

University of Nevada, Reno

**Functional Role of ClC-3 Chloride Channels
in Myocardial Hypertrophy and Heart Failure**

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in
Cellular and Molecular Biology

by

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December, 2011



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THE GRADUATE SCHOOL

We recommend that the thesis
prepared under our supervision by

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entitled

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In Myocardial Hypertrophy And Heart Failure**

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requirements for the degree of

MASTER OF SCIENCE

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Abstract

Background: In hypertrophied cardiac myocytes the volume-regulated chloride channels (VRCCs) are activated constitutively. But the functional role and molecular mechanism of the constitutively activated VRCCs in myocardial hypertrophy and heart failure are not well known. In this study a novel heart-specific inducible CIC-3 knockout (*doxysClcn3^{-/-}*) mouse was developed to specifically test the hypothesis that CIC-3, a member of the CIC voltage-gated chloride channel superfamily, may be responsible for the constitutively activated VRCCs and may play a protective role against heart failure.

Methods: A pressure overload model through a minimally invasive transverse aortic banding (MTAB) was established in the *doxysClcn3^{-/-}* mice when doxycycline in the diet was either maintained (on-Doxy, to preserve *Clcn3* gene expression) or removed (off-Doxy, to activate Cre recombinase and inactivate the *Clcn3* gene expression). Changes in cardiac function and whole-cell VRCCs in cardiac myocytes of the *doxysClcn3^{-/-}* mice during on-Doxy and off-Doxy were monitored using echocardiograph and whole-cell voltage-clamp techniques.

Results: 1) Compared to age-matched on-Doxy control mice native cardiac VRCCs current densities of the off-Doxy mice were significantly reduced at week 1.5 and were completely eliminated at week 3. The changes in VRCCs activities were closely correlated to the changes in molecular expression of CIC-3 and cardiac function. 2) Global anatomy and histological analysis of isolated hearts revealed dramatically enlarged hearts from the off-Doxy *doxysClcn3^{-/-}* mice at week 3 compared to the age-matched on-Doxy mice. 3) Echocardiography revealed a significant increase in left

ventricular mass (LVM) and chamber dimensions and a decrease in left ventricular ejection fraction (LVEF) and fractional shortening (%FS) in the *doxyhsCICn3^{-/-}* mice at both 1.5 and 3 weeks off-Doxy. 4) While on-Doxy preserved the compensatory myocardial hypertrophy and cardiac function for ~10 weeks after MTAB, off-Doxy accelerated the MTAB-induced increase in LVM (within 1 week) and the progression from myocardial hypertrophy to dilated heart failure (in < 3 weeks) during pressure overload.

Conclusions: CIC-3 is a key component of native VRCCs in mammalian heart and plays a significant cardioprotective role against myocardial hypertrophy and heart failure.

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Abbreviation

2D	Two-Dimensional
ATP	Adenosine Triphosphate
ANOVA	Analysis of Variance
BPM	Beats Per Minute
BW	Body Weight
CaCCs	the Ca ²⁺ Activated Cl ⁻ Channels
cDNA	Complementary DNA
CFTR	the Cystic Fibrosis Transmembrane Conductance Regulator
CIC	Voltage-Gated Chloride Channel
<i>Clcn3</i>	<i>exon 3</i> of CIC-3 Gene
<i>Clcn3</i> ^{-/-}	the <i>Clcn3</i> Global Knockout
<i>Clcn3</i> ^{+/+}	the <i>Clcn3</i> Wilt Type
DCM	Dilated Cardiomyopathy
DMEM	Dulbecco's Modified Eagle Medium
<i>doxysClcn3</i> ^{-/-}	Heart-specific Inducible <i>Clcn3</i> Knockout
EcCIC	CIC Channels from <i>Escherichia coli</i>
ECHO	Echocardiogram
<i>E_{Cl}</i>	the Equilibrium Potential for Cl ⁻
EF	Ejection Fraction
EGTA	Ethylene glycol tetraacetic Acid
ES cells	Embryonic stem cells
FS	Fractional Shortening
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GE	General Electric
gpCIC-3	CIC-3 cDNA from guinea-pig
<i>I_{Cl,vol}</i>	Volume-Regulated Chloride Current
<i>I_{Cl,ATP}</i>	Extracellular ATP Activated Chloride Current
<i>I_{Cl,b}</i>	the Basally-Activated Chloride Current
<i>I_{Cl,Ca}</i>	Intracellular Ca ²⁺ Activated Chloride Current
<i>I_{Cl,ir}</i>	Inwardly Rectifying Chloride Current
<i>I_{Cl,PKA}</i>	Protein Kinase A Activated Chloride Current
<i>I_{Cl,PKC}</i>	Protein Kinase C Activated Chloride Current
<i>I_{Cl,swell}</i>	Cell Swelling-Induced Chloride Current
IVS	Inter Ventricular Septum
LV	Left Ventricular
LVD	Left Ventricular Diameter

LVID	Left Ventricular Chamber Dimension
LVM	Left Ventricular Mass
LVPW	Left Ventricular Posterior Wall
MTAB	Minimally Invasive Transverse Aortic Banding
NMDG-Cl	N-methyl-d-glutamine Chloride
off-Doxy	Maintain without Doxycycline Diet
on-Doxy	Maintain with Doxycycline Diet
PCR	Polymerase Chain Reaction
PKA	Protein Kinase A
PKC	Protein Kinase C
ROS	Reactive Oxygen Species
RVD	Regulatory Volume Decrease
RVI	Regulatory Volume Increase
StClC	ClC Channels from Salmonella Typhimurium
VRCCs	the Volume-Regulated Chloride Channels
α -MHC	the Heart-Specific α -Myosin Heavy Chain

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Chapter 1 Introduction

1.1 Myocardial Heart Failure and Myocardial Hypertrophy

Cardiovascular disease is the leading cause of death globally. Heart failure, a cardiovascular disease condition characterized by dyspnea, fluid retention and decreased functionality, is associated with particularly high rates of morbidity and mortality (Velagaleti et al., 2009) and affects over 5.8 million individuals in the United States and over 23 million worldwide (Fonarow, 2011). Phases of chronic compensation and acute decompensation alternate during the progression of heart failure. The clinical status gets worse during the acute phase (Sato et al., 2008). The pathophysiology of both chronic compensated and acute decompensated heart failure remains incompletely understood, especially with respect to the differences between patients with heart failure and a decreased ejection fraction (EF) and those with heart failure and a normal ejection fraction. It is shown in recent studies that patients with heart failure have structural and functional changes consistent with remodeling and dominant abnormalities in cardiac function (Aurigemma and Gaasch, 2004; Aurigemma et al., 2006; Kitzman et al., 2002; Baicu et al., 2005; Lam et al., 2007; Melenovsky et al., 2007; Ahmed et al., 2006; Zile et al., 2001; Zile et al., 2004). Functional measurements made in these studies used noninvasive Doppler and tissue Doppler estimates of left ventricular diastolic pressure in patients with heart failure (Ahmed et al., 2006; Lam et al., 2007; Melenovsky et al., 2007). However, details of the mechanism or mechanisms of congestive heart failure remain unknown (Zile et al., 2008; Bozeat, 2007).

Myocardial hypertrophy is an early stage during the clinical course of heart failure. It is also an important risk factor causing subsequent cardiac morbidity and mortality. In response to various mechanical, hormonal, hemodynamic, and pathologic stimuli, the heart adapts to increased demands for cardiac work by increasing muscle mass through the initiation of a hypertrophic response. At the cellular level, cardiac myocytes respond to biomechanical stress by initiating several different processes that lead to hypertrophy (Figure 1). The physiologic hypertrophy that typically occurs in elite athletes is reflected in proportional increases in the length and width of cardiac myocytes. By contrast, the assembly of contractile-protein units in series characterizes the eccentric hypertrophy occurring in patients with dilated cardiomyopathy, with a relatively greater increase in the length than in the width of myocytes. During pressure overload, new contractile-protein units are assembled in parallel, leading to a relative increase in the width of individual cardiac myocytes and therefore to concentric hypertrophy (Hunter and Chien, 1999).

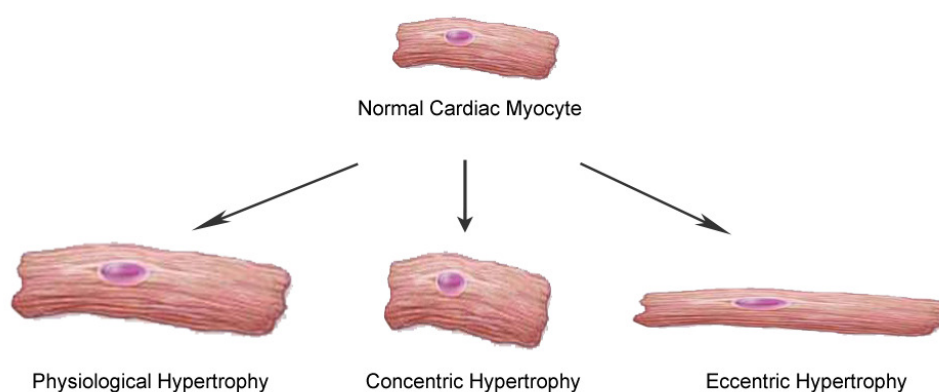


Figure 1 Morphology of ventricular muscle cells in cardiac hypertrophy and failure.

There are three kinds of myocardial hypertrophy. Concentric hypertrophy increases only in width but not length of myocytes while eccentric hypertrophy that happens in patients with dilated cardiomyopathy shows greater increase in length than in width of myocytes. Physiologic hypertrophy that happens with physical exercise shows proportional increases in both length and width of myocytes. (Revised from Hunter and Chien 1999.)

Myocardial hypertrophy and its progression to dilated cardiomyopathy or heart failure are characterized by structural remodeling (hypertrophic cell volume increase or growth of cardiac myocytes and changes in the cytoskeleton and extracellular matrix) and electrical remodeling (abnormal expression and/or function of ion channels) (Laser et al., 2000; Weber, 1995; Nass et al., 2008; Nattel et al., 2007; Tomaselli and Marban, 1999; Cutler et al., 2007). It should be pointed out that ionic remodeling during the progression of hypertrophy to heart failure provides not only substrates for arrhythmias but also cellular mechanisms for structural remodeling through regulation of many cellular functions including apoptosis.

Although recent studies have provided evidence that ionic remodeling of several cation channels, such as K^+ , Ca^{2+} , and stretch-activated non-selective channels, plays an important role in the pathology of heart failure (Fatkin and Graham, 2002; Lehmann-Horn and Jurkat-Rott, 1999; Nass et al., 2008; Nattel et al., 2007) very little is currently known about the functional role of anion (Cl^-) channels in human cardiac diseases (Hume et al., 2000a; Duan, 2009; Duan et al., 2005). Recent studies found that a volume-regulated Cl^- current ($I_{Cl,vol}$) are constitutively activated in human (Patel et al., 2003) and animal (Benitah et al., 1997; Clemo et al., 1998; Clemo et al., 1999; Clemo et al., 2001) failing hearts but it is unknown how activation of $I_{Cl,vol}$ would affect the structural remodeling at the ionic and molecular levels.

