintain cardiac structure and function, while the integrity of the SH2 domain is required to augment cardiac function. However, the specific TKs that ShcA interacts with are unknown. One obvious candidate is the ErbB2 receptor, as cardiac ablation studies phenocopy the loss of ShcA or PTB binding to upstream TKS in the myocardium, and the presence of multiple ShcA consensus binding motifs (Introduction section 3.2). To investigate the TK – ShcA pTyr interactions, various approaches could be undertaken. An unbiased and systematic approach would utilize mass spectrometry to identify TK-ShcA interactions. Whole heart lysates with Flag IPs allows for identification of TKs binding to either domain of ShcA. Contamination of other cell types is a drawback, especially given the abundance of fibroblasts in the myocardium. To circumvent this, pooling isolated single myocytes could also be done, however introduction of contamination during the isolation procedure, low yields and the lack of cellular adherence could obscure physiological interactions. After various attempts, I feel the best approach would be through isolation of ShcA^{flx/flx} neonatal myocytes. This would ensure an endogenous Flag epitope to IP and also allows for removal of the ShcA floxed allele through Cre recombinase (α-MHC MerCreMer) as a control. As an adjunct or alternative approach, isolated neonatal myocytes could be stimulated with various growth factors, such as Neuregulin, or plated on various ECM (collagen or fibronectin) and harvested for mass spectrometry. These studies would enable a myocyte specific ShcA-pTyr network to be established.
6.2.2  What is the effect of ErbB2-ShcA signaling in the myocardium?

Given the potential for ShcA to be signaling downstream of the ErbB2 receptor in the myocardium, one must use a genetic approach to precisely isolate the ErbB2-ShcA pathway. I propose using a genetic strategy employed in these studies and generation of a KI ErbB2 mutant allele. Mice heterozygous for the ErbB2 floxed allele (Crone et al. 2002) and a KI ErbB2 allele with point mutations of the ShcA consensus binding sites, when coupled with Cre recombinases expressed from the MLC2v promoter, will allow for direct interrogation of this signaling axis in the myocardium. Previously, a hypomorphic ErbB2 KI mouse with one mutated ShcA consensus binding site was generated (Chan et al. 2004), however no cardiovascular phenotype was uncovered. Schulze and colleagues (Schulze et al. 2005) identified additional ShcA binding motifs on the ErbB2 receptor, suggesting that the introduction of multiple point mutations to the ErbB2 receptor may be required to generate a phenotype. Readouts could include apoptosis, matrix interactions and contractility, in addition to full cardiovascular phenotypic analysis. This study will have the highest potential to translate directly to address clinical concerns of TKIs.

6.2.3  What are the clinical correlates to the loss of ShcA or TK signaling in the myocardium?

Dilated cardiomyopathy is a major reason for pediatric cardiac transplantation, along side complex congenital heart disease. While mutations in myofilament, cytoskeletal, and renin-angiotensin-aldosterone system (RAAS) proteins have been documented, it would be of interest to screen for mutations in TKs and ShcA (Fatkin et al.). These studies could be done in collaboration with the Pediatric Cardiology Transplant Team and the Hospital for Sick Children’s Molecular Genetics Program. Single nucleotide polymorphisms within ShcA have been evaluated in early onset cardiovascular disease, based on the p66 ShcA literature, but no significant polymorphisms were detected (Sentinelli et al. 2006). Screening for novel TK mutations or ShcA mutations could demonstrate novel causes of pediatric dilated cardiomyopathies.
6.2.4 How does the loss of ShcA regulate myofilament homeostasis?

The hypercontractile cardiomyocytes found in the ShcA CKO mice was unexpected and revealed a cell autonomous effect of ShcA in regulating myofilament homeostasis. The actual molecular mechanism by which ShcA does this is not apparent. Initial probing experiments did not show alterations in Serine 23/24 phosphorylation of troponin I, consistent with enhanced calcium sensitivity. To further screen and identify changes, proQ diamond staining (Pierce) of myofilament preparations was carried out, but no obvious changes were noted. Many different phosphatases and kinases regulate myofilament interactions (de Tombe et al. 2000; Solaro 2008), and therefore studies addressing the underlying interactions could be carried out in collaboration with Dr. P. deTombe. To address this initially, I infused various inhibitors and activators (PKC, MAPK) in an ex vivo isolated heart perfusion system to assay changes in phosphorylation of troponin I. No consistent changes were seen. An alternative approach would be incubating the isolated skinned myocytes of the tamoxifen-inducible ShcA MCKO with inhibitors or activators and looking for shifts in force-calcium parameters; this could potentially be a more robust measure. Initial candidates could include inhibitors/activators of Erk-MAPK pathway, PKC, and various phosphatases (Solaro 2008).

