7-25-2016 12:00 AM

Cardiovascular Characterization of Cx40/Panx1 Single and Double Knockout Mice

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Graduate Program in Anatomy and Cell Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

Abstract

Connexins (Cxs) and pannexins (Panxs) are protein families that form large-pore channels which exist at the plasma membrane for both intracellular and extracellular signaling. Given their potential for overlapping cellular signaling functions we proposed that mice lacking both a connexin and a pannexin would have a severe phenotype. To investigate this possibility we crossed Panx1 null mice with Cx40 knockout mice and characterized the first global connexin/pannexin double knockout mouse. Intriguingly, the combined ablation of both Cx40 and Panx1 caused decreased prenatal and newborn survival, but did not affect the fertility or lifespan of surviving mice. $Cx40^{-/-}$ and $Cx40^{-/-}Panx1^{-/-}$ mice had cardiac hypertrophy, and furthermore, combined channel ablation in double knockout animals led to increasing severe hypertension and decreased endothelium dependent vasodilation. Overall, these studies suggest that even though Panx1 and Cx40 act via differential mechanisms, they have a co-regulatory role in certain physiological processes such as vascular response.

Keywords

The following keywords can be used to describe the thesis entitled “Cardiovascular Characterization of the Cx40/Panx1 Single and Double Knockout Mice”

Large-pore channel proteins, gap junctions, connexins, pannexins, Cx40, Panx1, cardiovascular system, hypertension, heart hypertrophy, endothelial dysfunction.

Co-Authorship Statement

Thank you to Dr. Feng who was involved in the editing of Chapter 2.

Hypertension testing and vasomotor reactivity studies were performed utilizing Dr. Gros' experimental setups and he was an integral part of experimental design due to his vascular expertise and knowledge of cardiovascular system function.

Kevin Barr was the original breeder of the $Cx40^{-/-}Panx1^{-/-}$ mouse line.

Dr. Isakson was a valued consultant on this project and offered his insights.

Acknowledgments

As a Master's student in the Laird laboratory I have many individuals to thank for my success;

Firstly, I would like to thank Dr. Laird for being an incredible mentor and teaching me invaluable lessons both within and outside of the laboratory. His continuing support and guidance has helped me thrive and grow as a student, and pushed me to pursue a career in academia.

Secondly, I would like to thank Dr. Robert Gros for his aid, guidance, and willingness to teach me experimental techniques. During my time in his lab he was incredibly supportive and offered a friendly work environment that allowed me to excel.

Qing Shao and Kevin Barr were instrumental resources throughout the course of my Master's degree, and aided in my experimental technique training.

Thank you to my committee members, Dr. Bai, Dr. Feng, Dr. Gros, and Dr. Allan for providing constructive criticism and helpful suggestions as my project progressed.

Finally, I'd like to thank all members of the Laird lab and the ACB community for providing a fun academic learning experience.

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List of Abbreviations

AP	action potential
AV	atrioventricular
ATP	adenosine triphosphate
BSA	bovine serum albumin
Ca^{2+}	calcium
CT	carboxyl terminal
Cx	connexin
Cx40	connexin 40
Cx43	connexin 43
Cx45	connexin 45
E	embryonic day
EDHF	endothelium derived hyperpolarizing factor
EL	extracellular loop
ECM	extracellular matrix
GPCR	G-protein coupled receptor
GJ	gap junction
GJIC	gap junctional intercellular communication
IP_3	inositol triphosphate
IL	intracellular loop
MCh	methacholine
NT	amino terminus
NO	nitric oxide
Panx	pannexin
Panx1	pannexin 1
Panx3	pannexin 3
PE	phenylephrine
PLC	phospholipase C
K^+	potassium
PKG	protein kinase G
RAAS	renin-angiotensin-aldosterone system
SNP	sodium nitroprusside
SGC	soluble guanyl cyclase
TDA	thoracodorsal artery
TPC	triggered propagating contractions

Chapter 1

1 Introduction

1.1 Large-pore protein channels in cellular signaling

Normal cellular function, organ development, homeostasis, and repair after damage relies heavily on the initiation of a myriad of critical cellular signaling networks and the interaction of extracellular, intercellular, and intracellular cascades[1]. The connexin (Cx) and pannexin (Panx) families of large-pore forming channel proteins facilitate the passage of ions, adenosine triphosphate (ATP), and secondary messenger molecules such as calcium, inositol triphosphate (IP_3) either between cells or in the case of pannexins between a cell and the extracellular milieu[2]. Connexins may act in either intracellular signaling as functional hemichannels, or more often form dihexameric gap junction (GJ) channels for direct intercellular communication[3]. On the other hand pannexins exist at the membrane as large-pore channels that function solely in extracellular signaling[4]. It is the interplay between these key cellular signaling modalities that allows critical biochemical cascades to occur within systems that rely heavily on gap junction and paracrine communication such as the heart and vasculature[5].

1.2 Connexins form gap junctions for direct intercellular communication

Connexins are expressed throughout the body, in many different mammalian cell types. There are 21 connexin species in humans (20 in mice) and these polytopic membrane proteins are named according to the Cx prefix, followed by their predicted molecular weight in kDa[6]. Each connexin protein has a similar structural topology, and consists of four membrane-spanning domains, two extracellular loops (EL), an intracellular loop (IL), and an N-terminus (AT) and C-terminus (CT)[6]. Six Cx subunits oligomerize to form a connexon (Figure 1-1A), which may then be transported to the plasma membrane and act as a functional hemichannel in paracrine signaling[7]. Hemichannels are able to pass secondary messenger molecules such as ATP, IP_3, Ca^{2+}, and NAD^+ [7]. However, more often a connexon docks with its counterpart on an opposing

cell to form a gap junction (GJ) channel[8]. Gap junctions allow for intercellular communication via the joining of cytosolic compartments between adjacent cells[8]. Areas of high membrane apposition that contain a large number of GJ channels are termed GJ plaques[9]. GJ plaques allow for a diverse range of intercellular signaling events including the passage of ions, electrical signal, and secondary messenger molecules less than 1000 Da[9].

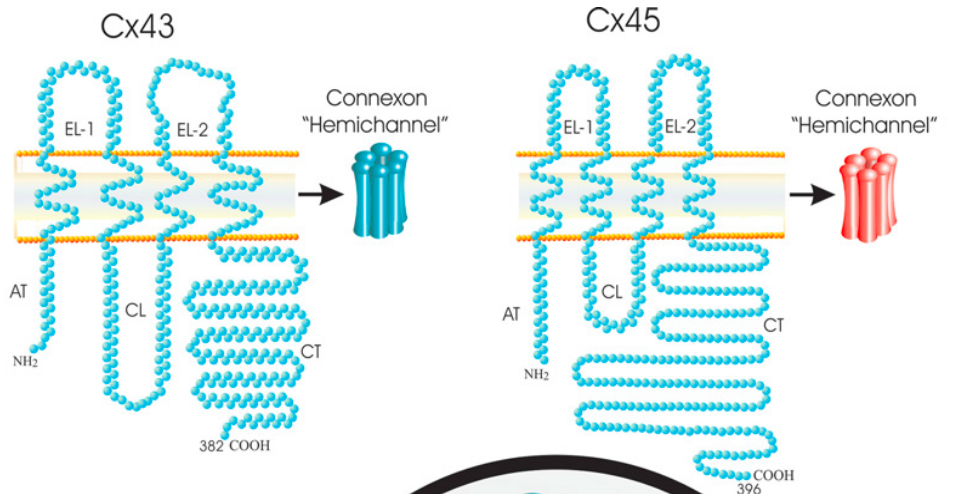
Connexons can be homomeric or heterotypic depending on if they are composed of the same Cx isoform or are mixed[10]. Furthermore GJ channels are defined as homotypic if they are comprised of the same connexon subtype, or heterotypic if they are composed of two different homomeric or heteromeric connexons (Figure 1-1B)[10]. Each connexin channel has different GJ intercellular communication capabilities regulated by variations in pore size, conductance, open channel probability, pH gating, and charge selectivity[11].

In the human population just under half of the connexin family members (10 Cx subtypes) have been linked to genetically inherited diseases[12]. These diseases range from mild to severe, and include skin abnormalities, deafness, oculodentodigital dysplasia, hypertension and cardiac arrhythmias[12]. This thesis will focus on Cx40, a connexin family member linked to hypertension and cardiac arrhythmias[13], and its roles within the cardiovascular system.

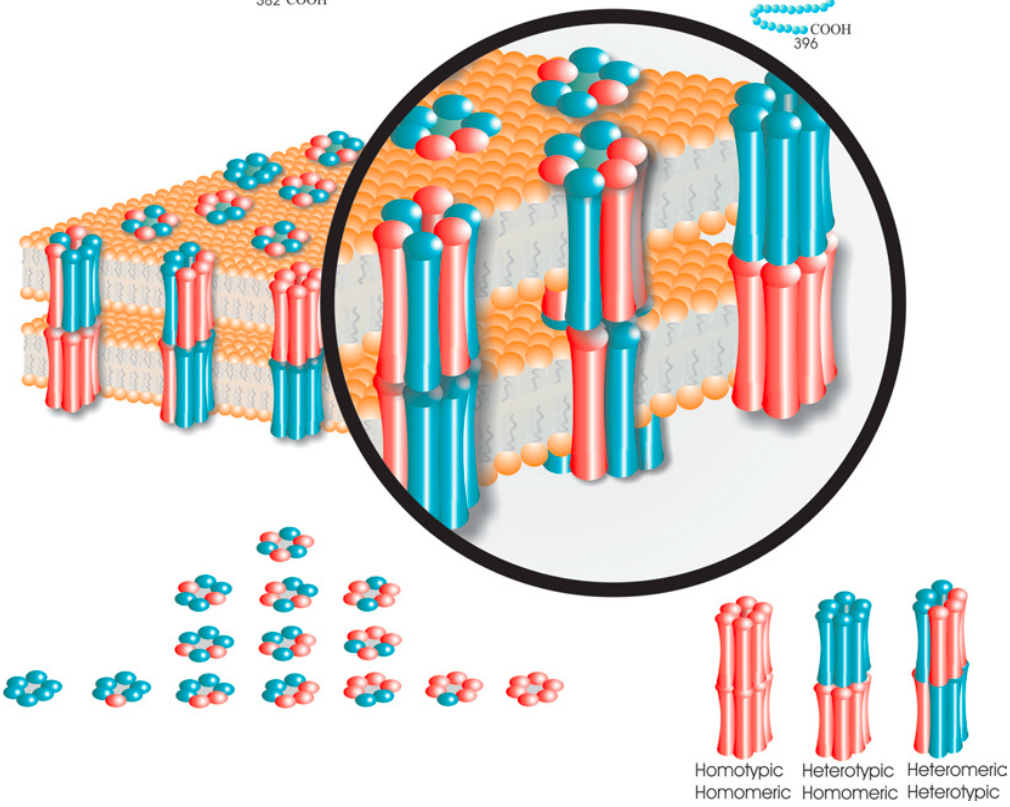
Figure 1-1. Connexins are subunit proteins that form GJs for intercellular communication

(A) A typical GJ protein contains 4 membrane spanning domains, two ELs, an IL, and an AT and CT. Six Cxs oligomerize to form a connexon. (B) Two connexons from apposing cells may dock to form GJs which may be homotypic, heterotypic, or mixed. Reused with permission: Laird DW. Life cycle of connexins in health and disease. The Biochemical Journal 2006;394:527–543.