1.2 Chloride Channels in the Heart

Anion channels are transmembrane proteins in biological membranes, which form functional pores and allow the diffusion of negatively-charged ions along the electrochemical gradients. Chloride (Cl^-) is the most abundant anion and it passes across the cell membranes mainly through Cl^- channels. The contribution of Cl^- channels to the cardiac action potential was first described in 1961 (Carmeliet, 1961). Since 1989 patch-clamp studies have described a variety of Cl^- channels with different single-channel conductance, anion selectivity, and mechanism of regulation in both plasma membrane and intracellular organelles (Hume et al., 2000). Functional and molecular studies have provided substantial evidence for the expression of the following Cl^- channels in cardiac myocytes: 1) the cystic fibrosis transmembrane conductance regulator (CFTR) encoded Cl^- channels, which can be activated by protein kinase A (PKA) ($I_{\text{Cl.PKA}}$) (Bahinski et al., 1989; Harvey and Hume, 1989; Harvey, 1993; Hume et al., 1994), protein kinase C (PKC) ($I_{\text{Cl.PKC}}$) (Collier and Hume, 1995; Walsh, 1991; Yamazaki et al., 1999; Zhang et al., 1994), and extracellular ATP ($I_{\text{Cl.ATP}}$) (Duan et al., 1999b; Levesque and Hume, 1995; Yamamoto-Mizuma et al., 2004a); 2) the ClC-2 encoded inwardly rectifying Cl^- channels, which can be activated by hyperpolarization and cell swelling, ($I_{\text{Cl.ir}}$) (Britton et al., 2005; Duan et al., 2000; Huang et al., 2009); 3) the ClC-3 encoded outwardly rectifying Cl^- currents, which can be regulated by changes in cell volume ($I_{\text{Cl.vol}}$) (Duan et al., 1997b), including the basally-activated ($I_{\text{Cl.b}}$) (Duan et al., 1992) and swelling activated ($I_{\text{Cl.swell}}$) (Duan et al., 1995; Duan et al., 1997a; Tseng, 1992) components. An increase in intracellular Ca^{2+} can also activate a Cl^- current ($I_{\text{Cl.Ca}}$) (Duan et al., 1995; Duan et al., 1997a; Collier et al., 1996; Kawano et al., 1995; Li et al., 1995; Li et al.,

2003; Li et al., 2004; Wang et al., 1995; Zygmunt and Gibbons, 1991; Zygmunt and Gibbons, 1992), but the molecular identity for the Ca^{2+} activated Cl^- channels (CaCCs) in the heart remains elusive (Duan, 2009). The current understanding of the physiology and pathophysiology of Cl^- channels in the human cardiovascular system is very limited due to the lack of specific pharmacological and molecular tools. With the recent identification of the molecular entities responsible for Cl^- channels in heart and the genes mapped to specific human chromosomal locations, it is now possible to use gene targeting and transgenic techniques to identify the structure of the channel proteins, to investigate the molecular and functional properties of these Cl^- channel genes, and to illuminate the physiological and clinical significance of these channels (Duan, 2009; Huang, 2009).

Previous *in vitro* experimental evidence has suggested that Cl^- channels in mammalian cells participate in a wide variety of cell and intracellular organelle functions, including regulation of electrical activity, pH, volume, and the transport of osmolites and metabolites, and may even play a role in the control of immunological responses, cellular excitability, intracellular organelle acidification, cell volume homeostasis, cell migration, proliferation, differentiation and apoptosis (Hume et al., 2000). Thus, they may have important physiological and pathological significance in cardiac function under normal and pathological (hypoxia, ischemia, myocardial infarction, hypertrophy, and heart failure) conditions (Duan, 2009). As a matter of fact, mutations in several Cl^- channels have been known to result in human inherited diseases (Lehmann-Horn and Jurkat-Rott, 1999). Because the equilibrium potential for Cl^- (E_{Cl}) is within a membrane potential range (usually -65 to -40 mV) that can be either negative or positive to the actual membrane potentials during the normal cardiac cycle, activation of cardiac Cl^- channels

can generate both inward and outward currents. Thus, compared with cationic channels, Cl^- channels have the unique ability to cause both depolarization as well as repolarization during the action potential and produce significant effects on pacemaker activity and action potential characteristics (Duan, 2009; Huang, 2009; Bozeat, 2007).

1.3 CIC Gene Family and CIC-3 in the Heart

The CIC gene family of chloride channels was identified over 25 years ago (White and Miller, 1979) and later verified through cloning CIC-0, a chloride channel enriched in the electric organ of the marine ray *Torpedo marmorata* (Jentsch et al., 1990). CIC proteins form a family of Cl^- transport proteins that are expressed in nearly all phyla. These proteins may be separated into two functional groups: 1) voltage-gated chloride channels, and 2) $\text{Cl}^- : \text{H}^+$ exchangers (Duran et al., 2010). While evolutionarily these proteins are very similar, the mechanisms by which they perform their functions are quite different. The functional unit of the CIC protein family is usually a homodimer (Suzuki et al., 2006). In mammalian systems, CIC proteins mediate Cl^- flux across the plasma and intracellular membranes in most cell types and take part in maintenance of cell volume regulation, resting membrane potential, and acidification of intracellular compartments such as lysosomes and endosomes (Zifarelli and Pusch, 2007). Based on the original hydrophobicity data and quantitative single channel analysis, it was hypothesized that these channels contain two parallel, independent pores (i.e., a double-barrel channel) with 12 transmembrane spanning domains, with intracellularly located amino (N) and carboxyl (C)-termini (Miller and White, 1984). In 2002, the crystal structure analysis demonstrated

that CIC channels from *Salmonella typhimurium* (StCIC) and *Escherichia coli* (EcCIC) contains 18 α -helices, which creates a homodimer each with its own ion conduction pore (Figure 2). Important amino acids from four separate regions are juxtaposed next to the membrane center to form an ion-binding site. These regions are highly conserved in all CIC Cl^- channels found to date; they include the sequences G(K/R)EGP, GSGIP, and GXFXP, as well as Tyr 445. Anion selectivity in CIC channels is achieved through partial positive dipoles, which creates a favorable electrostatic environment for Cl^- conductance (Dutzler et al., 2002; Huang, 2009; Bozeat, 2007).

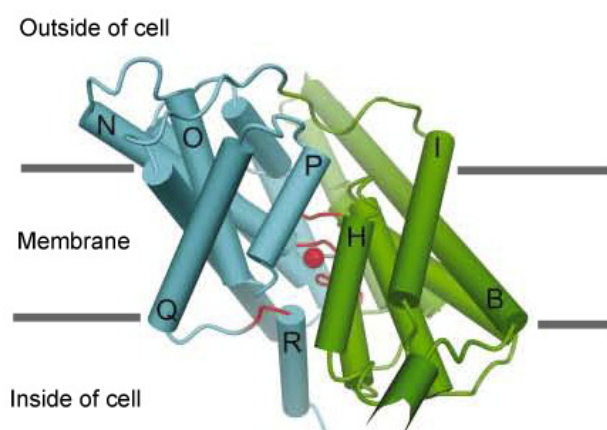


Figure 2 Structure of the CIC subunit.

This is a side-on view of one of the two subunits of a CIC channel, looking at the side that interacts with the other subunit. The two halves of the subunit are green and cyan, and regions forming the Cl^- selectivity filter are red. The α -helices are drawn as cylinders with the extracellular region above and the intracellular region below. (Adapted and revised from Dutzler et al. 2002.)

CIC-3 is ubiquitously expressed in many tissues, including but not limited to the heart, brain, and kidneys (Remillard and Yuan, 2005). CIC-3 has been previously identified as a member of the CIC chloride channel family (Jentsch et al., 1995) but it was first cloned and functionally expressed from rat cDNA. Functional expression of CIC-3 resulted in a basally activated Cl^- current that was strongly inhibited by PKC stimulation

and Ca^{2+} (Kawasaki et al., 1995). CIC-3 has also been cloned from human (Huang et al., 2001), mouse (van Sleightenhorst et al., 1994), and guinea pig (Duan et al., 1997; Huang, 2009; Bozeat, 2007).

CIC-3 cloned from guinea-pig cardiac ventricular myocytes is believed to be the molecular candidate responsible for $I_{\text{Cl,vol}}$ found in many tissues. When CIC-3 cDNA from guinea-pig (gpCIC-3) was expressed in NIH3T3 cells it raised a large basally active chloride conductance which was strongly modulated by cell volume and exhibited many properties identical to those of $I_{\text{Cl,swell}}$ in native cells. A mutation of asparagine to lysine at position 579 at the end of the transmembrane domains of CIC-3 abolishes the outward rectification and changes the anion selectivity from $\text{I}^- > \text{Cl}^-$ to $\text{Cl}^- > \text{I}^-$ but would otherwise leave the swelling activation intact (Duan et al., 1997b). Using site-directed mutagenesis and patch clamp techniques it was demonstrated that CIC-3 had a conserved N-terminal PKC phosphorylation site, which acted as a volume sensor in native cardiac cells and NIH/3T3 cells when overexpressed. CIC-3 channels were opened by cell swelling, but inhibited by PKC activation, phosphatase inhibition, or elevation of intracellular Ca^{2+} . A serine residue at 51 within the consensus PKC-phosphorylation site in the intracellular N-terminus is an important amino acid which functions as a volume sensor of the channel, as mutagenesis abolished swell activation (Duan et al., 1999). Separate research groups have confirmed the expression of CIC-3, and found the same anion permeability ($\text{I}^- > \text{Cl}^-$, outward rectification under hypotonic stress and inhibition by PKC (Duan et al., 1999a; Kawasaki et al., 1995; Srinivas et al., 1999). It should be noted that several research groups have found differences in voltage dependent inactivation and pharmacological profiles between the native volume sensitive current

and ClC-3 (Stobrawa et al., 2001; Weylandt et al., 2001). These discrepancies may arise due to experimental techniques, cell types used, protein-protein interactions, and the difficulty in controlling expression levels. The exact candidate for the native $I_{Cl,vol}$ may differ in each tissue type so conclusions made in one model system may not directly transfer to others systems (Huang, 2009; Bozeat, 2007).

1.4 Cell Swelling-Induced Cl⁻ Current ($I_{Cl,swell}$) in the Heart

One of the major homeostatic functions of all cells is volume regulation when stimulated by either intracellular or extracellular anisotonic conditions. This is normally achieved through the activation of specific channels within the plasma membrane. These volume regulatory mechanisms are termed regulatory volume increase (RVI) and regulatory volume decrease (RVD) (O'Neill, 1999). Besides maintaining cell volume, volume-regulatory mechanisms are also employed to initiate changes in cell volume, as required for cell growth and differentiation. There appears to be a direct association between volume-regulatory transporters, cell volume, cell growth and metabolism (O'Neill, 1999; Okada et al., 2001). All cell volume regulatory mechanisms involve the movement of ions across the cell membrane and/or changes in either anabolic or catabolic metabolism. These mechanisms are modified in pathophysiological changes such as ischemia (O'Neill, 1999; Okada et al., 2004; Baumgarten and Clemo, 2003; Huang, 2009; Bozeat, 2007).

In a swelling emergency most cell types respond by releasing intracellular KCl mainly via K⁺ and Cl⁻ channels leading to regulatory volume decrease (RVD) (Figure 3).

The volume-regulated chloride channels (VRCCs) which is believed to be responsible for the native $I_{Cl,swell}$ current, plays an important role in cell volume regulation. Blockage of Cl^- channels has been shown to suppress RVD in many cell types (Okada, 1997; Okada et al., 2004). Oppositely RVI occurs following cell shrinkage, and this involves the uptake of solutes and the passive diffusion of water to restore cell volume (Okada, 1997; Huang, 2009; Bozeat, 2007).