6.2.5 What are the downstream signaling consequences attributed the loss of ShcA in cardiomyocytes?

In vitro experiments have established ShcA in mitogenic Erk activation; however, no changes in the phosphorylation of Erk was noted at 6 months of age. As well, other pathways were interrogated and showed no consistent changes. To try to define the effects of ShcA on MAPK signaling in general, I used the ShcAflx/flx mouse embryonic fibroblasts and excised ShcA using tamoxafen inducible Cre recombinase mouse (Badea et al. 2003). Initial experiments show nice excision with little adverse effects to cells. This system allows for detection of signal transduction changes in response to a variety of stimuli. A more robust measure could be changes in c-fos or c-myc either through lucificerase assays or real time RT PCR. This system could be translated then to the neonatal cardiomyocyte culture system mentioned above to shed insight into cardiomyocyte specific changes. In particular, neuregulin, EGF, PDGF, and collagen stimulation would be of great interest.
6.2.6 What is the molecular mechanism by which the loss of ShcA affects the mechanical integrity of the myocardium?

The experiments mentioned above will provide insight into ShcA interactions potentially impacting on mechanical integrity. For example, mass spectrometry could identify interaction of the discoidin domain receptor, while matrix adhesion experiments could identify abnormalities in ShcA-deficient cardiomyocytes. Alternatively, GF stimulation could reveal differences in secretion of molecules important in regulating the integrity of the ECM. Initial studies in MEFs did not reveal an increase in MMP activity (by zymography) with generic stimulation using Concanavalin A or neuregulin, however further optimization and the use of real time RT PCR readouts could be more robust. One particular candidate of interest would be the regulation of hyaluronic acid/proteoglycans in the myocardium by ShcA. Microarray analysis of ShcA-deficient hearts identified the downregulation of Hyaluronidase synthase 2 (Has2), that was verified with real time RT PCR. Loss of Has2 in the embryonic period, results in hypoplastic endocardial cushions and a dilated heart, similar to the ShcA null mouse (Camenisch et al. 2000). Subsequent studies showed that Has2 induction is part of an ErbB2-ErbB3 signaling pathway (Camenisch et al. 2002). I initially stained for proteoglycans, but little staining was seen in the myocardium. To enhance retention of proteoglycans post fixation, cetyl pyridinium chloride is required during histological processing thereby improving the ability to quantify differences using Alcian blue stain. While it is unknown how proteoglycans affect the mechanical properties of the myocardium, much can be derived from cartilage literature. Proteoglycans are required to give tensile strength and are the major water carrying molecule, thereby contributing to the protective properties against mechanical force (Fomovsky et al.). Given the high amount of proteoglycans in papillary muscles, it would be of interest to see if ShcA is involved in a Has2-ErbB2/B3 pathway, especially given the embryonic similarities.

6.2.7 What is the role of Grb2 signaling in the myocardium?

To expand the adaptor protein literature in the myocardium, generation of a ventricular specific Grb2 KO should be carried out. While Grb2 null mice are embryonic lethal at early time points
(Cheng et al. 1998), heterozygotes are viable and have been evaluated in myocardial hypertrophy signaling (Zhang et al. 2003). In response to hemodynamic overload, Grb2 heterozygotes demonstrate an absence of hypertrophy, without dilation, that correlates with deficiencies in p38 MAPK activation. Therefore, it would be of great interest to more fully appreciate the role of Grb2 in the myocardium. The Grb2 floxed allele was generated in the Pawson laboratory and crossing it to various cardiac specific Cre mice will demonstrate the role for Grb2 in maintaining cardiac function.

6.3 **Concluding Remarks**

These studies demonstrate the requirement of ShcA in pTyr signaling in the myocardium. In the postnatal myocardium, ShcA is able to use distinct signaling pathways to impact on isolated cardiomyocyte and global heart function. ShcA can signal through the pTyr sites in the CH1 region to regulate cardiomyocyte contractility and coordinate signals in response to hemodynamic stress. In addition, ShcA is also able to use alternative modes to propagate signals, once docked to activated TKs, as seen with the dilated cardiomyopathy phenotype. These data show that ShcA is an integral docking protein for TKs in the postnatal myocardium and enhance our understanding of the complex signaling networks underlying cardiac physiology.
References


Graus-Porta, D., et al. (1997). "ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling." EMBO J 16(7): 1647-55.


Appendices (if any)
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