A


Cx43
EL-1
EL-2
Connexon
"Hemichannel"
AT
CL
CT
NH2
382 COOH
Cx45
EL-1
EL-2
Connexon
"Hemichannel"
AT
CL
CT
NH2
COOH
396

B


Homotypic
Homomeric
Heterotypic
Homomeric
Heteromeric
Heterotypic

1.3 Pannexins form large-pore channels for extracellular communication

The pannexin family has three isoforms: Panx1, Panx2, and Panx3. Panx1 expression is ubiquitous, and it is found in many organs including the brain, heart, skin, spleen, lungs, blood vessels, and kidney[14, 17]. Panx2 has a more restricted expression, being localized to the central nervous system, particularly in brain structures such as the hippocampus and olfactory bulbs[14]. Finally, Panx3 has been discovered in varying levels in the arteriole networks, skin, cartilage, liver, lung, thymus, and spleen[14]. All three pannexin isoforms demonstrate similar predicted structural topology, but no sequence homology to their connexin cousins[15]. Panxs have four membrane-spanning domains, two EL's, one IL, and AT- and CT[15]. Panx1 and Panx3 are more structurally homologous, whereas Panx2 exhibits a longer C-terminus[15]. Similarly to connexins, pannexins oligomerize to form pannexons that undergo initial posttranslational modifications in the endoplasmic reticulum (N-glycosylation) before they are transported through the Golgi apparatus to the plasma membrane. There they may act as large-pore channels to facilitate extracellular signaling of small nucleotides and secondary messengers such as ATP and Ca^{2+}[16]. Interestingly, unlike connexins, pannexin 1 has a long half-life of up to 32 hours[17]. Panx1 is known to play a role in several human pathologies including cancer[18] and hearing loss[19]. Unfortunately its role as a large-pore channel in the cardiovascular system, and its potential interactions with GJs in cellular signaling cascades within these systems has yet to be fully explored.

1.4 Connexin hemichannels in paracrine signaling

Since the discovery of pannexins the idea of connexin hemichannels, or connexons, remaining undocked at the plasma membrane to participate in paracrine cellular signaling events, and particularly in the release of ATP, has been a subject of debate[12]. Under standard physiological conditions connexin hemichannels are usually found in a closed conformation[20]. However these hemichannels have been induced to open experimentally, and act in the release of ATP as a secondary messenger. A caveat to these findings is that many experiments relied on artificial cellular environments to achieve their results[21]. For example, in most cases the release of ATP was only achieved through overexpression of connexin molecules in unpaired cells at a very low

extracellular Ca^{2+} concentrations, an environment which enhanced channel open probability[21]. In contrast, a few connexin subtypes, namely Cx26, Cx43, Cx46, and Cx50 have been found to form functional hemichannels under normal physiological conditions, although the extent to which they participate in extracellular signaling remains unclear[22-24].

Interestingly, both connexin hemichannels and pannexin channels have been reported to open under cellular stress conditions including ischemia, hypotonic stress, low oxygen tension, and mechanical stimulation (topic extensively reviewed by Lohman and Isakson)[21]. Hemichannels under pathological conditions experiencing uncontrolled opening are often referred to as leaky hemichannels[25]. Moreover, along with cellular stress responses, mutations in human connexin genes have also been linked to the formation of leaky hemichannels, and subsequent cell death[25]. An example of this phenomenon was demonstrated when researchers overexpressed human Cx40 gene mutants associated with idiopathic atrial fibrillation in N2A cells and found that one mutation (G38D) led to a gain-of-function in forming leaky hemichannels[26]. Unfortunately, Cx40 hemichannels have yet to be identified in native cell systems.

1.5 An overview of inter/intracellular communication in cardiovascular physiology

The heart is composed of two main functional components: the conduction system, and the working myocardium[27]. It is the junctions within the heart, and in particular GJs, that allow for the spread of electrical activity from the conduction system to the working myocardium and consequently the synchronized contraction of the cardiac tissue[28]. Without coordinated contraction the heart is at risk for atrial and ventricular arrhythmias, which could ultimately lead to ischemic events and heart failure[27]. Furthermore, the regulation of paracrine signaling within the heart in normal physiology and under pathological conditions is of key importance because aberrant signaling may lead to triggered propagated contractions and arrhythmogenic phenotypes[58]. To date members of connexin and pannexin gene families have been implicated in cardiac physiology due to their roles in GJ intercellular communication and purinergic signaling[29].

Another important part of the cardiovascular system in which both connexins and pannexins have been shown to participate in integral physiological processes is the vasculature[30, 31]. Arteries regulate blood flow and may be divided into distinct subcategories based on their size and function[32]. Conducting arteries, such as the aorta, are the largest vessels within the body, and are elastic in nature[32]. Conduit arteries are medium sized arteries that stem from conducting arteries and branch into resistance arteries to allow for the perfusion of organ systems and maintenance of adequate blood pressure. Resistance arteries, like the thoracodoral artery (TA) are muscular in nature[33]. Depending on their size arteries are composed of varying levels of smooth muscle and endothelial cells, and consequently display differential connexin/pannexin expression profiles[34.35]. Notwithstanding, both smooth muscle and endothelial cell layers within arteries require both intracellular and extracellular signaling for the spread of secondary messenger molecules, current, depolarization and hyperpolarization waves for vasoconstriction and vasodilation[36]. Intriguingly, both Cx40 and Panx1 channels have been found to play pivotal roles in the regulation of these processes[37, 38] as will be explored in detail in subsequent sections.

Finally, large-pore channels within the renal system contribute significantly towards cardiovascular homeostasis and the regulation of blood pressure[39]. In fact, connexins are highly involved in two central feed-back mechanisms instrumental to the control of arterial pressure[40]. Surprisingly, through its role as a large-pore ATP release channel, Panx1 has recently been implicated in these vital physiological processes as well[41].

1.5.1 *The role of cardiac connexins in normal and diseased physiology*

The conduction system of the heart relies heavily on GJ intercellular communication for the spread of action potentials (AP) from one cardiomyocyte to the next beginning with the pacemaker cells in the sinoatrial node and propagating to the cristae terminalis and atrial tissue[42]. Next the AP travels through the atrioventricular (AV) node, atrioventricular bundle, bundle branches, purkinje fibers, and finally the ventricles[42]. Without the myocardium functioning as a synchronous unit the heart cannot pump blood throughout the body in an efficient manner and is prone to fibrillations and irregular rhythm[43].

Connexin family members that play a role in GJ communication in the heart are Cx43, Cx45, and Cx40, which are found throughout the heart and at the intercalated discs of cardiomyocytes[43].

The presence of three different connexin family members within the heart allows for an increasingly diverse GJ composition and thus regulation of the speed and direction of impulse propagation[43]. Cx43 is the most abundant isoform present in cardiac tissue and is located at the junctions between cardiomyocytes in the working myocardium of the atria and ventricles[44]. Interestingly, although the ablation of this connexin in mice is neonatally lethal, it has been found that cardiomyocyte-specific ablation of Cx43 does not lead to alterations in heart morphology, however mice die spontaneously due to ventricular arrhythmias[45]. In addition, mice heterozygous for the Cx43 gene display no changes in cardiac conduction[46]. Cx45 is essential for early embryonic myocardial development and its ablation in global and cardiac specific knockout mouse models causes embryonic lethality[47]. Interestingly, using a tamoxifen-inducible, cardiac specific, knockout mouse model it was shown that Cx45 is non-essential in the heart of older mice, as gene ablation produced only minor alterations in AV nodal propagation[47]. Finally, Cx40 is localized to the working myocardium in the atria and cardiomyocytes of the conduction system of the heart (Figure 1-2)[44].

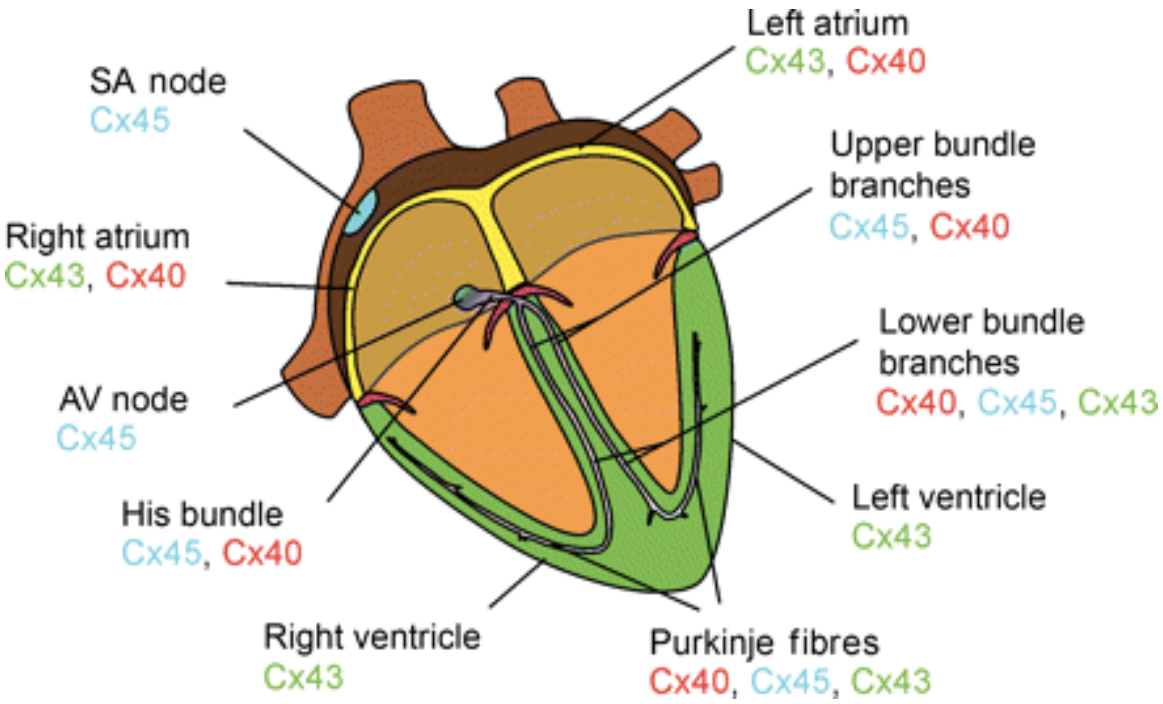
The role of Cx40 within the heart is quite diverse as it is the only cardiac connexin expressed in both the working myocardium and the conduction system. Cx40 knockout mice are viable making them an amenable model to study the function of GJs within the cardiac system[48]. Not only does Cx40 function in the propagation of action potentials, slow wave calcium (Ca^{2+}) signaling, and synchronous beating of atrial cardiomyocytes[48], but it also plays a role in cardiac development. Previous studies performed on wild type mice show that both the Cx40 transcript and protein are dynamically regulated within the heart during development[49]. At embryonic day (E) 11 Cx40 is widely distributed throughout the atria and ventricle primordia in the unorganized embryonic conduction system[49]. However by E14 the ventricular conduction system begins to differentiate and Cx40 recedes from the ventricular trabecular network to take on a more restricted distribution in the proximal segment ventricular conduction system, namely the His-bundle and the upper parts of the bundle branches, and throughout the atria[49]. Intriguingly, Cx40 is also involved in the structural development of the heart. Assessment of the morphology of young and adult $Cx40^{-/-}$ mouse hearts revealed that this mutant possessed several structural anomalies including septation defects, and cardiac hypertrophy[50]. Cx40 also plays a crucial role in the propagation of action potentials within the cardiac conduction system, and throughout the

atria[51]. Functional studies performed on Cx40 knockout mice revealed that the absence of this protein caused decreased automaticity, slower AP propagation throughout the atria and AV-node, and finally upper bundle branch block associated with first degree AV block[51].