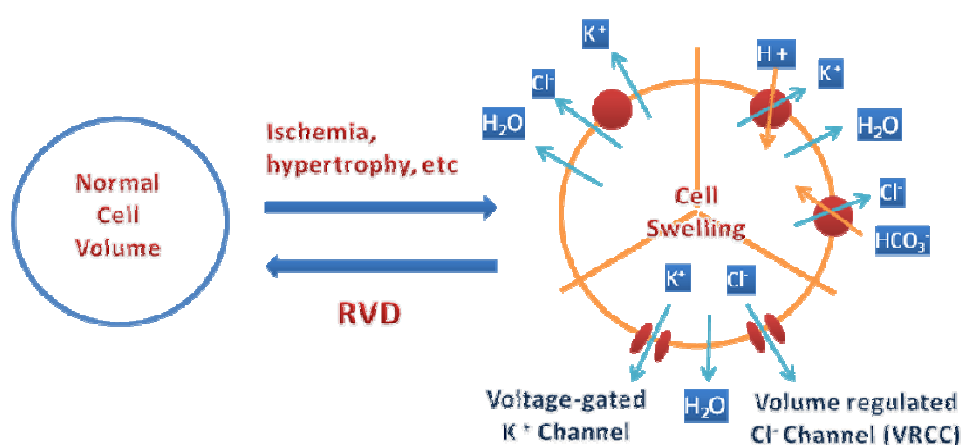


Figure 3 Schematic model for the molecular mechanism of Regulatory Volume Decrease (RVD).

Under ischemia or hypertrophy condition, the cells become swelled. Cardiac cells attempt to maintain their normal cell volume through activating multiple mechanisms, which move K^+ and Cl^- out of the cell, and therefore bring water out of the cell. The volume-regulated K^+ channels and volume-regulated Cl^- channels are involved in this process.

$I_{Cl,swell}$ was first identified in canine atrial and ventricular myocytes when the myocytes were subjected to hypotonic solutions to induce cell swelling (Benitah et al., 1997). Similar currents were also observed later in other species including human, dog and rabbit (Patel et al., 2003). A basally active Cl^- current ($I_{Cl,b}$) was first discovered in rabbit atrial myocytes. Later studies found that $I_{Cl,b}$ can be further activated by hypotonic cell swelling and inhibited by extracellular hypertonicity. $I_{Cl,swell}$ and $I_{Cl,b}$ share similarities in I-V relationship, single-channel conductance, and pharmacological

properties, suggesting that they may be the same VRCCs (Patel et al., 2003; Huang, 2009; Bozeat, 2007).

The significance of $I_{Cl,swell}$ for cardiac function under physiological and pathophysiological conditions remains unclear. On the basis of the magnitude and voltage dependence of the current, activation of $I_{Cl,swell}$ is thought to modulate cardiac electrical activity (Vandenberg et al., 1997; Du and Sorota, 1997). Another situation where $I_{Cl,swell}$ may be important is heart failure. The hemodynamic perturbations responsible for the development of heart failure and cellular hypertrophy produce mechanical stress on myocytes, leading to complex cellular remodeling (Sadoshima and Izumo, 1997) and activation of multiple intracellular signaling systems (Packer, 1996; Huang, 2009; Bozeat, 2007).

Clemo's study demonstrated that a Cl^- current is active under isosmotic conditions in canine heart failure myocytes (Clemo et al., 1999). Benitah reported that a 9-AC sensitive Cl^- current was present in rat ventricular myocytes acquired from a pressure-overload model (aortic constriction) of hypertrophy but not in normal cells (Benitah et al., 1997). In hypertrophied cardiac myocytes VRCCs are constitutively activated. Persistent activation of $I_{Cl,swell}$ during the myocardial remodeling associated with hypertrophy and failure is a common feature of several models in several species. VRCCs were previously found to be constitutively activated not only in hypertrophied ventricular myocytes of rat (Benitah et al., 1997), dog and rabbit heart failure models (Clemo et al., 1998; Clemo et al., 1999; Clemo et al., 2001) but also in human atrial myocytes obtained from patients with elevated left ventricular end-diastolic pressure and right atrial enlargement (Patel et al., 2003). Therefore, it is possible that upregulation of VRCCs activity is a common

adaptive response of cardiac myocytes to hypertrophy or heart failure-induced remodeling although it has not been reported whether VRCCs are also constitutively activated in the mouse heart during the development of myocardial hypertrophy or heart failure in the face of pressure-overload. (Huang, 2009; Bozeat, 2007)

1.5 the *DoxyhsClcn3*^{-/-} Genotype

VRCCs are broadly distributed throughout the heart and other tissues. In hypertrophied cardiac myocytes the VRCCs are constitutively activated, implicating a potential functional role of VRCCs in heart failure (Clemo et al., 1999). Many studies have provided strong evidence for CIC-3 as the gene encoding VRCCs in various cell types (Duan et al., 1997b; Huang et al., 2001; Ogura et al., 2002; Schmieder et al., 2001; Isnard-Bagnis et al., 2003; Olsen et al., 2003; Olsen et al., 2003; Wang et al., 2003; Olsen et al., 2003; Stobrawa et al., 2001). Both VRCCs and CIC-3 have been suggested to play an important role in modulating physiological functions including cell volume regulation, progression of cell cycle, cell proliferation and apoptosis.

The original demonstration of the presence of native VRCCs in hepatocytes and pancreatic acinar cells from the *Clcn3* global knockouts (*Clcn3*^{-/-}) mice (Stobrawa et al., 2001) has been considered strong evidence arguing against a significant role of CIC-3 as a molecular candidate responsible for native VRCCs (Wills and Fong, 2001; Jentsch et al., 2002; Li and Weinman, 2002; Nilius and Droogmans, 2003). There are numerous examples of activation of compensatory mechanisms in response to conventional gene inactivation, which can complicate the accurate assessment of the phenotypic impact of

the gene in question. Yamamoto-Mizuma et al compared the properties of native VRCCs in atrial myocytes from *Clcn3*^{+/+} and *Clcn3*^{-/-} mice to determine whether the presence of VRCCs in myocytes from *Clcn3*^{-/-} mice can be attributed to lack of involvement of CIC-3 as an endogenous protein responsible for native VRCCs, as previously suggested (Stobrawa et al., 2001), or whether the properties of VRCCs in these cells may differ. The altered properties of VRCCs in *Clcn3*^{-/-} mice may be due to compensatory expression of a protein unrelated to the CIC Cl⁻ channel family, which may act to maintain the fundamental role of VRCCs in cell volume regulatory mechanisms in *Clcn3*^{-/-} mice (Arreola et al., 2002). Conventional methods to produce targeted inactivation of the murine *Clcn3* gene result in complex compensatory changes in the expression of a variety of membrane proteins. These changes must be carefully considered when interpreting the physiological consequences of loss of CIC-3. Thus the exact physiological role of CIC-3 is still controversial (Yamamoto-Mizuma et al., 2004b).

To overcome the shortcomings of the global knockouts a novel heart-specific inducible CIC-3 knockout (*doxyhsClcn3*^{-/-}) mouse was developed to use. Our group took advantage of the heart-specific α -myosin heavy chain (*α -MHC*) promoter. The Nevada Transgenic Center produced *doxyhsClcn3*^{-/-} mice, by breeding *Clcn3*^{-/-} mice with tetO-*Cre* and *α -MHC-tTA* animals. The *doxyhsClcn3*^{-/-} mice are regularly maintained on a special doxycycline diet (on-Doxy) to preserve *Clcn3* gene expression in the heart. Removal of doxycycline food (off-Doxy) activates *Cre* recombinase and starts to inactivate the *Clcn3* gene only in the heart in adult mice. This special transgenic design allows the study of possible phenotypic changes after the heart-specific deletion of the *Clcn3* gene within a well-controlled time frame. Compared to the conventional global

gene inactivation method, our newly generated novel transgenic mouse lines with the *doxysClcn3^{-/-}* manipulations have the great advantage of avoiding the possible complex compensatory phenotypic alterations which have been demonstrated to occur in the conventional *Clcn3^{-/-}* animals (Yamamoto-Mizuma et al., 2004a). Thus they can serve as better animal models and represent new approaches to resolve the current controversies concerning the CIC-3 chloride channel as a molecular contender for native VRCCs in mammalian heart.

1.6 Hypothesis and Objective of Thesis

This study will obtain substantial evidence and gain crucial knowledge on the novel function and mechanistic basis of CIC-3/VRCCs in myocardial hypertrophy and heart failure, which will significantly advance our understanding of the integrated physiology and pathophysiology of the underappreciated Cl⁻ channels in the heart. The identification of CIC-3/VRCCs as novel molecular interventional target that mitigates pressure-overload induced heart failure may reveal a previously unknown cardioprotective mechanism and set the stage for the development of new therapeutic strategies and translate these preclinical findings into clinical treatment of heart failure.

The objective of this study is to test the hypothesis that CIC-3 gene may be involved in the molecular mechanism of the constitutively activated VRCCs in hypertrophied cardiac myocytes and to test the hypothesis that CIC-3 plays an important role in the structural remodeling of the heart during pressure-overload and the development of myocardial hypertrophy and its progression to heart failure.

Chapter 2 Materials and Methods

The present study conformed to the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and was approved by the University of Nevada Institutional Animal Care and Use Committee.

2.1 Generation of the *DoxyhsClcn3*^{-/-} Mice

A genomic clone containing a 10.8 kb fragment of the mouse *Clcn3* gene was isolated from a mouse 129 genomic DNA library. The short arm, *exon 3* and the long arm were amplified from this genomic clone using *Pfu turbo* (Stratagene, La Jolla, CA). A 1,141bp fragment constituting the short arm was cloned into the *XhoI* sites of *pFloxP-flp-neo* vector (Dr. James Shayman, University of Michigan, MI). A 957bp fragment containing *exon 3* was cloned into the *BamHI* sites. Finally, a 3,903bp fragment containing *exon 4* and *exon 5* was cloned into the *SwaI* sites of the *pFloxP-flp-neo* vector. The *pFloxP-flp-neo-Clcn3* targeting vector was verified by DNA sequencing and in the resulting targeting construct *exon 3* was flanked by *loxP* sites.

Fifty micrograms of linearized plasmid was electroporated into R1 mouse embryonic stem (ES) cells maintained on a feeder layer of irradiated mouse fibroblasts. R1 ES cells were grown in DMEM supplemented with 15% FCS and 1000U/ml recombinant leukemia inhibitory factor (Chemicon, Temecula, CA) and selected with medium that contained 0.3 mg/ml G418 (Invitrogen). DNA was extracted from G418 resistant ES cell colonies which were then digested with *PstI* and the resultant Southern blot probed using

a DNA probe located outside the targeted region. The wild-type allele generated a 6kb band while the target allele produced a 3.4kb band (Figure 4B). Positive ES cell clones were microinjected into C57BL/6J blastocysts. Chimeric mice were mated with C57BL/6J animals to produce agouti pups that showed germline transmission of the conditional targeted *Cln3* allele by PCR (Figure 4C). To remove the *PGK-neo* cassette from the targeted *Cln3* allele, *Cln3*^{-/-} animals were crossed with *FLPe* mice (Jackson Laboratories, Bar Harbor, ME). Loss of the neomycin gene was confirmed by PCR (Figure 4D). Resultant *Cln3*^{-/-} mice that lacked the neomycin gene were bred with *tetO-Cre* (Dr. Jeffrey Gordon, Fred Hutchinson Cancer Research Institute, Seattle, WA) and *α-MHC-rTtA* (Jackson Laboratories, Bar Harbor, ME) and transgenes were followed by PCR (Figure 4E). *Cre* expression was driven by the heart-specific promoter, *α-MHC*, which restricts homologous recombination to cardiomyocytes (Moga et al., 2008). Resultant *Cln3*^{-/-}; *tetO-Cre*; *α-MHC-rTtA* mice were maintained on chow containing doxycycline (Bio-Serve, Frenchtown, NJ) which prevented expression of *Cre* recombinase and maintained normal expression of *Cln3* gene in the heart. Mice were placed on chow without doxycycline to activate *Cre* recombinase expression and inactivate the *Cln3* gene only in the heart.