Much like the mouse, in the human population Cx40 also plays a role in cardiac health[52]. Patients with idiopathic paroxysmal or chronic atrial fibrillation (AF) display up to a 77% reduction of Cx40 protein in atrial tissue compared to tissues obtained from a non-diseased heart[52]. As well, it has been found that a few idiopathic AF patients display either somatic and germline mutations in *GJA5,* the gene encoding Cx40. So far, 6 germline mutations, and 3 somatic mutations in the *GJA5* gene have been identified in a total of 23 patients[53]. Functional studies on several of these mutations revealed compromised Cx40 GJ and hemichannel function, pointing towards the importance of this connexin family member in heart health[53]. All in all, the ablation of Cx40 in a transgenic mouse model seems to be the most attractive method to study the role of GJs in cardiovascular physiology, not only because these mice are viable and still display an irregular cardiac phenotype, but also because the knockout of this gene may result in similar pathologies to those seen in loss of function *GJA5* gene mutations within the human population.

Figure 1-2. Localization of connexins within the heart

Cx40, Cx43, and Cx45, can be found throughout the heart, in both the working myocardium of the atria and ventricles and the cardiac conduction system. Reused with permission: Severs NJ, Bruce AF, Dupont E, Rothery S. Remodelling of gap junctions and connexin expression in diseased myocardium. Cardiovascular research 2008;80:9–19.

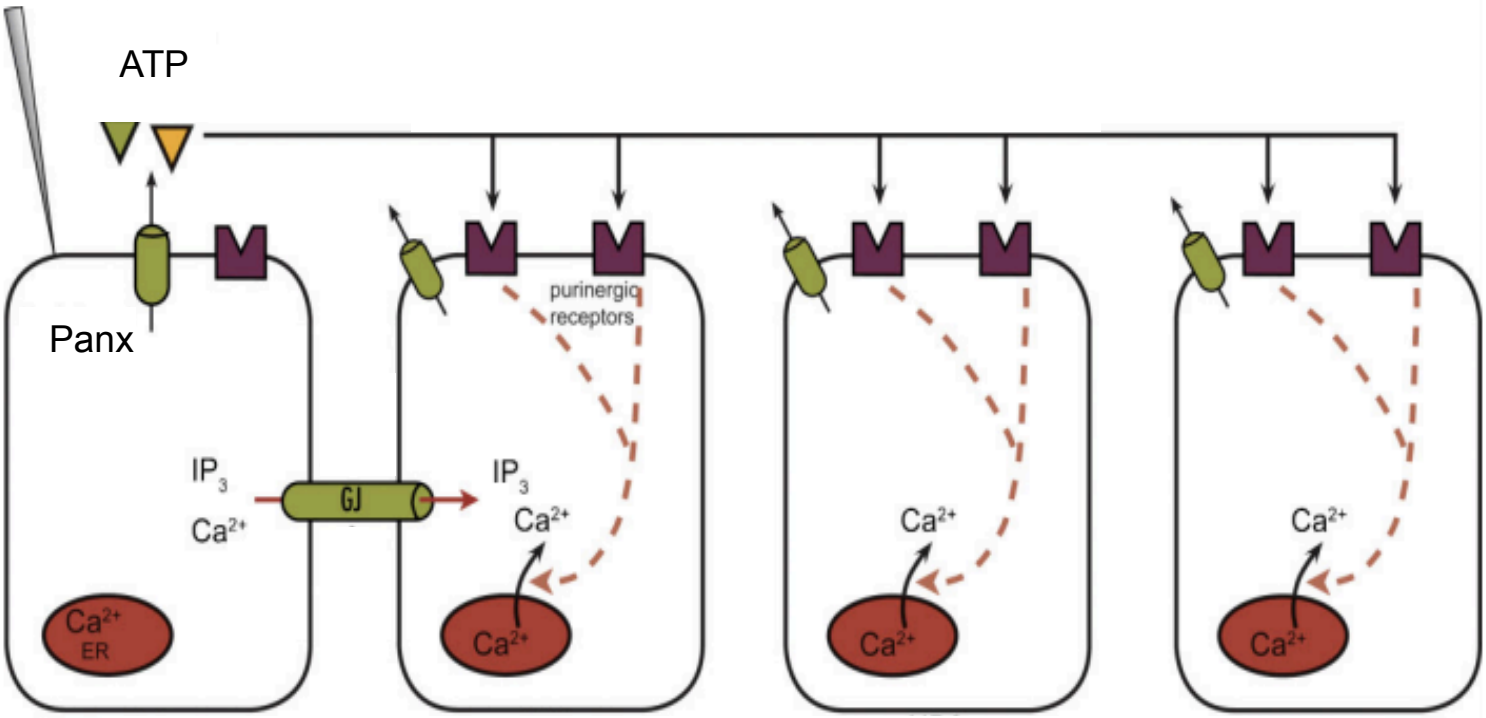

Left atrium
Cx43, Cx40
SA node
Cx45
Upper bundle branches
Cx45, Cx40
Right atrium
Cx43, Cx40
Lower bundle branches
Cx40, Cx45, Cx43
AV node
Cx45
Left ventricle
Cx43
His bundle
Cx45, Cx40
Right ventricle
Cx43
Purkinje fibres
Cx40, Cx45, Cx43

1.5.2 *Panx1, slow wave Ca^{2+} propagation, and atrial arrhythmias*

Panx1 is the most prevalent pannexin family member expressed in the cardiovascular system, and the only Panx localized to the heart[54]. Panx1 mRNA is present throughout the heart, in both the atria and ventricle with slightly higher transcript levels being localized to the atria[54]. It has also been identified in various cardiovascular cell types including cardiomyocytes, endothelial cells, and smooth muscle cells[55]. Its function within the cardiac system remains somewhat unclear, however, it is known that Panx1 acts as a major ATP release channel in cardiomyocytes following injury or inflammation[56]. As well, it has been hypothesized by several groups that Panx1 may contribute to inter- and intracellular slow wave calcium signaling via purinergic signaling and subsequent Ca^{2+} release from intracellular stores that then diffuses from cell to cell via GJs[57] (Figure 1-3). Slow wave calcium signaling within the heart typically occurs at a rate of 0.1 mm/s, and can lead to a depolarization wave and triggered propagating contractions (TPCS)[58]. TPCs may lead to an arrhythmogenic phenotype[58]. Remarkably, $Panx1^{-/-}$ mice also exhibit increased susceptibility to atrial fibrillation, a prolonged QT wave phenotype, and a higher incidence of AV block[54]. Although Cx40 GJ channels and Panx1 single membrane channels play different roles in cardiac physiology as previously outlined, they are both localized similarly, in the atria, participate in similar signaling cascades such as slow wave calcium signaling, and their ablation within knockout mouse models leads to a similar arrhythmogenic cardiac pathology.

Figure 1-3. Cx40 and Panx1 large-pore channel calcium wave propagation

Proposed mechanism concerning the participation of Cx40 and Panx1 large-pore channels in paracrine and GJ signaling interactions involving the passage of ATP and secondary messenger molecules IP_3 and Ca^{2+} in order to cause intracellular calcium store release and contribute to slow wave calcium propagation in some cell types. Modified with permissions from: D'hondt C, Ponsaerts R, Smedt H De, Bultynck G, Himpens B. Pannexins, distant relatives of the connexin family with specific cellular functions? BioEssays 2009;31:953–974.


ATP
Panx
purinergic
receptors
IP3
Ca2+
GJ
IP3
Ca2+
Ca2+
ER
Ca2+
Ca2+
Ca2+
Ca2+

1.5.3 *Connexins in vascular physiology*

The regulation of vascular tone and coordination of vasomotor response is essential for the control of blood pressure in normal physiology. Often, these responses are regulated by the passage of ions and secondary messengers between coupled vascular cells[59]. Endothelial-endothelial, smooth muscle-smooth muscle, and myoendothelial cell coupling is accomplished by Cx37, Cx40, Cx45, and Cx43[60]. More specifically, Cx40 allows for coupling between endothelial cells and the spreading of vasodilatory signals, Cx43 and Cx45 for the coupling of smooth muscle cells, and finally Cx37, Cx40, and Cx43 for the formation of myoendothelial junctions[60]. In large arteries, such as the aorta or coronaries, Cx37 and Cx40 are the most abundant connexin subtypes in the endothelium, and Cx43 the most abundant in smooth muscle[61]. Contrastingly, in small resistance arteries Cx40 and Cx45 are more abundant in the smooth muscle cells than Cx43[61]. Certain pathologies such as hypertension may cause alterations in the connexin expression profile in a tissue specific fashion, and this effect has been demonstrated both within the blood vessels and within the kidneys[62]. For example in mouse and rat models in which renal hypertension is experimentally induced it has been found that Cx43 and Cx40 transcript and protein levels are increased in the kidneys[62]. In contrast, when hypertension is induced by inhibiting endothelial nitric oxide synthase, an enzyme associated with the production of nitric oxide in the vasculature, Cx37 and Cx43 levels are reduced within the vessels and Cx40 remains unaffected[62].

Unfortunately Cx37 null mice mainly exhibit phenotypes that are non-vessel related[63]. Contrastingly, Cx45 knockout mice exhibit a severe vascular phenotype and embryonic lethality (E9.5-E10.5) characterized by abnormally enlarged or narrowed vessels, diminished yolk sack vessel formation, and hindered smooth muscle cell differentiation around large arteries[63]. Endothelial cell specific knockouts of Cx43 have demonstrated that this connexin protein may be important in vasodilation as select knockout models exhibit hypotension that was hypothesized to be due to increased nitric oxide (NO) production[64]. Finally, transgenic mice in which the Cx40 gene has been ablated show impairments in vasomotor responses including altered contraction and relaxation profiles[65]. Mice lacking Cx40 display blunted conducted vasodilation responses in both arterioles and larger arteries in response to endothelium dependent vasodilators like acetylcholine[65]. Interestingly, receptor dependent vasocontractile responses to phenylephrine

agonists are enhanced in some $Cx40^{-/-}$ mouse stuides[66]. This was hypothesized to be due to endothelial dysfunction leading to a lack of coordinated contraction/relaxation balance within the vessels in question[66].

1.5.4 *The functional role of Panx1 within large and small arteries*

Although there are three Panx family members, Panx1 is the primary species expressed in the vasculature[67]. Panx1 has been localized to both endothelial cells and smooth muscle cells[68]. Intriguingly, the distribution of Panx1 in smooth muscle cells is graded, and vessels greater than 300 μm in diameter do not express Panx1 in smooth muscle[67]. Therefore, in large arteries like the aorta, Panx1 is predominately localized to the endothelium. As well, interestingly, it has been reported that global Panx1 knockout mice show compensatory upregulation of Panx3 channels in the thoracodorsal artery (TDA)[68].

In the vasculature Panx1 has been implicated in altered vasomotor responses in conduit arteries by regulating endothelium derived hyperpolarizing factor (EDHF)[38] and in small resistance arteries by modulating vasoconstriction[68]. The ability of the Panx1 channel to regulate vasomotor responses is speculated to be due to their ability to act as an ATP release channel in order to modulate slow wave Ca^{2+} signaling under differential physiological conditions[55, 56, 69]. For example in conduit arteries Panx1 participates in the relaxation response by mitigating EDHF responses. In fact, global $Panx1^{-/-}$ mice display increased resistance due to the blunted response of arterioles to muscarinic receptor agonist acetylcholine[38]. Meanwhile in small resistance arteries like the TDA it has been found that Panx1 shares a functional link with α1D-adrenergic receptors (α1D-AR)[70]. When norepinephrine is released from sympathetic nerves to activate α1D-ARs there must be concomitant activation of purinergic receptors or the contractile response of the artery is blunted[70]. Furthermore, it was found that Panx1 played a role in purinergic receptor activation because when phenylephrine, a α1D-AR agonist was applied to the TDA after Panx1 channel inhibition vasoconstriction was blunted[70].

Contrastingly, in two separate studies it has been found that a) Panx1 does not mediate α1D-AR vasoconstriction in resistance arteries, and b) that ATP is not required for α1D-AR-mediated constriction[71, 72]. Their protocols utilized both TDA and mesenteric small resistance arteries, and a variety of Panx1 inhibitors[71, 72]. Therefore due to the controversy in this field concerning the

roles of Panx1 in small resistance arteries, and the exhaustive amount of research that has already been performed on the contributions of Panx1 to vasoconstriction in the TDA, we propose instead to assess the role of Panx1 in endothelial cell function. As mentioned, endothelium integrity was previously measured in conduit arteries of $Panx1^{-/-}$ mice and found to be dysregulated. We therefore postulate that in an increasingly large artery, such as the aorta, which contains higher levels of endothelial Panx1, the severity of the phenotype would be increased.

Much like in the atria of heart, the roles of Panx1 and Cx40 in large arteries and consequently, the aorta seem to overlap. Within larger arteries both of these key large-pore channels are localized to the endothelium[61, 67], play a role in vasodilation albeit via differential mechanisms, and their ablation leads to endothelial dysfunction[38, 65, 66]. It is therefore worth considering what the effect of the combined ablation of Cx40/Panx1 large-pore channel proteins would have not only on the heart, but also within the vasculature.

1.5.5 *Connexins and pannexins in the renal system*

The kidneys play a pivotal role in modulating cardiovascular system response by regulating blood pressure and peripheral resistance through two central processes: pressure natriuresis regulation and by releasing neurohormonal vasoactive factors[73]. Interestingly, both of these processes are in part modulated by connexins[76]. Pressure natriuresis is a feedback mechanism in the renal system used to control blood pressure by closely regulating sodium and water balance[74]. In short, rises in arterial pressure lead to increased perfusion pressure within the renal artery. This then causes increased sodium and water excretion, and decrease in the level of extracellular fluids ultimately resulting in decreased blood pressure[73]. Pressure natriuresis is a regulatory mechanism that occurs irrespective of the stimulus that provoked the initial increase in arterial pressure[73].

The renin-angiotensin-aldosterone system (RAAS) in the kidney works alongside the pressure natriuresis mechanism to maintain a normalized blood pressure by releasing neurohormonal factors[74]. Increases in arterial pressure lead to the inhibition of the RAAS system, a reduction of angiotensin II production, reduced vasoconstriction on the afferent arterioles of the kidney, and finally decreased sodium reabsorption[74]. Contrastingly, the activation of RAAS through the secretion of renin from the kidneys provokes angiotensin converting enzyme to convert

angiotensin I into angiotensin II which then acts to cause increased vessel constriction, increases in blood pressure, and ultimately hypertension through increased fluid reabsorption[75].

Connexins have been localized to the cortex and medulla of the kidney, and throughout the renal vasculature[76]. Importantly, within the nephron Cx43, Cx40, Cx30, Cx37, and Cx45 are found[77]. Physiologically within the kidney connexins participate in several regulatory mechanisms including the RAAS, in tubuloglomerular feedback and pressure natriuresis[76]. More specifically Cx40 is found within the endothelial cells of the juxtaglomerular apparatus, and other parts of the renal microcirculation[78]. As a prominent member of the kidney microcirculation Cx40 may act as a mediator of slow wave Ca^{2+} signaling in order to modulate vascular conductance and endothelium mediated vasodilation of the arteries in order to control glomerular filtration rates[77]. Studies have also shown that Cx40 plays an important role in renin synthesis and release, contributing to autoregulation mechanisms (RAAS feed-back) within the kindey[78, 79].

In addition, Panx1 and Panx3 channels have been localized within the kidney. Panx1 is present in both cortical and medullary tubule segments of the kidney and in the renal vasculature[80], while Panx3 has a more restricted distribution pattern and is found only within the renal vasculature[77]. Unfortunately little is known about the role of Panx1 in the renal system. However based on its similar localization profile to Cx30 within the apical collecting ducts, and its role in purinergic signaling, it has been hypothesized that Panx1 may play a role in pressure natriuresis[80]. It has also been postulated that $Panx1^{-/-}$ mice might possess a salt retention phenotype that would consequently lead to hypertension[80].

1.6 Signaling in receptor-dependent and independent vasomotor responses

Vascular tone is an important part of system homeostasis and is maintained by local vasoconstriction and dilatory responses[81]. In general vasoconstriction may be either receptor-dependent or independent and is regulated by smooth muscle cell contraction[82]. Contrastingly, vasodilation is mediated by the endothelium which releases a variety of vasoreactive factors in the regulation of smooth muscle relaxation such as nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF)[81]. The assessment of the vasoreactivity of a vessel to various

agonists causing constriction/relaxation is often used as a measure of endothelial function[83], and therefore this approach will be utilized in this thesis to study the effects of the ablation of Panx1/Cx40 in the aorta. Because these methodologies will be employed, key signaling pathways involved in these responses are delineated.

α-adrenergic receptor mediated contraction is one of the central pathways that mediate vasoconstriction, and one that has been utilized in this thesis to examine the contractile responses of the aorta. Adrenergic receptors are G-protein coupled receptors (GPCR) that are activated by catecholamine agonists[84]. They are found within the in the cardiovascular system and have been divided into two main subtypes: α-adrenergic receptors (α-ARs) and β-adrenergic receptors[84]. In the vasculature α-ARs are surface receptors located on smooth muscle cells that play a role in vasoconstriction[85]. α1-ARs are activated by the release of neurotransmitters from the sympathetic nervous system or by drug agonists such as phenylephrine (PE) and methoxamine[85]. The GPCR then initiates an intracellular cascade by activating the membrane bound phospholipase C protein (PLC). Once activated PLC stimulates the production of IP_3 and diacylglycerol, two secondary messengers which act on intracellular Ca^{2+} stores and cause their release. The rise in intracellular Ca^{2+} ultimately leads to vasoconstriction of the artery[84, 85].

Similarly potassium (K^+) channels are important for the regulation of smooth muscle cell membrane potential and excitability. In brief the phenomenon of K^+ mediated vasodilation and vasoconstriction will be explained[86]. The opening of these channels leads to K^+ efflux, hyperpolarization of the membrane, closure of voltage gated Ca^{2+} channels, and ultimately smooth muscle cell relaxation[86]. In contrast, the closing of these channels causes membrane depolarization, opening of voltage gated Ca^{2+} channels, and ultimately vasoconstriction[87]. In total there are four different types of K^+ channels including: calcium activated-K^+ channels, voltage dependent K^+ channels, ATP-sensitive K^+ channels, and inwardly rectifying K^+ channels[87].

Nitric oxide is a potent vasodilator and acts through activation of intracellular cascades which ultimately cause shifts in actin/myosin chain dynamics, muscle relaxation, and thus vasodilation[88]. In endothelial cells nitric oxide is synthesized from amino acid L-arginine by endothelial nitric oxide synthase[88]. NO can then diffuse from endothelial cells into SMCs and activate membrane bound receptor soluble guanylyl-cyclase (sGC)[89]. Activated sGC produces

secondary messenger cyclic guanylyl monophosphate, which induces protein kinase G (PKG). PKG, causes the reuptake of cytosolic Ca^{2+}, promotes the opening of Ca^{2+} dependent K^+ channels, and activates myosin light chain phosphatases[89]. These factors cause the dephosphorylation of the myosin light chain, and the hyperpolarization of SMCs, which inhibits muscle contraction and subsequently leads to vasodilation[88, 89].

Sodium nitroprusside is a NO donor, often used to study nitric oxide mediated vasodilation in an endothelium independent manner[90]. SNP acts by breaking down into its molecular components and releasing nitric oxide into the blood stream[90]. Nitric oxide can then act directly on SMCs to induce a vasodilatory response[91]. The use of SNP along with other endothelial dependent vasodilators such as methacholine allows researchers to assess endothelium function[91].

Muscarinic (M_3) receptor mediated vasodilation is a pathway that causes smooth muscle cell relaxation in the vasculature[92]. M_3 receptors are GPCRs located on the endothelium that may be stimulated by various non-selective agonists such as methacholine and acetylcholine[92]. Once activated these GPCRs act to initiate PLC and IP_3 secondary messenger signaling cascades that lead to rises in intracellular Ca^{2+}, and the formation of NO[93]. Nitric oxide may then diffuse from the endothelium to SMCs in order to exert a vasodilatory effect as previously mentioned. Intriguingly, this pathway is often used to assess endothelial function, as the NO mediated vasodilatory function of the endothelium is often deregulated upon damage[92].

Ca^{2+} is required for the contraction of smooth muscle cells, where the extent of contraction is dependent on the intracellular levels of this ion. Intracellular rises in Ca^{2+} cause decreases in membrane polarization and activation of voltage-gated calcium channels and influxes of this molecule into the cell[94]. Interestingly, voltage-gated calcium channels are expressed in a graded manner the vasculature, wherein channel number increases as vessel size decreases[93]. Although calcium is vital to vasoconstriction, it is not thought that this secondary messenger itself could cause synchronized contraction over long distances, rather that it acts through GJs as a messenger molecule along the length of vessel or from cell to cell to amplify or maintain the contractile response[93].

Calcium also plays an important role in endothelium mediated vessel relaxation. Endothelial cells lining the lumen have a variety of surface receptors that are stimulated upon interaction

with ATP, circulating hormones or by changes in blood flow[95]. Many of these receptors such as metabotrophic P2Y receptors, are G-protein coupled receptors, which will then initiate intracellular cascades to cause rises in IP_3 and subsequently Ca^{2+} concentration[95]. Rises in endothelial cell Ca^{2+} will activate ion channels and promote synthesis of secondary messenger molecules, such as prostacyclin, nitric oxide (NO), and endothelium derived hyperpolarizing factor[96]. These signaling molecules are termed endothelium derived relaxing factors, and ultimately cause the relaxation of the smooth muscle cell layer of the vessel[96].

1.7 The role of Cx40 and Panx1 in blood pressure regulation

Previous studies have shown that the ablation of the Cx40 gene in the mouse creates a hypertensive phenotype[37, 79]. Research has found that this increase in systolic/diastolic pressure is not a consequence of Cx40 channel loss from the vasculature[97]. This was determined when Cx40 was selectively deleted from the endothelium and transgenic mice remained normotensive despite blunted arteriole relaxation[97, 98]. Remarkably, it was found that the hypertensive phenotype in the global Cx40 knockout mouse was not salt sensitive but rather due to alterations in the renin-angiotensin-aldosterone system (RAAS)[99]. More specifically, the ablation of Cx40 impairs the negative feedback loop of pressure on renin release, resulting in excess renin excretion[99]. To elaborate on these findings, hypertensive $Cx40^{-/-}$ mice were rendered normotensive when the RAAS was inhibited via ACE or angiotensin II receptor blockers[100]. When this hypertension was further explored it was found that selective ablation of Cx40 from the renin producing cells of the kidney created the same phenotype found in the global Cx40 knockout mice[100]. Furthermore $Cx40^{-/-}$ mice have increased plasma renin and aldosterone concentrations[100, 79].

In humans, single nucleotide polymorphisms in both the intron and promotor region of the Cx40 gene (*GJA5*) have been associated not only with idiopathic atrial fibrillation but also hypertension and left ventricular hypertrophy in males[101, 102]. More specifically, polymorphisms were located in the promoter region of the minor Cx40 allele at position S44AA/R71GG and within the intron at RS791295[101, 102].

Previous reports have demonstrated a role for Panx1 in blood pressure regulation and peripheral resistance through various mechanisms. One involves Panx1 channels on erythrocytes being activated to release ATP in conditions where there is low blood pO_2[69]. This nucleotide would then induce vasodilation in the artery in question[69]. Similarly, the carotid body, known for its role in maintaining blood pO_2 balance, possesses Panx1 channels, and has been shown to act as an ATP amplifier by releasing of ATP onto receptor type II cells which then propagates this release onto afferent nerve terminals[103]. Afferent nerve terminals would ultimately relay this signal to cardiorespiratory centers in the medulla oblongata for the regulation of blood pressure and respiratory rate[103]. Therefore, it has been shown that Panx1 plays a significant role in hypoxia-induced vasodilation[69, 103].