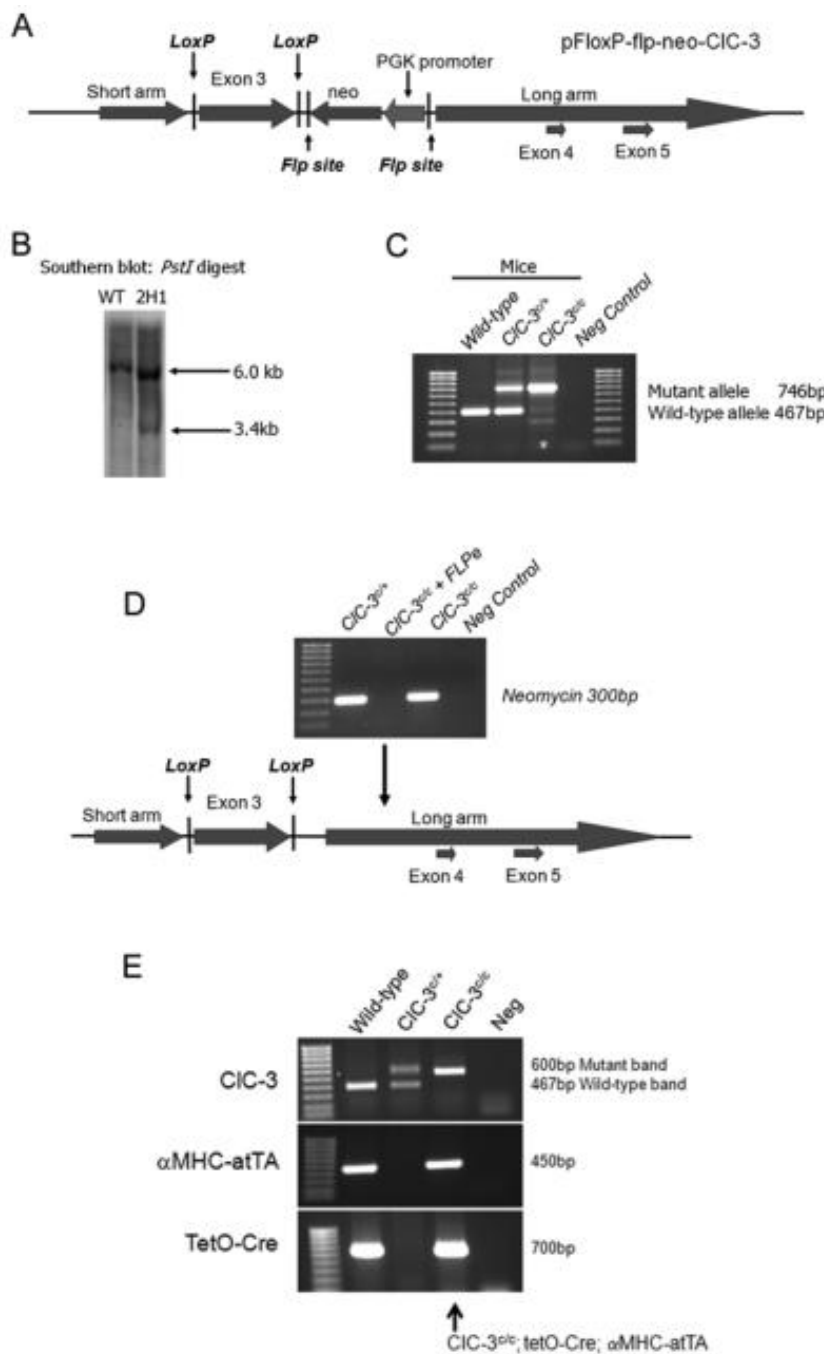


Figure 4 Production of the *doxycycline-Clcn3^{-/-}* mice.

(A) Diagrammatic representation of the *pFloxP-flp-neo-CIC3* targeting construct with *exon 3* flanked by *loxP* sites. (B) Southern blot of genomic DNA from non-transfected (wild-type) and transfected (2H1) ES cells digested with *PstI*. A single 6.0kb was detected in wild-type DNA, while a 6.0 and 3.4kb band was detected in the 2H1 targeted cell line. (C) Resultant mice were genotyped for targeted *Clcn3* gene. The wild-type *Clcn3* allele produced a 467bp band while the targeted allele produced a 746bp band. (D) Loss of the neomycin gene after *FLPe* recombination was confirmed by PCR. (E) PCR was used genotype mice at the *Clcn3* locus and for the *α MHC-atTA* and *TetO-Cre* transgenes. (Adapted from Xiong et al. 2010.)

2.2 Echocardiography

Left ventricular (LV) function was assessed by two-dimensional (2D) and M-mode echocardiography using a GE Vingmed System Five Ultrasound with a 10 MHz sector transducer (EchoPAC software version 6.2, GE Vingmed Ultrasound A/S, Horten, Norway). Briefly, mice were lightly anesthetized with isoflurane until the heart rate was maintained within the range of 550-600 beats per minute (BPM). When clear 2D images of the LV in short-axis and long-axis view were obtained, M-mode images were recorded for the measurement of end-diastolic (d) volume and end-systolic (s) volume, inter ventricular septum (IVS), left ventricular diameter (LVD), and left ventricular posterior wall (LVPW) using the leading edge-to-leading edge convention adopted by the American Society of Echocardiography (Sahn et al., 1978). The left ventricular ejection fraction ($LVEF = [(LVDd)^3 - (LVDs)^3]/LVDd^3$) and left ventricular fractional shortening ($\%FS = [(LVDd - LVDs)/LVDd] \times 100\%$) were calculated with the analysis program incorporated on the ultrasound machine. At each time point, the measurements were repeated 3 times during 3 different cardiac cycles and a mean value was used. All animals were sacrificed after the 2nd echocardiography study; the heart was excised and weighted before stored in the formalin solution for histology studies. Heart/body weight ratio was calculated and compared.

2.3 Westerns Blot Experiments

For Western blotting, whole hearts and whole brains from age-matched *doxysClcn3^{-/-}* mice, (6 on-Doxy, 6 off-Doxy for 3 weeks), were individually

homogenized in 10 mM Tris, 0.5 KCl, pH 7.4, and centrifuged for 5 min at 1,500 xg. The supernatants were centrifuged at 100,000 xg for 15 minutes. The pellets were resuspended in 10 mM Tris, 0.3 M sucrose, pH 7.4, repelleted and resuspended in the same buffer. 50µg of each crude heart and brain membrane prep was used for Western analysis, and probed with an anti-CIC3 antibody. Blots were reprobed with an anti-GAPDH antibody to confirm equal loading of protein in wells.

2.4 Whole-Cell Voltage-Clamp Experiments

Mouse atrial myocytes were enzymatically isolated from age-matched (17–21 weeks old) *doxysClcn3^{-/-}* mice and their on-Doxy control animals as described previously (Yamamoto-Mizuma et al., 2004b). Current–voltage relationships for $I_{Cl,vol}$ were generated by voltage steps ranging from –100 mV to +100 mV in 20 mV increments. To study the time-course of changes in $I_{Cl,vol}$, repetitive voltage steps at ±80 mV were applied every 30 seconds from a holding potential of –40 mV. Symmetrical chloride concentrations were used in the bath and pipette solutions. The hypotonic bath solution (220 mOsmol) contained (in mmol/L): NaCl 90; tetraethylammonium chloride 10; BaCl₂ 2.0; CaCl₂ 1.0; MgCl₂ 0.8; CdCl₂ 0.2; HEPES 10; glucose 5.5, adjusted to pH 7.4. The isotonic solution (300 mOsmol) was prepared by adding mannitol to the hypotonic solution. The pipette solution contained (in mmol/L): N-methyl-d-glutamine chloride (NMDG-Cl) 108; EGTA 5; γ-ATP 5; HEPES 5, with pH adjusted to 7.3 and osmolarity to 290 mOsmol with mannitol. Patch pipettes (1.5mm o.d. borosilicate glass electrodes) had tip resistances of 1–3Ω when filled with pipette solutions. Cell membrane

capacitances were measured by application of 2.5 ms duration 5 mV voltage steps and the area of the capacitive transient was calculated using pCLAMP software (Molecular Devices, Sunnyvale, CA, USA) following membrane rupture. Due to the small size and slow kinetics of the measured $I_{Cl,vol}$, no series resistance or capacitance compensation was utilized.

2.5 Minimally Invasive Transverse Aortic Banding (MTAB)

Mice are anesthetized with 1% isoflurane delivered via nose cone. A topical depilatory agent is applied to the neck and chest and the area is cleaned with betadine and alcohol. Mice are placed supine and temperature maintained at 37⁰C with a heating pad. A horizontal skin incision ~ 0.5 - 1.0 cm in length is made at the level of the suprasternal notch. The thyroid is retracted and a 2 - 3 mm longitudinal cut is made in the proximal portion of the sternum. This allows for visualization of the aortic arch under low power magnification. A wire with a snare on the end is passed under the aorta between the origin of the right innominate and the left common carotid arteries. A 6-0 silk suture is snared with the wire and pulled back around the aorta. A bent 27-gauge needle is then placed next to the aortic arch and the suture is snugly tied around the needle and the aorta. Following ligation, the needle is quickly removed. The skin is closed and mice are allowed to recover on a warming pad until they were fully awake. The sham procedure is identical except that the aorta is not ligated.

2.6 Data Analysis

Data will be obtained from age matched litter mates serving as control for transgenic mice and pathological models. All results will be expressed as mean \pm S.E. Statistical comparisons will be performed either by analysis of variance (ANOVA) with Scheffé contrasts for group data, or by Student's t test when only two groups are compared. A two-tailed probability of $<5\%$ is taken to indicate statistical significance.

Chapter 3 Results

3.1 Western Blot Studies in the *DoxyhsClcn3*^{-/-} Mice

To verifying the authenticity of the deletion of the *Clcn3* gene expression in cardiac myocytes of the *doxyhsClcn3*^{-/-} mice, the CIC-3 protein expressions were examined. Enzymatically isolated atrial and ventricular myocytes were collected from the age-matched *doxyhsClcn3*^{-/-} mice at 3 weeks off-Doxy and their relevant on-Doxy control mice for western blot analysis to exam the targeted CIC-3 protein expression levels. CIC-3 protein expression was decrease in whole hearts from the *doxyhsClcn3*^{-/-} mice 3 weeks off-Doxy compared to on-Doxy control hearts (Figure 5), but no differences were found in mouse brain.



Figure 5 CIC-3 protein expression in heart and brain of the *doxyhsClcn3*^{-/-} mice maintained on-Doxy, or off-Doxy for 3 weeks. Note whole heart was used for the Western blot, so non-cardiomyocytes cell types are present. (Adapted from Xiong et al., 2010).

3.2 Electrophysiological Studies in the *DoxyhsClcn3*^{-/-} Mice

To investigate the functional properties of native $I_{Cl,vol}$ in this novel gene technology mouse, the whole-cell patch clamp technique was used in enzymatically dispersed ventricular myocytes from the *doxyhsClcn3*^{-/-} mice at 3 weeks off-Doxy and from

control mice maintained on-Doxy. Freshly isolated single mouse ventricular myocytes were placed in an isotonic bath solution (300 mOsm) for ~ 5 minutes to stabilize before the perfusion of hypotonic solution (220 mOsm), which was used to swell the cell and activate native $I_{Cl,vol}$. At 3 weeks off-Doxy, the hypotonic-induced $I_{Cl,vol}$ were completely eliminated in ventricular myocytes from the *doxyhsClcn3^{-/-}* mice (Figure 6B&C), compared to the hypotonic-induced $I_{Cl,vol}$ present in ventricular cells from on-Doxy control mice (Figure 6A&C).

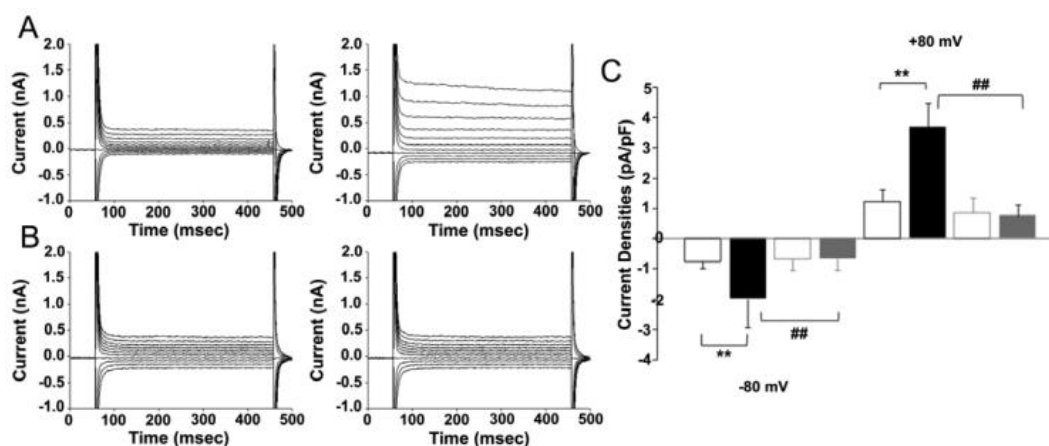


Figure 6 current traces in isotonic condition and under hypotonic challenge in the freshly isolated ventricular myocytes from age-matched *doxyhsClcn3^{-/-}* mice.

(A) Mice maintained on-Doxy, and (B) mice after 3 weeks off-Doxy. (C) is a summary of current densities under isotonic and hypotonic solutions, recorded at +80 mV and -80 mV, in freshly isolated atrial myocytes from age-matched *doxyhsClcn3^{-/-}* mice, either maintained on-Doxy (n=13, 4 mice), or off-Doxy for 3 weeks (n=20, 4 mice). Open boxes, under isotonic conditions, filled boxes under hypotonic conditions. Black boxes on-Doxy, and grey boxes off-Doxy for 3 weeks. ** p<0.01, hypotonic-induced $I_{Cl,vol}$ densities compared to isotonic conditions. ## p<0.01, hypotonic-induced $I_{Cl,vol}$ densities compared between, on-Doxy, and 3 weeks off-Doxy using ANOVA. (Adapted from Xiong et al., 2010.)

3.3 Echocardiograph Studies in the *DoxyhsClcn3*^{-/-} Mice

To understand better the morphological changes of the *doxyhsClcn3*^{-/-} mice, the whole heart were collected from the age-matched *doxyhsClcn3*^{-/-} mice at 3 weeks off-Doxy and their relevant on-Doxy control mice. Visual inspection of hearts revealed dramatically enlarged hearts from the *doxyhsClcn3*^{-/-} mice 3 weeks off-Doxy compared to age-matched on-Doxy control mice (Figure 7).

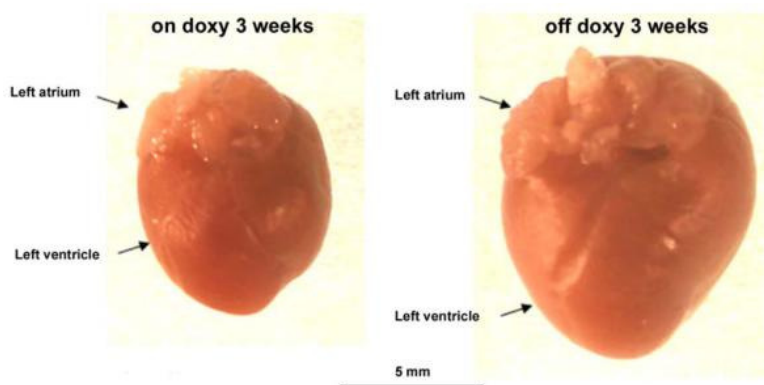


Figure 7 Comparison of hearts from age-matched *doxyhsClcn3*^{-/-} mice. (Left) 3 weeks on-Doxy, and (right) 3 weeks off-Doxy. (Adapted from Xiong et al., 2010.)

To investigate whether *doxyhsClcn3*^{-/-} resulted in any abnormal cardiac function, The M-mode echocardiogram was used to examine the LV wall thickness and cardiac function of these age-matched 3 weeks off-Doxy compared to age-matched on-Doxy control mice. At the time point of 3 weeks off-Doxy, serious signs of myocardial hypertrophy and heart failure were revealed by echocardiography recordings (Figure 8 and Table 1).

To eliminate the influence of changes in the heart rate on cardiac performance under anesthetized status a small dose of isoflurane was used to maintained within the range of

550-600 beats per minute (BPM) during echocardiograph recordings. Evaluation of systolic and diastolic LV wall thickness (IVS and LVPW), chamber dimension (LVID), and contractile function (LVEF and %FS) were recorded at the 0 week, 1.5 weeks and 3 weeks off-Doxy compared to age-matched on-Doxy control mice. These parameters revealed no significant differences in the *doxyhsClcn3^{-/-}* mice before off-Doxy. However, as shown in Fig. 7, after 3 weeks off-Doxy, significantly reduced cardiac function parameters (left ventricular ejection fraction (LVEF) and fractional shortening (%FS)) were observed in the *doxyhsClcn3^{-/-}* mice compared to the age-matched control mice maintained on-Doxy. Additionally, heart mass of the *doxyhsClcn3^{-/-}* mice turned out to be significantly increased at 3 weeks off-Doxy, compared to the age-matched on-Doxy control animals. While *Clcn^{-/-}* mice had a significant increase in heart mass, there was no statistically significant difference in body weight between the *doxyhsClcn3^{-/-}* mice and their age-matched control animals. In addition, the heart mass/body weight ratio was also significantly increased in the *doxyhsClcn3^{-/-}* mice at 3 weeks off-Doxy. To study the time-course of *doxyhsClcn3^{-/-}* on mouse heart function, we also performed echocardiography examination at an intermediate time point of 1.5 weeks off-Doxy.

Table 1, as detailed, showed that M-mode echocardiographic evaluation of systolic and diastolic left ventricular (LV) wall thickness (IVS and LVPW), chamber dimension (LVID), mass (LVM) , LVM/body weight (BW) ratio and contractile function (LVEF and %FS) revealed a significant increase in the chamber cavity (LVIDs and LVIDd), LVM, LVM/BW ratio, and a marked decrease in LVEF and %FS in the *doxyhsClcn3^{-/-}* mice off-Doxy for 1.5 and 3 weeks while no significant changes were observed in the age-matched control mice with doxycycline kept on-Doxy (on-Doxy control). These data

suggest that mice off-Doxy for 1.5 weeks are likely to have a reduction of *Cln3* gene expression that may be responsible for the observed dilated cardiomyopathy (DCM). The development of DCM was more prominent in mice off-Doxy for 3 weeks.

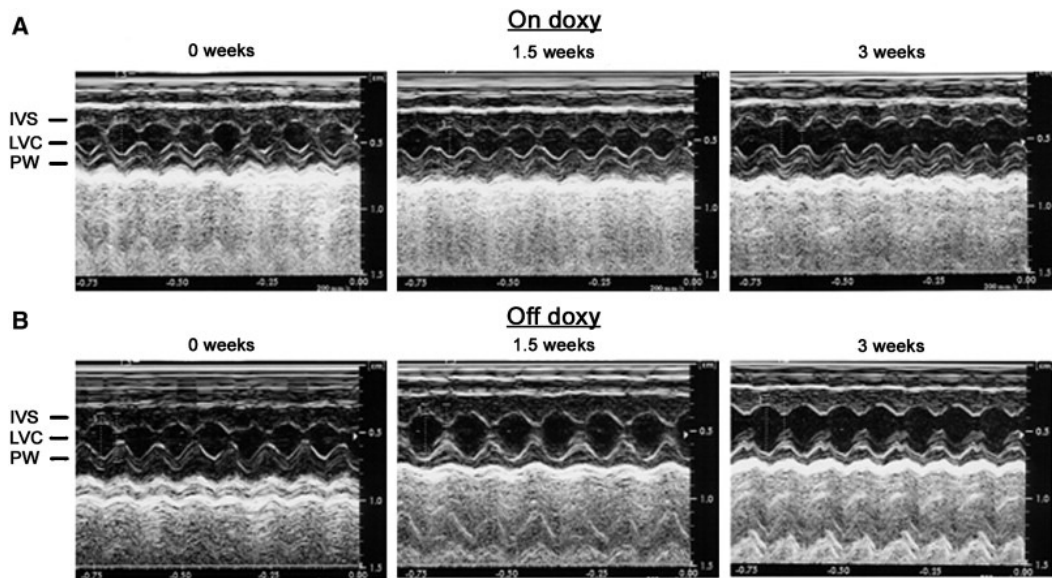


Figure 8 Representative M-mode echocardiograms of age-matched *doxyhsCln3^{-/-}* mice.

(A) Maintained on doxycycline (on doxy) or (B) without doxycycline (off doxy) for 1.5 and 3 weeks. IVS: interventricular septum; LVC: left ventricular chamber; PW: left ventricular posterior wall. The average parameters of the measurement are shown in Table 1. (Adapted from Xiong et al., 2010.)

	0 week		1.5 weeks		3 weeks	
	On Doxy	Off Doxy	On Doxy	Off Doxy	On Doxy	Off Doxy
	n=8	n=15	n=4	n=12	n=5	n=9
IVSs (mm) ¹	1.6±0.03	1.6±0.04	1.7±0.01	1.5±0.04 ^{*,##}	1.6±0.03	1.5±0.04 [*]
LVIDs (mm)	1.0±0.1	1.0±0.1	1.1±0.1	1.5±0.11 ^{***}	1.2±0.1	1.6±0.1 ^{***,#}
LVPWs (mm)	1.4±0.1	1.5±0.1	1.2±0.1	1.3±0.04	1.3±0.03	1.3±0.04
IVSd (mm)	0.8±0.02	0.8±0.02	0.8±0.02	0.8±0.02	0.8±0.02	0.8±0.02
LVIDd (mm)	2.7±0.2	2.9±0.1	2.7±0.1	3.3±0.1 ^{*,#}	2.9±0.1	3.2±0.1 ^{*,#}
LVPWd (mm)	1.1±0.1	1.1±0.03	1.0±0.04	1.1±0.04	1.0±0.1	1.1±0.1
FS (%)	64.1±1.7	67.1±1.8	59.3±1.9	54.3±1.9 ^{***}	60.5±1.8	51.6±1.8 ^{***,##}
LVEF (%)	95.1±0.7	96.0±1.6	93.0±0.9	89.8±1.2 ^{**}	93.6±0.1	88.2±1.3 ^{***,##}
Mass (mg/mm ²)	80.0±4.3	92.9±3.42	72.1±4.1	102.5±4.4 ^{##}	87.8±5.3	102.1±3.0 [#]
Body Weight (g)	34.9±2.6	43.1±1.9 [#]	33.2±3.6	41.2±2.1	38.2±1.8	36.3±2.4
Mass/BW Ratio	2.4±0.2	2.2±0.1	2.3±0.2	2.5±0.1 [*]	2.3±0.1	2.9±0.1 ^{***,#}

Table 1 Time-dependent changes in M-mode echocardiogram of age-matched *doxyhsClcn3*^{-/-} mice.