In addition, a previous mouse study shows that when Panx1 is specifically ablated from smooth muscle cells, those partial knockout animals are slightly hypotensive[104]. This effect was hypothesized to be due to the role that Panx1 channels play in mediating α-1D-adrenergic receptor signaling in vasocontraction in small resistance arteries[104]. As well, as previously delineated Panx1 may play a role in pressure natriuresis[80] and $Panx1^{-/-}$ mice might possess a salt retention phenotype and consequently hypertension[80].

Therefore not only have Cx40 and Panx1 channels been implicated in similar localization profiles within the atria[44, 54] and aorta[31, 67], and similar functions within the heart and vessels, but they are also both linked to blood pressure regulation[80, 100, 104]. As this is a widespread physiological process that occurs via the interaction of multiple organ systems and mechanisms within the body it would be interesting to observe the effect of the combined ablation of these two large-pore channel proteins on blood pressure regulation.

1.8 Hypertension and its associated risk factors

Arterial pressure depends on two main variables: peripheral resistance and cardiac output[105]. Peripheral resistance is mediated by small arteries, whereas cardiac output is influenced by blood volume, and is thus under renal control[105]. Hypertension is defined as abnormally high blood pressure, and is a rising epidemic that affects more than 25% of the global population and is one of the leading causes of mortality in developed countries[106]. Hypertensive vessels are

characterized by increased medial and reduced luminal area, along with increased ECM proteins[107]. This disease if often asymptomatic until later in life and is associated with several risk factors such as cardiac hypertrophy, myocardial infarction, aortic dissection, endothelial dysfunction, atherosclerosis, stroke, and chronic kidney disease[108]. In relation to the role that hypertension plays in the development of myocardial hypertrophy, endothelium dysfunction will be delineated as well as the roles that both connexin and pannexin large-pore channels play in the development of this multifactorial disease[109].

1.8.1 *Cardiac hypertrophy induced by pressure overload*

Cardiac hypertrophy is an adaptive response, and a pathophysiological condition that arises due to increased volume/pressure load, sarcomeric mutations or dysfunction, and decreased myocyte contractility after tissue injury[110]. Hypertrophic growth induced by increased pressure/volume load is secondary to several pathologies including hypertension, ischemia, valvular disease, and heart failure[110]. Pressure overload hypertrophy is a topic that is central to this thesis project and will therefore be discussed in detail.

Arterial hypertension is the most common cause of pressure overload within the heart and may lead to pronounced left ventricular hypertrophy (LVH)[111]. Pressure induced hypertrophy is believed to occur as a physiological compensation mechanism that the myocardium undergoes in order to decrease left ventricular wall stress and oxygen consumption[111]. The left ventricle is often the most affected cardiac region as it has the greatest workload[111]. At the molecular level muscle wall thickening is due to biomechanical stress potentiating changes in cardiac gene expression, the reactivation of the fetal gene program (α-myosin heavy chain, atrial natriuretic peptide, and brain natriuretic), and ultimately the growth of the cardiomyocytes[112]. Along with increases in cardiomyocyte area cardiac hypertrophy is often accompanied by augmented interstitial myocardial fibrosis, GJ and cytoskeletal remodeling, which may lead to a detrimental arrhythmogenic phenotype and ultimately heart failure[113].

More recently it has been suggested that hypertension associated LVH may not be due to pressure overload alone, but that neurohormonal factors can exert trophic effects and ECM deposition which may play an import part in the establishment of cardiac hypertrophy[114]. These neurohormones include angiotensin II, aldosterone, norephinephrine, and insulin[114]. They

directly stimulate cardiac hypertrophy and protein synthesis by stimulating the production of cytokines and growth factors[114].

Meanwhile, genetic factors have also been hypothesized to play a role in LVH because there is a considerable amount of interindividual variability associated with a hypertensive phenotype and increases in left ventricular mass[111]. For example, large cohort studies show that only 50% of the variability in left ventricular mass can be explained by increased systolic blood pressure and other associated risk factors[110]. Furthermore, it has been found that the extent of cardiac growth in response to pressure load is not uniform among patients experiencing hypertension. Therefore, these data, as reviewed by Drazner *et al*. suggest the presence of an unknown, unmeasured risk factor[111].

Another important consideration is the regulation and/or suppression of cardiac hypertrophy via different effector molecules. Increasing amounts of evidence show that NO may act through the Ca^{2+}/calcineurin-NFAT cascade to suppress the hypertrophic signal that causes cardiomyocyte enlargement[112]. As well, it has been shown that cardiac remodeling in response to pressure overload is inhibited by the NO-cGMP pathway[112].

1.8.2 *Hypertension induced endothelium dysfunction*

The endothelium is the innermost lining of a blood vessel and plays a critical role in maintaining normal physiology. It is involved in a number of processes including barrier function, regulation of vascular tone, neutrophil recruitment, and hormone trafficking[115]. Endothelial dysfunction may be defined as a diminished capacity of the endothelial cells to produce nitric oxide and other vasodilators and/or an increase in the production of endothelium-derived vasoconstrictors. Functionally, this imbalance leads to impairments in endothelium-mediated vasodilation[115]. At the molecular level endothelial dysfunction is characterized by pro-thrombotic, pro-inflammatory, and pro-constrictive changes in the endovascular tissue[116]. Hypertension and endothelial function are entwined, wherein hypertension may lead to endothelium dysfunction or vice versa. In fact, it has been previously reported that increasingly hypertensive phenotypes presented with decreased endothelial function and that this response was graded[116]. These experiments were performed in both men and women by measuring brachial artery reactivity while co-administering antihypertensive therapy[116]. It is also worth noting a study performed by

Hamasaki *et al*. that found that only patients with both hypertension and LVH presented with coronary endothelial dysfunction, however endothelial responses in patients with hypertension without LVH were similar to those of normotensive individuals[117].

Several mechanisms have been implicated in the development of hypertension mediated endothelium dysfunction including: excessive reactive oxygen species (ROS) production, and local vascular and systemic inflammation[118, 119]. In brief, endothelium dependent vasodilation was assessed while mouse carotid arteries were exposed to increasing intraluminal pressure[118]. It was found that under increasing pressure there was a parallel decrease in vasodilation and an upregulation in nicotinamide adenine dinucleotide phosphate oxidase activity[118]. In addition it has been found that the anti-inflammatory interleukin-10 diminishes endothelium dysfunction in experimentally induced hypertensive animals advocating for the adverse role of inflammation in the development of this pathology[119].

1.9 Synopsis of the cardiovascular phenotypes found in Cx40 and Panx1 null mice

1.9.1 *Cx40 null mice*

There are two global Cx40 knockout mouse models central to the cardiac gap junction field, which were engineered in the laboratories of Dr. Klaus Willecke, and Dr. David Paul. Both of these Cx40 null mice possess similar cardiac physiology including decreased conduction velocities due to increased P-wave and QRS-complex duration, and a normal basal heart rate[49, 50, 120, 121]. The altered cardiac physiology found in these mice has been hypothesized to be due to the inability of the atria and conduction system to fully compensate for the loss of this GJ channel protein[120, 121]. Furthermore, Cx40 ablation leads to altered electrical foci and the inability of the myocardium to correctly propagate action potentials, Ca^{2+} waves, and function as a syncytium[122]. However there is one caveat as only the Willecke $Cx40^{-/-}$ mouse model has been reported to have an arrhythmogenic phenotype[49]. Although the mice were designed in the same manner, this discrepancy could be due to slightly different mouse backgrounds, the amount of backcrossing that was performed, or due to the fact that the measurements performed not sufficiently sensitive (3-lead ECG and 6-lead ECG)[49, 50].

The cardiac structure of the Cx40$^{-/-}$ mouse was assessed and a mild ventricular septation defect, and decreased pre and postnatal survival were reported. It was found that offspring of single Cx40 knockout dams had increased in utero death between E11.5 and E13.5 and that 16% of newborns died shortly after birth[49]. The authors hypothesized that embryonic death was due to functional rather than structural factors, and that postnatal fatalities were due to septatation defects and the improper establishment of pulmonary circulation at birth[49].

Along with cardiac abnormalities, Cx40 knockout mice (both global and endothelial/kidney specific knockout models) present with pathophysiological alterations in the vasculature and renal system. To summate previous studies it was found that Cx40 ablation led to a perturbed renin-angiotensin-aldosterone system, increased renin release, and consequently arterial hypertension[98, 99, 100]. As well, Cx40 transgenic mice have irregular perturbed smooth muscle calcium dynamics and irregular arteriole vasomotion, characterized by decreased vasorelaxation in response to endothelium derived agonists[41, 79].

The Cx40$^{-/-}$ mouse model generously provided to our laboratory was engineered using a targeted vector approach. A herpes simplex virus thymidine kinase cassette driven off of a phosphoglycerate kinase promotor targeted a portion of exon 2 of the Cx40 gene for deletion[120]. A neomycin resistance cassette driven by the phosphoglycerate kinase promotor was inserted in its place[120]. We decided to proceed with the David Paul knockout mouse due to the fact that this model has been extensively characterized in terms of both cardiac and vascular physiology.

1.9.2 *Panx1 mutant mouse models*

Over the past decade at least 5 different mutant mouse lines have been generated to knockout Panx1 expression as reviewed by Hanstein *et al*. [14, 123]. Our global Panx1$^{-/-}$ was generated via the deletion of exon 2 using Cre-Lox recombination technology by Dr. Vishva Dixit at Genentech[124] and has been utilized in multiple studies, although the cardiovascular system of this mouse has yet to be characterized[70,126,127,128]. As this thesis is concerned with the cardiovascular characterization of the Panx1 transgenic mouse line findings relevant to this aim will be discussed.

The cardiac physiology of the Panx1$^{-/-}$ (Monyer) mouse was recently defined by Petric *et al*. (2016), and is currently the only study to assess the roles of this large-pore channel protein in the heart. They found the hearts of Panx1$^{-/-}$ mice to be normal in weight, size, cardiomyocyte arrangement, free from excess fibrosis, and that all functional cardiodynamics as measured by echocardiography were similar to wild-type[54]. As well, electrocardiogram studies revealed that this mouse showed a normal heart rate, a prolonged QT interval, higher incidences of AV-block only at lower heart rates, and an increased susceptibility to atrial arrhythmias after burst stimulation[54]. This study is in line with previous speculations that Panx1 may contribute to slow wave calcium signaling within the heart, and that alterations in these dynamics could lead to arrhythmias[55].

An inducible model in which Panx1 was selectively ablated from smooth muscle cells was created and the effects of Panx1$^{-/-}$ on the vasoconstriction were studied. It was found that the deletion of Panx1 lowered blood pressure creating a slightly hypotensive phenotype, and that vasoconstriction to PE was reduced in the TDA indicating that Panx1 may be involved in α1-adrenoreceptor mediated vasomotor response in resistance arteries[70,104]. Alternatively conduit arteries have been found to have impaired vasorelaxation to endothelium derived relaxation factors such as acetylcholine due to a blunted EDHF response[38,125].

1.10 Rational, Hypothesis, and Objectives

Due to the overlapping roles of Cx40 and Panx1 large-pore channels in multiple processes in the cardiovascular system from slow wave Ca^{2+} signaling to the regulation of vascular tone, modulation of blood pressure, and involvement in arrhythmogenic pathology, it was postulated that these channel proteins might have co-regulatory roles. Interestingly, both Panx1 and Cx40 are localized to similar locations in the cardiovascular system including the atria and the endothelium of the aorta. Hence, due to their similar ability to regulate homeostatic processes and comparable localization profiles, we speculated that the ablation of these two channels could lead to severe cardiovascular phenotypes, as these channels appear to engage in important cross-talk mechanisms. Thus the engineering and characterization of a novel global knockout mouse model in which both Cx40 and Panx1 channels are ablated will allow for an increased understanding of the roles that these two large-pore channels play in the heart and vessels in both health and disease.