(on Doxy: with doxycycline in the diet, off doxy: withdraw of doxycycline from the diet. ^{*}P<0.05, ^{**}P<0.01, ^{***}P<0.001 vs off Doxy 0 week; [#]P<0.05, ^{##}P<0.01, ^{###}P<0.001 vs on Doxy at the same time point using ANOVA. ¹systolic and diastolic left ventricular (LV) interventricular septum and posterior wall thickness (IVS and LVPW), chamber dimension (LVID), mass (LVM), LVM/body weight (BW) ratio, and contractile function (LV ejection fraction (LVEF) and percent fractional shorting (%FS)) (Adapted from Xiong et al., 2010.)

3.4 MTAB in the *DoxyhsClcn3*^{-/-} Mice

To examine the function properties of Clc-3 gene in myocardial hypertrophy, the pressure-overload model were performed in the *doxyhsClcn3*^{-/-} mice.

Ping Hu et al reported the minimally invasive transverse aortic banding (MTAB) acutely and chronically increased LV systolic pressure, increased heart weight/body weight ratio, and induced myocardial fibrosis (Hu et al., 2003). The procedure can be performed rapidly and with low mortality. After mice were anesthetized with isoflurane, a bent 27-gauge needle was then placed next to the aortic arch between the origin of the

right innominate and left common carotid arteries, and a 6-0 silk suture was snugly tied around the needle and the aorta. After ligation, the needle was quickly removed. The procedure can be performed rapidly and with low mortality. The sham procedure was identical except that the aorta was not ligated.

An example of a mouse aorta after chronic aortic banding is shown in Figure 9. The location of the ligature between the innominate and left carotid origins is evident. Measurement of the narrowest diameter of the aortic cast (0.4 mm) was nearly identical to the diameter of the 27-gauge needle. Measurements of the cross-sectional areas of the latex cast suggested a ~90% reduction in the cross-sectional luminal area of the aorta.

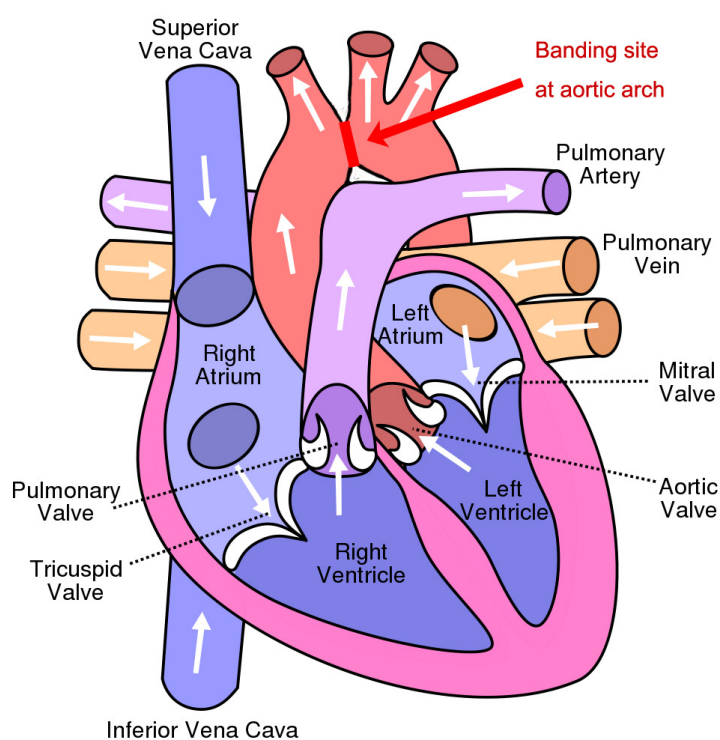


Figure 9 Banding site in aorta after minimally invasive transverse aortic banding (MTAB). Arrow demonstrates the location of constriction. (Adapted and revised from "Cardiology Doctor" website.)

The whole hearts were isolated from age-matched *Clcn3^{+/+}* and *Clcn3^{-/-}* mice after MTAB or sham operation 1 week or 10 weeks (Figure 10). After MTAB 10 weeks, the *Clcn3^{+/+}* mouse heart is significant larger than sham operation. Disruption of CIC-3 gene is significantly increased in this remodeling process. Both left ventricle and atrium were extremely enlarged after MTAB 10 weeks in *Clcn3^{-/-}* mice.

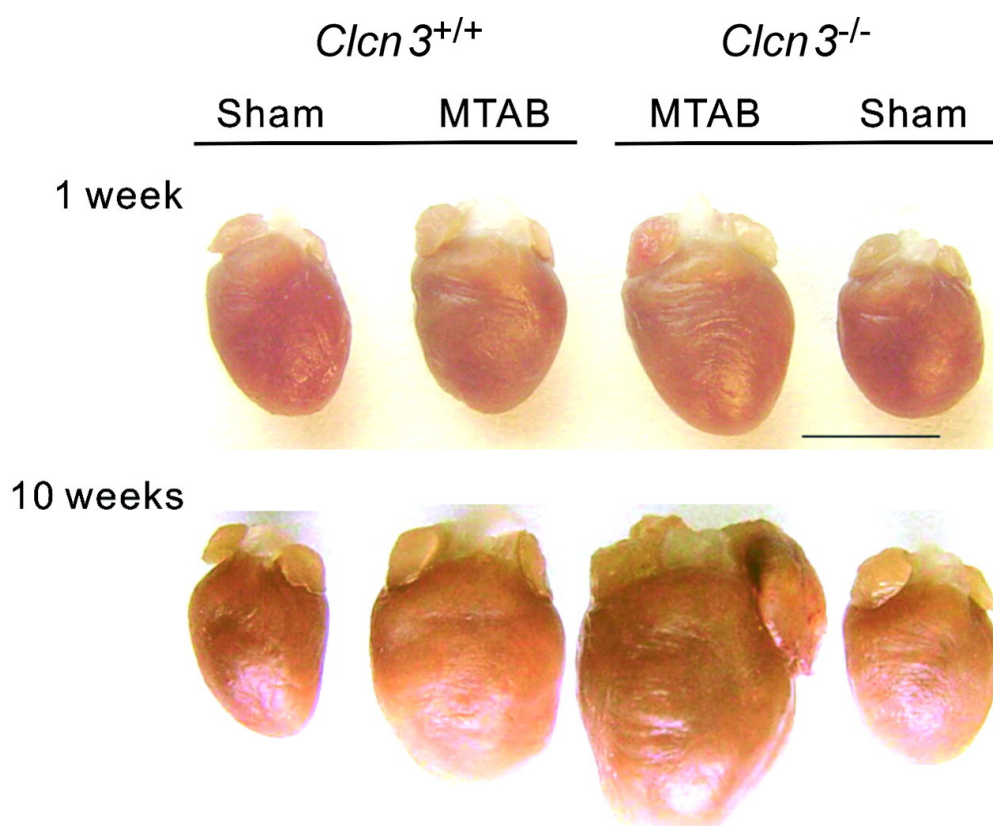


Figure 10 Comparison of pressure overload-induced heart remodeling of *Clcn3^{+/+}* and *Clcn3^{-/-}* mice after MTAB or Sham (no aorta banding) 1 week and 10 week.

Hearts after operation were cleaned up blood and connective tissues, and then weighed and fixed in 4% paraformaldehyde (a, Bar=5 mm). Disruption of CIC-3 gene significantly changed the remodeling process after MTAB. Both left ventricle and atrium were extremely enlarged after 10 weeks of MTAB in *Clcn3^{-/-}* mice (Adapted from Duan, J Physiol., 2009).

3.5 Echocardiograph Studies in *DoxyhsClcn3*^{-/-} Mice after MTAB

MTAB was applied to the *doxyhsClcn3*^{-/-} mice during on-Doxy (as control) and off-Doxy and the cardiac function of the mice was measured by echocardiography. Evaluation of systolic and diastolic LV wall thickness (IVS and LVPW), chamber dimension (LVID), and contractile function (LVEF and %FS) revealed no significant differences before surgery in *doxyhsClcn3*^{-/-} mice. When *doxyhsClcn3*^{-/-} mice were subjected to MTAB no significant increase in LVM and LVIDs or decrease in %FS was observed until 3 weeks of off-Doxy mice. After 3 weeks, the increase in LVIDs and LV mass became much more rapid accompanied with progressively reduced %FS in off-Doxy mice compare with the age-matched control mice with doxycycline kept on-Doxy (Figure 11).

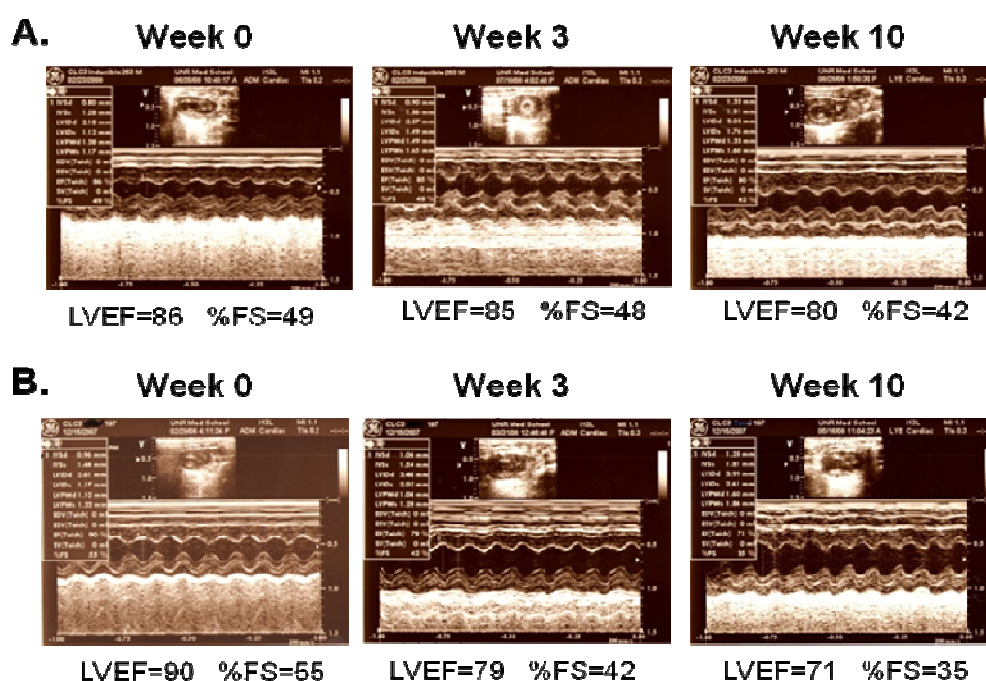


Figure 11 Representative echocardiograph of age-matched *doxyhsClcn3*^{-/-} mice maintained (A) with doxycycline (on doxy) or (B) without doxycycline (off doxy) before and after MTAB for 3 and 10 weeks.

(LVEF: left ventricular ejection fraction, %EF: left ventricular fraction shortening.)

The whole hearts were taken out from on-Doxy and off-Doxy *doxyhsClcn3^{-/-}* mice after MTAB 10 weeks (Figure 12). Both atrial and ventricular of off-Doxy *doxyhsClcn3^{-/-}* mice hearts were dramatically larger than on-Doxy controls.

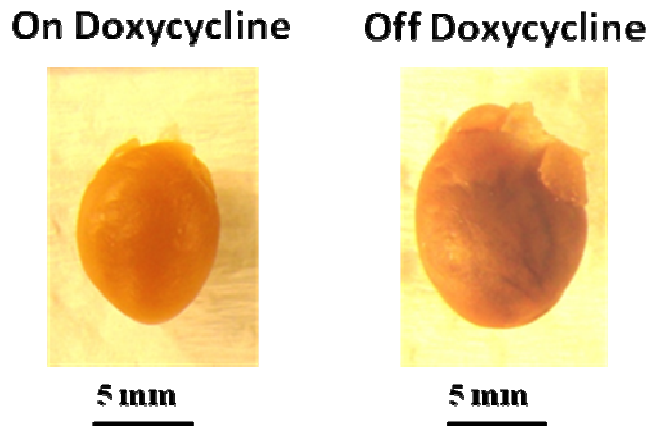


Figure 12 Representative isolated hearts of age-matched *doxyhsClcn3^{-/-}* mice maintained (left) with doxycycline (on doxy) or (right) without doxycycline after MTAB 10 weeks.

Figure 13 shows that with the time course, the LV mass were both increase in on-Doxy and off-Doxy *doxyhsClcn3^{-/-}* mice after MTAB. This increase is significantly faster in the off-Doxy mice than on-Doxy controls.

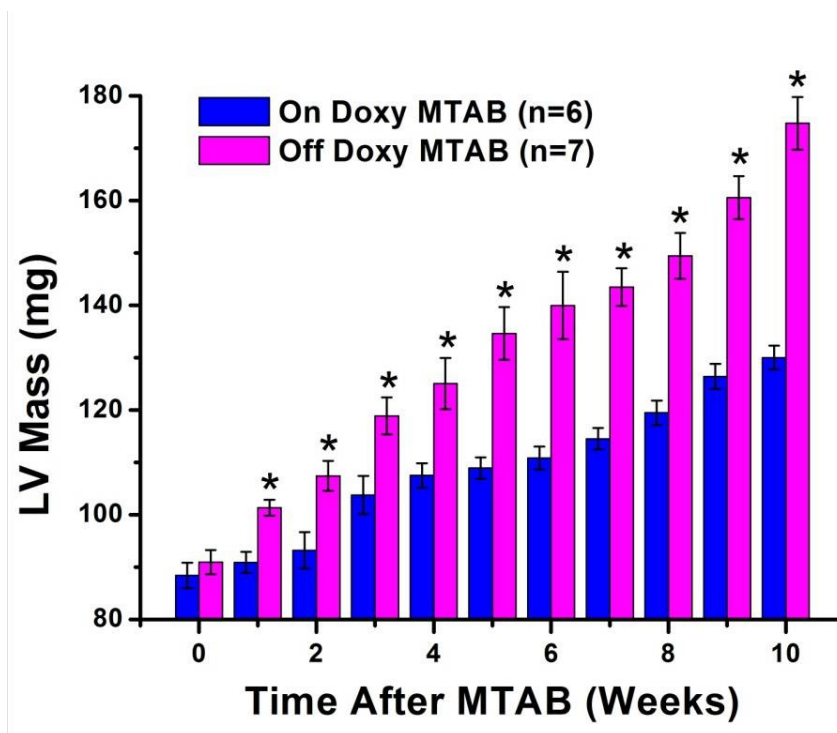


Figure 13 Time-course of changes in left ventricular mass after MTAB in age-matched *doxysClcn3^{-/-}* maintained (blue) with doxycycline (on doxy) or (pink) without doxycycline (off doxy).

Data are expressed as mean±S.E.* $P < 0.05$.

The left ventricular dimension was significantly increased in off-Doxy mice after MTAB 10 weeks, and no significant change was observed in on-Doxy mice (Figure 14).

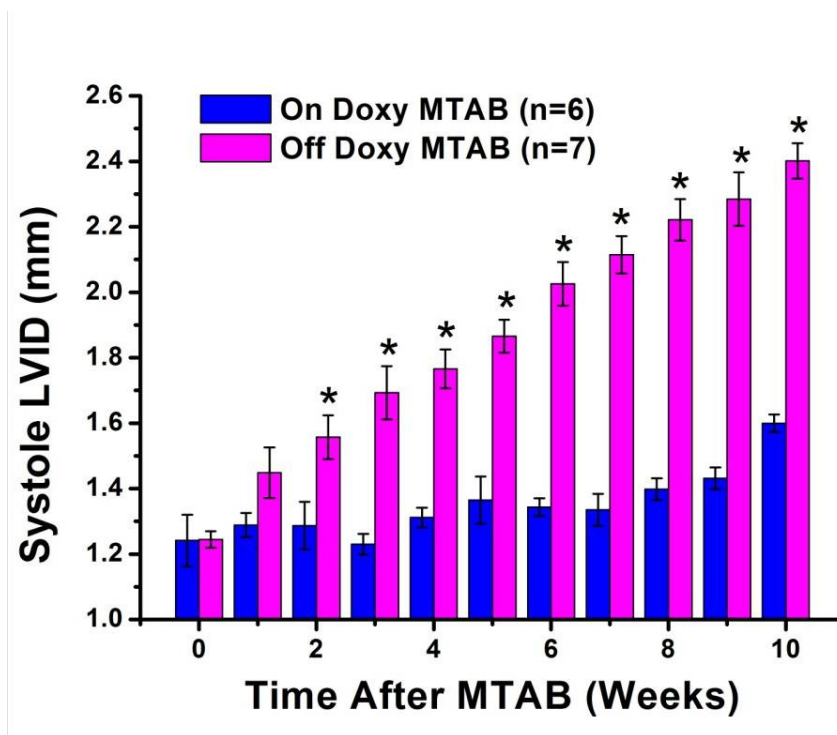


Figure 14 Time-course of changes in left ventricular dimension of age-matched *doxysClcn3^{-/-}* mice maintained (blue) with doxycycline (on doxy) or (pink) without doxycycline (off doxy) after MTAB 1 - 10 weeks.

Data are expressed as mean±S.E.* $P < 0.05$.

Left ventricular ejection fraction (LVEF) and fractional shortening (%FS) were both decreased in on-Doxy and off-Doxy of the *doxysClcn3^{-/-}* mice after MTAB 10 weeks (Figure 15). This decrease was significantly faster in the off-Doxy mice.

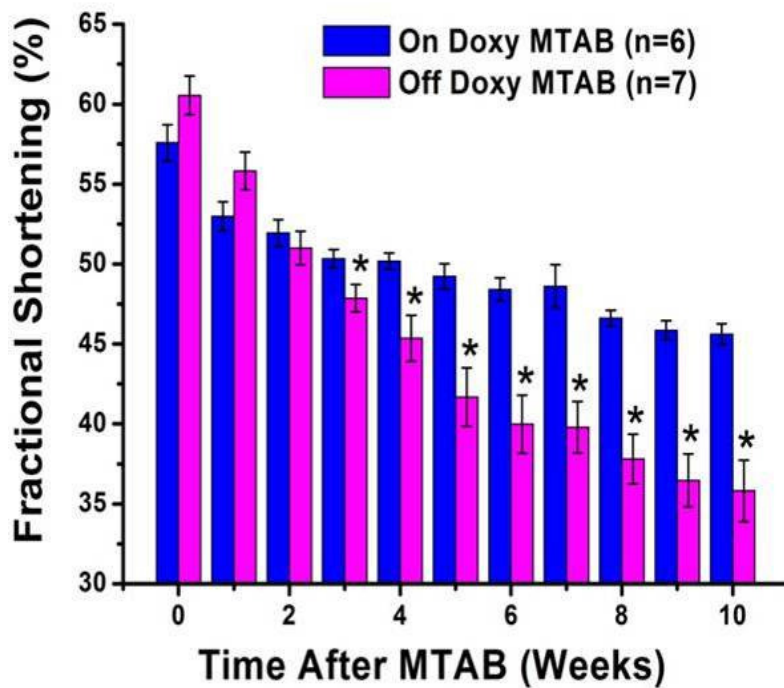
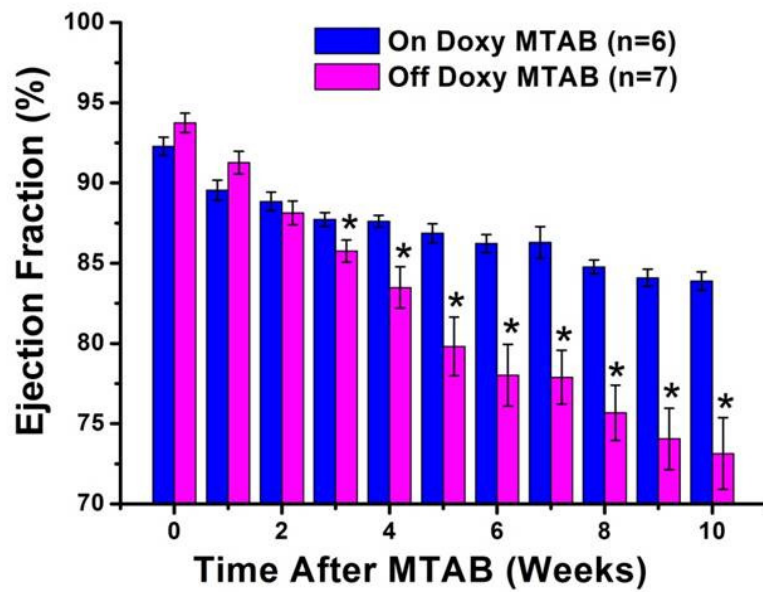


Figure 15 Time-course of changes in left ventricular function of the *doxysClcn3^{-/-}* mice after MTAB in age-matched (blue) on-Doxy and (pink) off Doxy.

Data are expressed as mean±S.E.* $P < 0.05$.

Chapter 4 Discussion

Previous studies of the potential role of CIC-3 as VRCCs based on a classic global CIC-3 knockout (*Clcn3*^{-/-}) mouse model are difficult to be interpreted due to the complicated compensational effects of gene knockout. Thus the exact physiological role of CIC-3 is still controversial. To overcome the shortcomings of the global knockouts a novel heart-specific inducible CIC-3 knockout (*doxysClcn3*^{-/-}) mouse was developed to determine the actual physiological role of CIC-3 in the heart. These novel line of transgenic mice were developed by the Nevada Transgenic Center by utilized the tetO-Cre approach to conditionally inactivate *Clcn3* gene in mouse heart. The advantages of this approach include elimination of possible confounding effects if *Clcn3* gene is required for mouse development, organ specific *Clcn3* gene inactivation, and temporal control of Cre expression and *Clcn3* gene deletion.

Western blot studies shows that CIC-3 protein expression is decreased in whole hearts from mice 3 weeks off-Doxy compared to on-Doxy control hearts, but no difference is found in mouse brain. CIC-3 protein expression was not completely eliminated in whole hearts at 3 weeks off-Doxy, since CIC-3 protein expression is not expected to be affected in other cell types in heart such as nerve, smooth muscle, fibroblasts.