Due to the lack of knowledge on this topic this thesis will aim to examine the function of Cx40 and Panx1 individually and elucidate their potential for overlapping functions within the cardiovascular system by using single Cx40 and Panx1 null mice and a novel Cx40/Panx1^{-}double knockout mouse. In addition the $Cx40^{-/-}Panx1^{-/-}$ mouse will be characterized for survival and any overt phenotypes, as it is the first global Cx/Panx knockout mouse model of its kind.

It is hypothesized that the ablation of Cx40 and/or Panx1 will lead to reduced mutant mouse survival and dysregulated cardiovascular function in vivo.

My aims are:

1. To characterize the phenotype and survival of mice lacking Panx1, Cx40 and Panx1/Cx40.
2. To determine if mice lacking Panx1 and/or Cx40 suffer from cardiac hypertrophy and fibrosis.
3. To assess if mutant mice are subject to hypertension and dysregulated vasomotor responses.

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Chapter 2

2 Decreased Survival and Increased Hypertension in Mice Lacking Both Cx40/Panx1

Connexins (Cxs) and pannexins (Panxs) are large-pore channel forming proteins that participate in cell signalling via the passage of small molecules. Given their potential for overlapping cell signaling functions, we proposed that mice lacking both a Cx and a Panx would have a severe phenotype. In particular, Cx40 and Panx1 have been reported to potentially share functional overlap in the cardiovascular system where they are both expressed. Thus, we crossed Panx1 null mice with Cx40 knockout mice and characterized the first global connexin/pannexin double knockout mice. All studies performed were carried out on $Cx40^{-/-}Panx1^{-/-}$ mice and compared to wild-type mice or mice lacking Cx40 or Panx1. Mice lacking both Cx40 and Panx1 were fertile but ~50% of the pups died either in utero or as newborns. Both $Cx40^{-/-}$ and $Cx40^{-/-}Panx1^{-/-}$ mice exhibited cardiac hypertrophy and a slight increase in cardiac fibrosis. Surprisingly, tail-cuff blood pressure measurements of conditioned or anaesthetized mice devoid of Panx1 or Cx40 were found to be hypertensive with hypertension being increasingly elevated in mice lacking both Cx40 and Panx1. Interestingly, mice null for Panx1 did not exhibit the accompanying cardiac hypertrophy seen in $Cx40^{-/-}$ and $Cx40^{-/-}Panx1^{-/-}$ mice. Furthermore, the combined ablation of Cx40 and Panx1 decreased endothelium-dependent vasodilation characteristic of endothelial dysfunction. Overall, these studies suggest that even though Panx1 and Cx40 may act via differential mechanisms, they have a co-regulatory role in vascular response.

A version of this chapter will be submitted to Cardiovascular Research

Jelen M, Barr K, Feng Q, Isakson BE, Gros R*, Laird DW*. Decreased Survivability and Increased Hypertension in Mice Lacking Both Cx40 and Panx1 (in preparation for submission).

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2.1 Introduction

Normal cellular function, organ development, homeostasis, and repair after damage relies heavily on the initiation of a myriad of critical signaling networks and the interaction of extracellular, intercellular and intracellular cascades[1]. The connexin (Cx) and/or pannexin (Panx) families of large-pore forming channel proteins facilitate the passage of various ions, nucleotides and secondary messenger molecules such as calcium, adenosine triphosphate (ATP), and inositol triphosphate (IP_3) either between cells or between a cell and the extracellular milieu[2]. Connexins may act in either intracellular signaling as functional hemichannels, although this is not well documented *in vivo*, or more classically form gap junction (GJ) channels for direct intercellular communication[3]. On the other hand, pannexins typically exist at the cell surface as large-pore channels that function in extracellular signaling[4]. It is the interplay between these key-signaling modalities that allow critical cellular cascades to govern many functions of the heart and vasculature[5].

The heart requires cell-cell communication for the spread of electrical activity, the propagation of action potentials, and the synchronized contraction of the myocardial tissue[6]. The primary connexin family members present in the heart are Cx43, Cx40, and Cx45[7]. Interestingly in the murine heart, Cx40 is developmentally regulated with peak expression throughout the entire heart at embryonic day 14 before its expression is confined to the developing atria and conduction system[8]. Cx37, Cx40, and Cx43 present within the vasculature allow for the coupling of endothelial cells and vascular smooth muscle cells at the myoendothelial junction, the alteration of vasomotor responses, and the propagating slow Ca^{2+} waves[9]. For example in the renal vasculature, Cx40 and extracellular ATP are key mediators of slow wave calcium signaling between endothelial cells. Slow wave Ca^{2+} signaling acts not only in vasodilation of the vessels

to control glomerular filtration rate, but may also play an important role in renin synthesis and release[10, 11]. Characterization of a Cx40 global knockout mouse revealed myocardial hypertrophy, fibrosis, arrhythmias, and increased P-wave and QRS complex duration[12, 13]. Furthermore, these animals suffer from renin/angiotensin-mediated hypertension and perturbed smooth muscle calcium dynamics[14].

Panx1 is the most prevalent pannexin family member expressed in the cardiovascular system, and is found in cardiomyocytes, endothelial cells, and smooth muscle cells[15]. The abundance, localization and function of Panx1 within the heart are not well characterized. However, it is known that Panx1 acts as a major ATP release channel in cardiomyocytes following injury or inflammation[16]. As well, Panx1 may contribute to inter- and intra- cellular slow wave calcium signaling via extracellular purinergic signaling in order to amplify Ca^{2+} release from intracellular stores[17]. In the vasculature, Panx1 has been implicated in altered vasomotor responses in large arteries by regulating endothelium-derived hyperpolarizing factor[18] and in small resistance arteries by modulating vasoconstriction[19]. The ability of Panx1 channels to regulate vasomotor responses is due to their ability to act as an ATP release channel in order to modulate α-adrenergic and slow wave Ca^{2+} signaling under differential physiological conditions[20, 21, 22]. In the heart, it has been reported that $Panx1^{-/-}$ mice have an increased incidence of AV-block, a prolonged QT interval and an increased risk of atrial fibrillation after burst stimulation[23]. Furthermore, the Panx1 knockout mouse has been shown to possess decreased vasoconstriction in resistance arteries exposed to phenylephrine[19], and decreased muscarinic receptor mediated vasodilation of large arteries[18].

Given that Cx40 and Panx1 appear to play critical roles in the heart and blood vessels, the question arouse as to whether mice that globally lacked both of these large-pore channel proteins

would even survive, and if so, would they exhibit a severe cardiovascular phenotype. Currently there is only one other Cx/Panx double knockout mouse in existence where the conditional ablation of Cx43 and Panx3 lead to defective osteogenesis where both of these channel proteins are highly expressed[24]. In the current study we found approximately half the mice lacking both Cx40 and Panx1 did indeed survive, were fertile, exhibited a normal lifespan, and showed no overt phenotype. That being said, we discovered that $Cx40^{-/-}Panx1^{-/-}$ mice had fewer offspring due to death in utero, and decreased post-natal survivability. With respect to the cardiovascular system, $Cx40^{-/-}Panx1^{-/-}$ mice were found to have cardiac hypertrophy and mild fibrosis. Interestingly, tail cuff blood pressure measurements revealed that mice lacking Cx40, Panx1 or both Cx40 and Panx1 were hypertensive and exhibited a decreased endothelium-dependent vasodilatory response, which was exaggerated in mice devoid of both Cx40 and Panx1.

2.2 Materials and Methods

2.2.1 *Engineering and Characterization of Mice*

$Cx40^{-/-}Panx1^{-/-}$ mice were bred in house from $Cx40^{-/-}$ and $Panx1^{-/-}$ single knockout mice that had been previously backcrossed onto a C57BL/6N background for several generations. The Cx40 knockout mouse was generated by Simon *et al*. and was a generous gift from Dr. David Paul via Dr. Donglin Bai (University of Western Ontario, London, ON)[13]. Panx1 null mice were provided by Dr. Vishva Dixit at Genentech and have been previously characterized by Qu *et al*[25]. Wild-type mice of the C57BL/6N strain from Charles River were used as controls. Mice were housed 4 per cage, received food and water *ad libitum,* and were maintained on a 12 hour light/dark cycle at 24°C. PCR genotyping was performed to ensure the ablation of Cx40 and Panx1 as described previously using ear clip DNA as a template[25, 26]. For the characterization of male mouse weight/size, mutant and wild-type mice from 3-6 litters, with a minimum of 10 mice per

timepoint, were weighed periodically for up to a year. During tissue collection all animals were sacrificed via cervical dislocation, and organs were fixed in formalin or flash frozen and stored at -80°C. All studies performed were in accordance with the Animal Care Committee of Western University.

2.2.2 *q-PCR Analysis*

RNA was extracted using the Qiagen RNeasy kits (Qiagen) from the atria and ventricle of 3 month old wild-type and $Panx1^{-/-}$ mice. cDNA was synthesized using the first-strand cDNA synthesis kit (SuperScript VILO). Panx1 transcript levels were determined using mouse Panx1-specific primers (5'ACAGGCTGCCTTTGTGGATTCA3'; 5' GGGCAGGTACAGGAGTATG3') and the PowerUp SYBR green Mastermix (Life technologies) in a Bio-Rad CFX96 real-time system. Results were normalized to 18SrRNA. Brain tissue was used as a positive control as it expresses abundant levels of Panx1 transcript, and tissues from $Panx1^{-/-}$ mice were included with wild-type tissue as a negative control. N = 3 per group.

2.2.3 *Litter Size Characterization*

Litter sizes of wild-type and mutant mice were tracked by setting up multiple breeder cages for each strain and counting the number of offspring per litter at birth and 3 days post-natal (N=12 litters, approximately 5-10 mice/litter). Survivability was calculated wherein the amount of pups alive 3 days post birth was compared to the number of pups at birth. Furthermore fertility and incidences of in utero death were assessed in the $Cx40^{-/-}Panx1^{-/-}$ double knockout mouse by counting the number of implantations, resorption sites, and in utero death (N=7 dams) prior to day 14.5. For timed pregnancies, E0.5 was taken as the morning on which a vaginal plug was found.

2.2.4 *Heart Weight Characterization*

Three week and 3 month old mice were euthanized via cervical dislocation, and their hearts were excised. Dry heart weights were then recorded and normalized to the initial mass of the animal to obtain a normalized heart weight (mg/kg N=6 hearts per group).

2.2.5 *Western Blot Analysis*

Tissue lysates of mutant and wild-type hearts were prepared via homogenization of atria, or whole hearts on ice in lysis buffer (150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10 mM Tris-HCl) with protease and phosphatase inhibitors (Roche-Applied Sciences; 100 mM NaF and 100 mM Na_3VO_4). 30 μg of protein from lysates were resolved on a 10% SDS-PAGE gel and then transferred to a nitrocellulose membrane using an iBlot Dry Blotting system (Invitrogen). Membranes were blocked in a 3% bovine serum albumin/0.05% Tween20 PBS solution (PBST) for 30 minutes at room temperature. Membranes were then exposed to the following primary antibodies: rabbit anti-Cx43 (1:5000, Sigma, C 6219) goat anti-Cx40 (1:500, Santa Cruz, sc-20466); mouse anti-collagen 1 (1:500, Abcam, ab34710), rabbit anti-fibronectin (1:500, Abcam, ab2413), mouse anti-N-cadherin (1:200, BD Signal Transduction, 610920), and mouse anti-GAPDH (Santa Cruz, sc-365062), diluted in blocking solution at 4°C overnight. Membranes were then washed 3 times for 5-minute intervals with PBST. Fluorescent-tagged secondary anti-rabbit Alexa Fluor® 680 (1: 5000, LICOR Biosciences, ab175772) or anti-mouse IRdye 800 (1:5000, Rockland Immunochemicals, Inc., 610-132-003) were then used to detect primary antibodies. Blots were then imaged using Odyssey Infrared Imaging System (Li-Cor Biosciences) and densitometry analyses was performed wherein samples were normalized to GAPDH loading controls. N=4 samples per group.

2.2.6 *Immunofluorescence Microscopy*

Immunofluorescence was performed as previously described[27]. Briefly, 10% formalin fixed paraffin embedded heart sections (7 μm) were deparaffinized, antigen retrieved in Vector Antigen Unmasking Solution (Vector Labs), and then rinsed with PBS. For cryosections (10 μm) hearts were fixed in formalin fixative overnight and then cryoprotected in a 30% sucrose solution prior to sectioning. Sections were blocked in 3% BSA/0.1% Triton X-100 for 1 hour at room temperature. Sections were probed overnight at 4°C using the primary antibody dilutions: rabbit anti-Cx43 (1:1000, Sigma, C 6219); goat anti-Cx40 (1:200, Santa Cruz, sc-20466). Secondary Alexa Fluor® 555-conjugated anti-rabbit, anti-mouse or anti-goat (1:500, Molecular Probes, A21425, A21429 or A21431) and Alexa Fluor® 488-conjugated anti-rabbit or anti-mouse (1:500 dilution, Molecular Probes, A11008 or A11017) was applied for 1 hour at room temperature to detect primary antibody binding. Hoechst (1:10, 000) was applied for a 10-minute period to label nuclei (1:1000 in ddH_2O). Coverslips were mounted with Airvol and imaged using the Zeiss LSM 800 confocal microscope.

2.2.7 *Quantification of Cardiomyocyte Area*

Cross sections (20 μm) of formalin fixed left atria and ventricle of wild-type, $Cx40^{-/-}$, $Panx1^{-/-}$, and $Cx40^{-/-}Panx1^{-/-}$ mice were deparaffinized, and stained with wheat germ agglutinin (1:10, 000, ThermoFisher, W11261), diluted in PBS, to visualize sarcolemma membranes[28]. Airvol was used to mount coverslips and slides were then imaged using the Zeiss LSM 800 confocal microscope using a 40x lens. Images from all four genotypes were blinded to the investigator and quantified by measuring myofibril area by encircling cell membrane borders in Image J.

2.2.8 *Cardiac Fibrosis Assessment*

Longitudinally sectioned hearts all mouse genotypes (7 μm) were deparaffinized and stained with Masson's Trichrome using standard procedures to analyze the extent of cardiac fibrosis. Light microscopic images from the left atrial and left ventricular wall were analyzed for interstitial and perivascular collagen deposition[29].

2.2.9 *Heart Rate and Blood Pressure Recordings*

Heart rates, systolic and diastolic blood pressures were recorded from male 3-month-old conscious mice from all four genotypes using the CODA tail-cuff blood pressure system (Kent Scientific, Torrington, CT, USA) as previously described[30, 31](N=9). Briefly, mice were placed into restraint tubes and the tail cuff was positioned at the base of their tail. Mice were than given 30 minutes to acclimatize before initiating the measurements, and 3 trials of preconditioning were performed before measurements were used to account for anxiety effects. To further account for the influence of anxiety on blood pressure and heart rate, measurements were also taken on anesthetized mice (ketamine 100 mg/kg and xylazine 10 mg/kg intraperitoneal).

2.2.10 *Vasomotor Studies*

Vascular reactivity in wild-type, $Cx40^{-/-}$, $Panx1^{-/-}$, and $Cx40^{-/-}Panx1^{-/-}$ mice was assessed by performing isometric tension experiments on a wire myograph (DMT) as previously described[32, 33]. 3-4 month old mice were sacrificed via cervical dislocation, thoracic aortas were excised, cleaned, cut into 2 mm rings, and mounted in organ bath chambers containing 5 mL of 37°C oxygenated KREBS buffer (in mmol/l: 130 NaCl, 14.9 $NaHCO_3$, 10.0 glucose, 4.70 KCl, 1.17 $MgSO_4$, 1.18 KH_2PO_4, 1.60 $CaCl_2$, and 0.027 EDTA). Contractile responses to phenylephrine (1 nM to 30 μM), and KCl (10 to 100 mM) were recorded in vessels stretched to a passive resting

tension of either 1000 or 1250 mg (determined in preliminary experiments). Similarly, dilatory responses were assessed with either methacholine (0.1 nM to 30 μM) or sodium nitroprusside (0.01 nM to 30 nM) in phenylephrine (10 μM) pre-contracted rings.

2.2.11 *Statistical Analysis*

Results are provided as means ± SEM. Student's t tests, one-way ANOVA, and repeated measures ANOVA with a post hoc Tukey test were used to evaluate statistical differences between treatment groups for various experiments. All statistical analyses were performed using graphpad. Values where $p < 0.05$ were deemed statistically significant.

2.3 Results

2.3.1 *Mice lacking Panx1 and/or Cx40 are similar in size and weight*

To evaluate the levels of Panx1 present in the wild-type mouse heart, RNA was extracted from atrial and ventricular tissues and subjected to q-PCR analysis. Panx1 transcripts were abundant in both the atria and the ventricles, not unlike observations in brain tissue. Moreover, evaluation of Panx1 transcript levels from Panx1 null mice revealed that Panx1 was ablated from the heart and brain (Figure 2.1A). PCR genotyping further revealed that both Cx40 and Panx1 were ablated from the double knockout mouse generated from crossing Panx1 null mice with mice lacking Cx40. The presence of Cx43 mRNA in control and mutant mice confirmed the integrity of the samples used in genotyping (Figure 2.1B). In order to determine if mice lacking Panx1, Cx40 or both Panx1 and Cx40 exhibited any overt phenotypes, we tracked the size and weight of all mouse genotypes. It was found that all four-mouse genotypes exhibited no differences over a 1-year time frame (Figure 2.1C, D).

2.3.2 *Increased in utero and newborn pup death in $Cx40^{-/-}Panx1^{-/-}$ mice*

Although no overt anatomical phenotypes were found, the Panx1 and Cx40 double knockout mouse produced 33% fewer pups per litter than wild-type control animals (Figure 2.2A). The survivability ratio revealed that 36% more $Cx40^{-/-}Panx1^{-/-}$ pups die 1-3 days post birth than single knockout mice and wild-type controls (Figure 2.2B). Therefore, between embryonic and newborn pup death $Cx40^{-/-}Panx1^{-/-}$ litter sizes are decreased by 69%. Pregnant dams were dissected on embryonic day (E) 14.5 to delineate if the in utero death was due to resorption, early embryonic death (prior to E14.5) or late fetal death. We found that the $Cx40^{-/-}Panx1^{-/-}$ embryos had a similar number of implantations and percent resorption compared to wild-type mice (Figure 2.2C, D). Interestingly, 18% of in utero death in $Cx40^{-/-}Panx1^{-/-}$ mice was found to occur at E14.5 (Figure 2.2E). These results indicate that $Cx40^{-/-}Panx1^{-/-}$ mice die both prematurely in utero and soon after birth.

Figure 2-1 Characterization of Cx40/Panx1 knockout mice.

(A) Quantitative PCR revealed that Panx1 was highly expressed in both the atrial and ventricles of wild-type (WT) mice, similar to that found in brain tissue, but absent in Panx1 null mice. (B) PCR genotyping confirms that both Panx1 and Cx40 are ablated in double knockout mice as reveled in lanes 2-5. Cx43 was present in all mice and the insertion of the Cx40 neocassette (Cx40-NC) reaffirms the ablation of Cx40. (C) Photograph shows that the overall size of control and mutant mice are approximately the same. (D) Assessment of mouse weight over 1 year revealed that all control and mutant mice have similar weights and weight gain (N= 3-6 litters, 10 mice/timepoint).

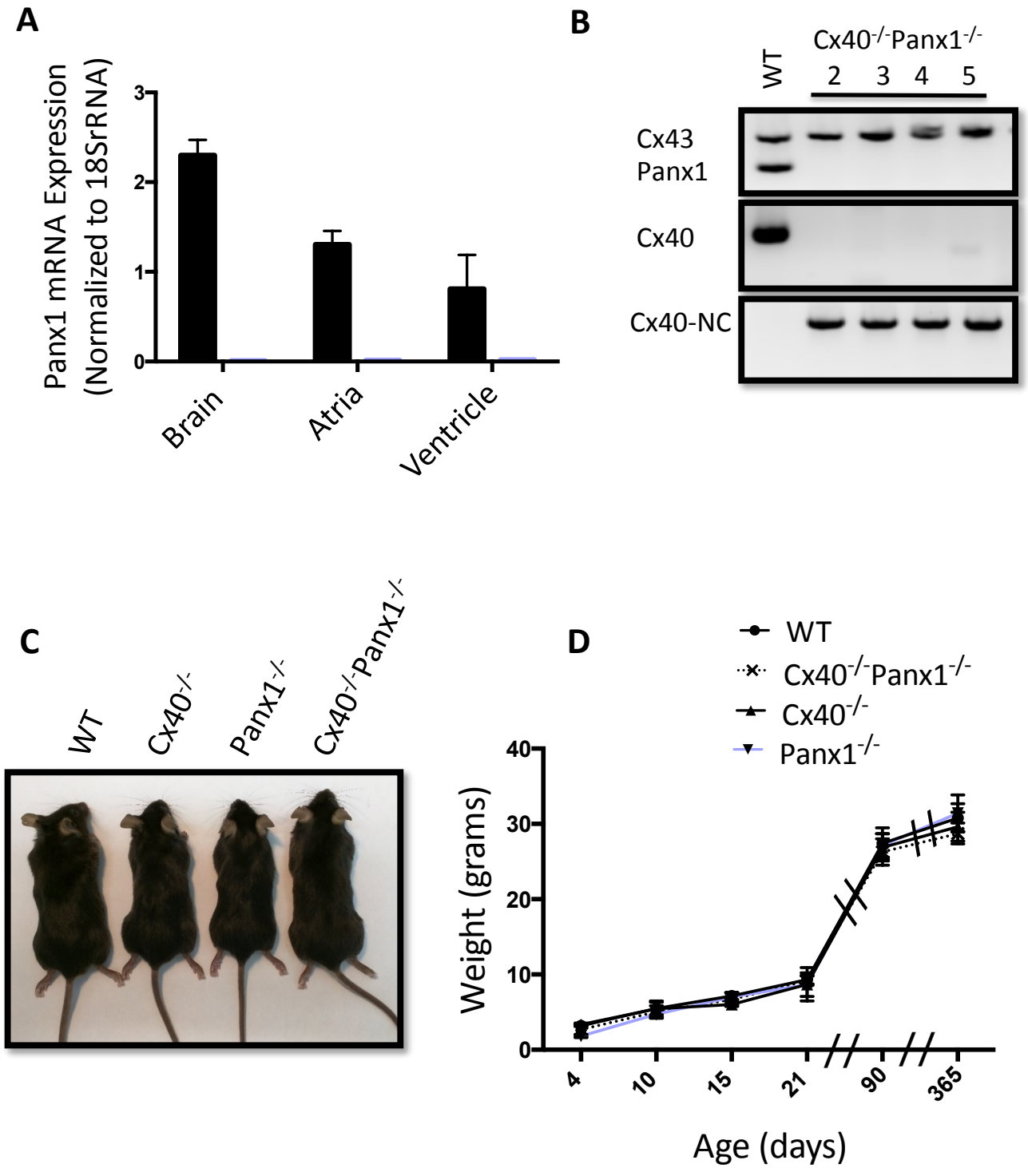
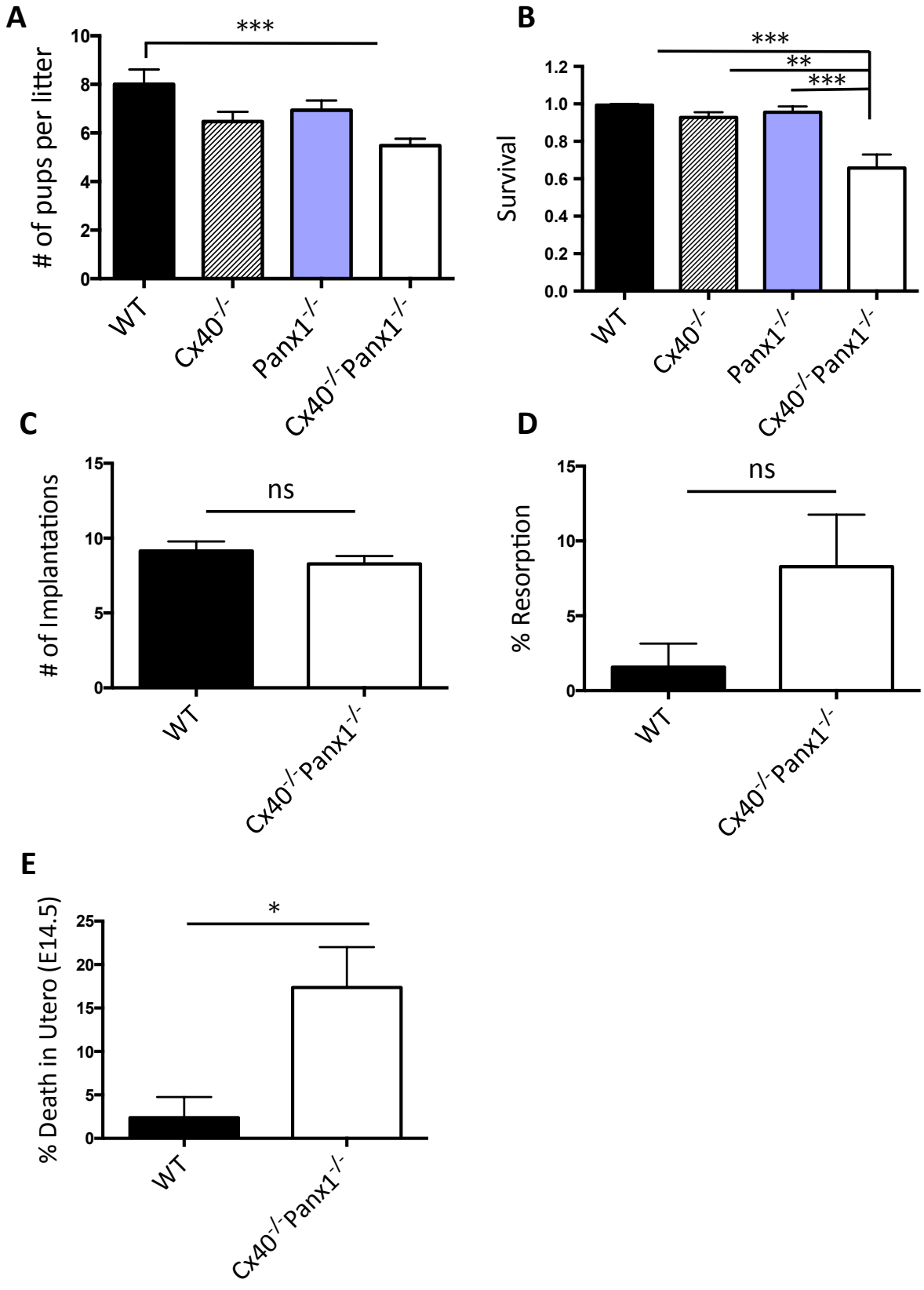