Electrophysiological examination of native $I_{Cl,vol}$ in isolated ventricular myocytes 3 weeks off-Doxy revealed a complete elimination of the currents, whereas at 1.5 weeks, $I_{Cl,vol}$ densities were significantly reduced compared to age-matched control mice maintained on-Doxy. The results suggest that, after 3 weeks off-Doxy, the hypotonic-induced $I_{Cl,vol}$ were completely eliminated compare with on-Doxy. CIC-3 may responsible

for $I_{Cl,vol}$ in mouse hearts. Myocardial cells are known to swell during ischemia, dilated cardiomyopathy and heart failure. Native $I_{Cl,vol}$ have been shown to be persistently activated during these conditions and likely play a protective role in helping maintain cardiac cell volume. The myocardial hypertrophy and dilated cardiomyopathy characteristic of the *doxyhsClcn3*^{-/-} mice 3 weeks off-Doxy is precisely the phenotype expected due to loss of an ion channel intimately involved in cell volume homeostasis.

Visual inspection of hearts revealed dramatically enlarged hearts from the *doxyhsClcn3*^{-/-} mice 3 weeks off-Doxy compared to age-matched on-Doxy control mice. The morphological changes indicate the structure remodeling of cardiac myocytes in the *doxyhsClcn3*^{-/-} mice after 3 weeks off-Doxy.

To investigate whether CIC-3 deletion resulted in abnormal hemodynamic performance and ventricular wall thickness M-mode echocardiogram (ECHO) was utilized. ECHO was used before off-Doxy and after off-Doxy 1.5 and 3 weeks in the *doxyhsClcn3*^{-/-} mice (Table 1). Due to the effects of the anesthetic on HR, recordings were made at 600±50 bpm. The results indicate that at 3 weeks off-Doxy, *doxyhsClcn3*^{-/-} in mice resulted in a significant increases in heart mass and heart mass/body weight ratio compared to the age-matched on-Doxy control mice. Serious signs of myocardial hypertrophy and heart failure were revealed by echocardiography in the *doxyhsClcn3*^{-/-} mice 3 weeks off-Doxy, compared to age-matched on-Doxy control mice. There was significantly reduced left ventricular ejection fraction and fractional shortening. These results suggested that CIC-3 normally plays a protective role against the development of myocardial hypertrophy.

MTAB was used to produce the pressure overload model in *Clcn3*^{-/-} mice. Both left ventricle and atrium were extremely enlarged after MTAB 10 weeks. Disruption of *Clcn3* gene significantly increased this remodeling process. This is consistent with a previous report that global deletion of the *Clcn3* gene in mice facilitated the pathological development of myocardial hypertrophy and heart failure in a pressure overload transverse aortic banding animal model.

Echocardiography was used before and after MTAB 10 weeks in the *doxyhsClcn3*^{-/-} mice both on-Doxy (as control) and off-Doxy groups. M-mode echocardiogram revealed a significant fast reduction in left ventricular ejection fraction (LVEF) in off-Doxy groups compared to on-Doxy groups after MTAB 3 weeks. Off-Doxy mice have a significant fast increase in LVEF compared to on-Doxy group. The LV mass in off-Doxy mice was increased significantly ($P < 0.05$) faster compared to on-Doxy group. These results indicate that activation of the CIC-3/VRCCs may be detrimental and make the pressure-overload-induced hypertrophic remodeling worse, and *Clcn3* knockout should protect the heart and prevent the heart against dilated cardiomyopathy.

Heart failure claims over 200,000 lives annually in the US alone. Myocardial hypertrophy and its progression to heart failure or dilated cardiomyopathy are characterized by structural remodeling and electrical remodeling. However the functional role of anion (Cl^-) channels in human cardiac diseases is unknown. VRCCs are broadly distributed throughout the heart and other tissues. Recent studies strongly suggest CIC-3, a member of the CIC voltage-gated Cl^- channel family, as the gene encoding VRCCs in various cell types. The properties described for CIC-3 channels are very similar to those of the constitutively-activated $I_{\text{Cl,vol}}$ in human and animal cardiac myocytes. Both VRCCs

and CIC-3 have been suggested to play an important role in modulating physiological functions including cell volume regulation, progression of cell cycle, cell proliferation and apoptosis. But it is unknown how activation of $I_{Cl,vol}$ would affect the structural remodeling in human and animal failing hearts. This study obtained substantial evidence and gained crucial knowledge on the novel function and mechanistic basis of CIC-3/VRCCs in myocardial hypertrophy and heart failure, which will significantly advance our understanding of the integrated physiology and pathophysiology of the underappreciated Cl^- channels in the heart. The identification of CIC-3/VRCCs as novel molecular interventional target that mitigates pressure-overload induced heart failure may reveal a previously unknown cardioprotective mechanism and set the stage for the development of new therapeutic strategies and translate these preclinical findings into clinical treatment of heart failure.

Chapter 5 Conclusions

In this study, we tested our hypothesis that CIC-3 is a key component of native $I_{Cl,vol}$ in mouse heart and activation of CIC-3/VRCCs provides a novel cardioprotective mechanism against decompensatory structural remodeling and progression to heart failure. A novel line of the *doxysCln3*^{-/-} mice greatly moves forward our previous research with mammalian cardiac CIC-3 chloride channel and helps to better elucidate the actual physiological role and pathological significance of CIC-3 chloride channel in mammalian cardiac function and its essential role in native VRCCs in mammalian cells.

Immunodetection methods were utilized to assess the CIC-3 protein expression in the *doxysCln3*^{-/-} mice. Whole-cell patch clamp techniques were used to investigate and characterize the basic properties of native $I_{Cl,vol}$ in freshly isolated mouse cardiac myocytes from the heart-specific CIC-3 transgenic mouse line and their relevant age-matched control animals. Echocardiography techniques were utilized to assess mouse heart function and to further evaluate the possible physiological role and pathophysiological significance of CIC-3 in mammalian heart with heart-specific CIC-3 transgenic manipulations.

Cardiac *Cln3* gene was inactivated after Off Doxy for 3 weeks, which also caused a marked myocardial hypertrophy and dilated heart failure under normal conditions without MTAB. MTAB induced the time course of the changes in ventricular chamber, wall thickness and cardiac function were closely correlated to the changes in $I_{Cl,vol}$ density, implicating that the structural remodeling and heart failure may be a result of inactivation of CIC-3/VRCCs function in the heart. Alternatively, MTAB induced myocardial

hypertrophy and progressive dilated heart failure a significantly faster pace, implicating an important protective role of CIC-3 in structural and functional remodeling of the heart in response to pressure overload.

Chapter 6 Future Directions

Chloride is the most abundant extracellular anion of different organisms and chloride transport proteins in mammalian heart have been shown to play many important functional roles in a great variety of different processes in cardiac physiology and pathophysiology. The CIC-3 chloride channel is a broadly expressed mammalian CIC gene family member, whose actual physiological function and biological role has been an issue of hot debate for many years. Although convincing data have been accumulated from different labs for years, the hypothesis that CIC-3 chloride channel is the molecular correlate of native VRCCs has been controversial. In this study, we investigated the molecular, electrophysiological and pharmacological properties of CIC-3 channels in the heart, as well as the molecular and functional role of CIC-3 channels in the heart. These studies not only provided compelling evidence for the important and novel physiological function of the newly identified CIC-3 channels in the heart but also opened a new field for future study of the cardiac physiology and for the development of new therapeutic approaches to the treatment of cardiac diseases such as myocardial hypertrophy and heart failure.

The mechanism responsible for the CIC-3/VRCCs protect the heart and prevent the heart against dilated cardiomyopathy needs further investigation. To accomplish this goal, we only used tissue-specific conditional and inducible “loss-of-function” strategies to specifically manipulate expression of CIC-3 Cl^- channel gene, which has been implicated to encode VRCCs, in the heart and apply established multiple approaches and disease models to the genetically-engineered mice to delineate the role and mechanism of

CIC-3/VRCCs in cardiac physiology and heart failure. One important alternative approach we will take to address this question is to overexpress *Clcn3* (“gain-of-function”) in the mouse heart and examine the MTAB-induced changes in $I_{Cl,vol}$ expression of CIC-3 in the heart.

To gain mechanistic insights into the effects of CIC-3 knock-out or overexpression on cardiac electrophysiology $I_{Cl,vol}$ and action potentials will be recorded from left atrial and ventricular myocytes isolated from on-Doxy and off-Doxy *doxyhsClcn3^{-/-}* mouse hearts subjected to MTAB or sham operations at different time points. The time-course of changes in APD will be analyzed to answer the question whether knockout *Clcn3* gene causes any changes in APD when compared with wild type mice subjected to MTAB.

Structural remodeling of hypertrophic and failing heart involves excessive cell volume increase and dilated myocyte membrane stretch, which alter not only cell volume homeostasis but also many cellular functions including cell proliferation, differentiation, and apoptosis. It has been well-established that VRCCs and CIC-3 Cl^- channels play a pivotal role in the RVD (Jin et al., 2003; Mao et al., 2008; Sardini et al., 2003; Wang et al., 2000; Xiong et al., 2009), a major mechanism for maintenance of cell volume homeostasis. Increased production of ROS and oxidative stress has also been implicated a central role of in the pathophysiology of failing human and animal hearts (Dai et al., 2005). Oxidative stress upregulates the CIC-3 expression and the VRCCs function (Ramana et al., 2004; Remillard and Yuan, 2005; Dai et al., 2005). CIC-3 up-regulation in pulmonary artery smooth muscle improved cell viability against oxidative stress and protected cells from oxidative stress-induced necrosis, thereby improving cell survival and promoting medial hypertrophy (Hawkins et al., 2007). In smooth muscle and

epithelial cells CIC-3 also regulates Nox, which is a ubiquitous generator of ROS involved in structural remodeling of the heart and vasculature during hypertension. It has been shown that superoxide can pass through CIC-3 channels and flux across vascular endothelial cell plasma membrane (Wei et al., 2004). VRCCs and CIC-3 channels are also important modulators of apoptosis (Lemonnier et al., 2004; Guan et al., 2006; Zhang et al., 2006; Sarkar et al., 2004). It has been suggested that apoptosis plays a driving role in the transition from compensatory myocardial hypertrophy to dilated cardiomyopathy and heart failure (Latif et al., 2000). Indeed, both pro- and anti-apoptotic Bcl-2 family proteins are upregulated in patients with end-stage heart failure (Lemonnier et al., 2004). The significant increases in the pro-apoptotic proteins Bak and Bax are highly correlated to the higher percentage of TUNEL-positive cells in the failing heart, suggesting actively ongoing apoptosis. However, the anti-apoptotic proteins, Bcl-2 and Bcl-xL, are increased in the hypertrophied myocardium, suggesting a possible concomitant, compensatory anti-apoptotic mechanism in the diseased heart. It is interesting that Bcl-2 also induces up-regulation of $I_{Cl,vol}$ by enhancing CIC-3 expression (Lemonnier et al., 2004). Therefore, we hypothesize that the constitutive activation of CIC-3/VRCCs during myocardial hypertrophy strengthens the ability of cardiac cells to handle hypertrophic cell volume increase and oxidative stress thereby diminish their pro-apoptotic potential and promote their survival thorough enhancing RVD and regulation of redox signaling (Lemonnier et al., 2004).

It will be ideal if specific compounds for CIC-3 can be developed as pharmacological tools to address the hypotheses and to develop drugs targeting the CIC-3 gene as novel

therapeutic tools for the treatment of many cardiac and vascular diseases such as myocardial hypertrophy, hypertension, ischemia, and heart failure (Duan, 2011).

Chapter 7 References

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