A
Panx1 mRNA Expression
(Normalized to 18SrRNA)
3
2
1
0
Brain
Atria
Ventricle
B
WT
Cx40-/-Panx1-/-
2 3 4 5
Cx43
Panx1
Cx40
Cx40-NC
C
WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-
D
WT
Cx40-/-Panx1-/-
Cx40-/-
Panx1-/-
Weight (grams)
40
30
20
10
0
4
10
15
21
90
365
Age (days)

Figure 2-2 Cx40/Panx1 null mice have decreased survival in utero and postnatal

(A) WT and genetically-modified mouse mean litter size at birth and (B) survivability 3 days postnatal (N=12 litters). (C) Mean number of implantations, (D) % fetal resorption, and (E) % fetal deaths in utero prior to E14.5 for WT and $Cx40^{-/-}Panx1^{-/-}$ mice (N=7 damns). *$p<0.05$, ***$p<0.001$, ns = not significant.


A
# of pups per litter
***
WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-
B
Survival
***
**
***
WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-
C
# of Implantations
ns
WT
Cx40-/-Panx1-/-
D
% Resorption
ns
WT
Cx40-/-Panx1-/-
E
% Death in Utero (E14.5)
*
WT
Cx40-/-Panx1-/-

2.3.3 *Ablation of Panx1 does not alter the expression/distribution of Cx40*

In order to determine if there was potentially regulatory cross-talk between Cx40 and Panx1 channels, we assessed if the ablation of Panx1 altered the expression or distribution of Cx40. Immunofluoresence revealed the presence of Cx40 in GJ structures in the atria of wild-type and $Panx1^{-/-}$ mice which was appropriately absent from hearts obtained from Cx40 knockout mice (Figure 2.3A). Western blots further revealed that there was no quantitative change in Cx40 levels in mice lacking Panx1 (Figure 2.3B, C). Thus, Cx40 levels or localization does not change in mice lacking Panx1.

2.3.4 *$Cx40^{-/-}$ and $Cx40^{-/-}Panx1^{-/-}$ mouse hearts are hypertrophic*

Given the enlarged appearance of $Cx40^{-/-}$ and $Cx40^{-/-}Panx1^{-/-}$ mouse hearts (Figure 2.4A), it was not unexpected that both these mutant mice had higher heart mass compared to $Panx1^{-/-}$ and wild-type mice at 3 weeks (Figure 2.4B) and 3 months (Figure 2.4C) of age. Left and right kidney weights were also assessed and found to be similar among all four genotypes (Figure 2.4D). To validate that the increased heart mass seen in the $Cx40^{-/-}$ and $Cx40^{-/-}Panx1^{-/-}$ mice was due to cardiomyocyte hypertrophy, myofibril area was measured after denoting the boundaries of the cells by labeling with wheat germ agglutinin. Representative micrographs of atrial (Figure 2.5A) and ventricular (Figure 2.5B) cross-sections of myofibril bundles in 3-month-old mice and quantitation revealed that cell area

Figure 2-3 Cx40 levels and localization are unchanged in Panx1 ablated mice.

(A) Fluorescent micrographs reveal similar localization of Cx40 (green) in WT and Panx1$^{-/-}$ mouse atria. The absence of Cx40 gap junction plaques in Cx40$^{-/-}$ and Cx40$^{-/-}$Panx1$^{-/-}$ confirms the ablation of Cx40. (B) Western blot and (C) quantification reveals similar levels of Cx40 in wild-type and Panx1$^{-/-}$ mice, and its ablation in Cx40$^{-/-}$ and Cx40$^{-/-}$Panx1$^{-/-}$ atria (N=3). Scale bar= 20 μm.

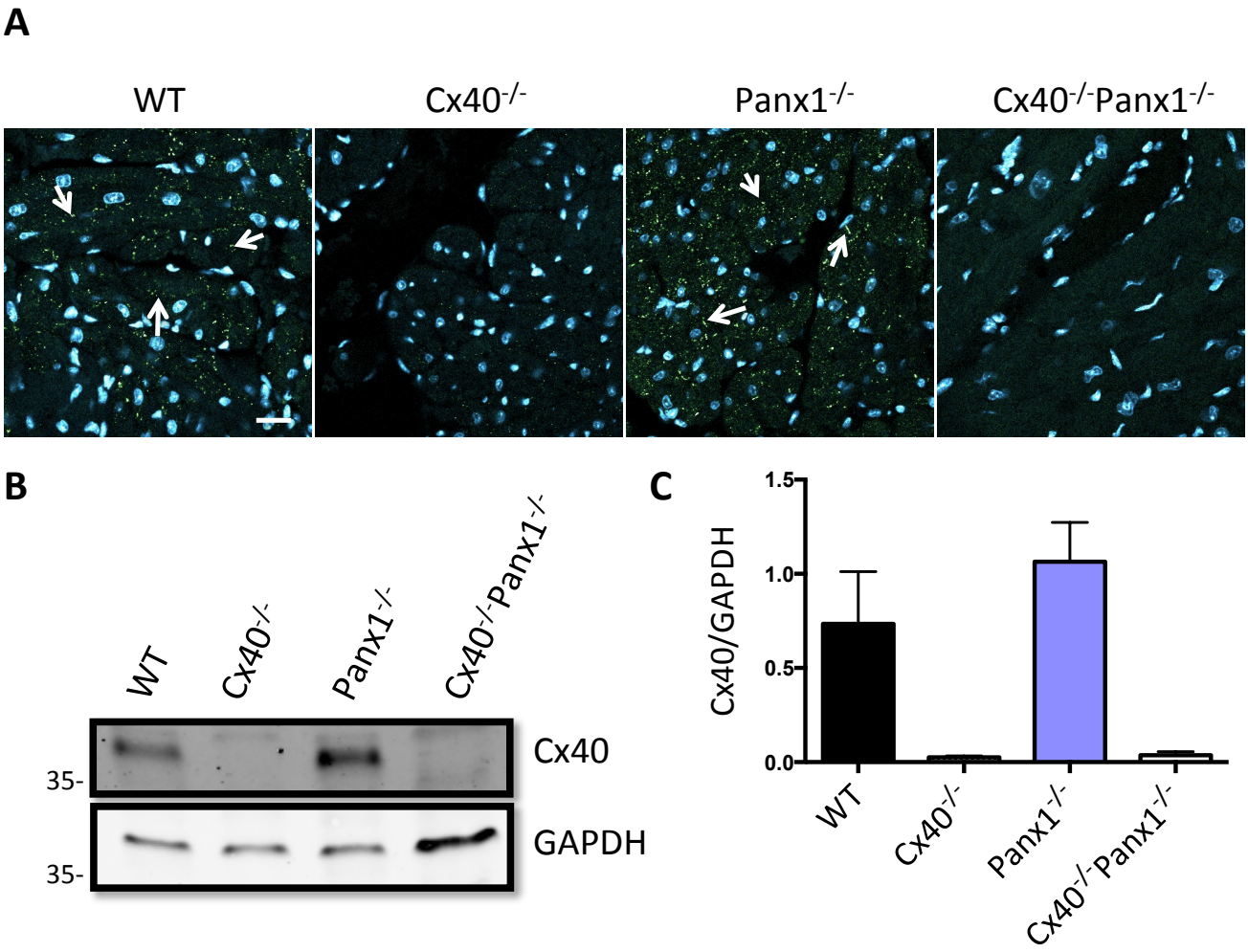

A
WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-
B
WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-
35-
Cx40
35-
GAPDH
C
Cx40/GAPDH
1.5
1.0
0.5
0.0
WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-

Figure 2-4 Increased heart mass found in Cx40 and Cx40/Panx1 knockout mice.

(A) Photographic examples of representative hearts from all mouse cohorts highlighting the enlarged hearts in $Cx40^{-/-}$ and $Cx40^{-/-}Panx1^{-/-}$ mice. When heart weights were examined it was found that $Cx40^{-/-}$ and $Cx40^{-/-}Panx1^{-/-}$ mice had increased heart weight relative to body weight at the ages of (B) 3 weeks (N=4) and (C) 3 months (N=6) as compared to WT and $Panx1^{-/-}$ mice. (D) Mean kidney weights were compared in 3 month old mice and found to be similar (N= 6). Different letters represent significant changes, $p<0.001$. ns = not significant

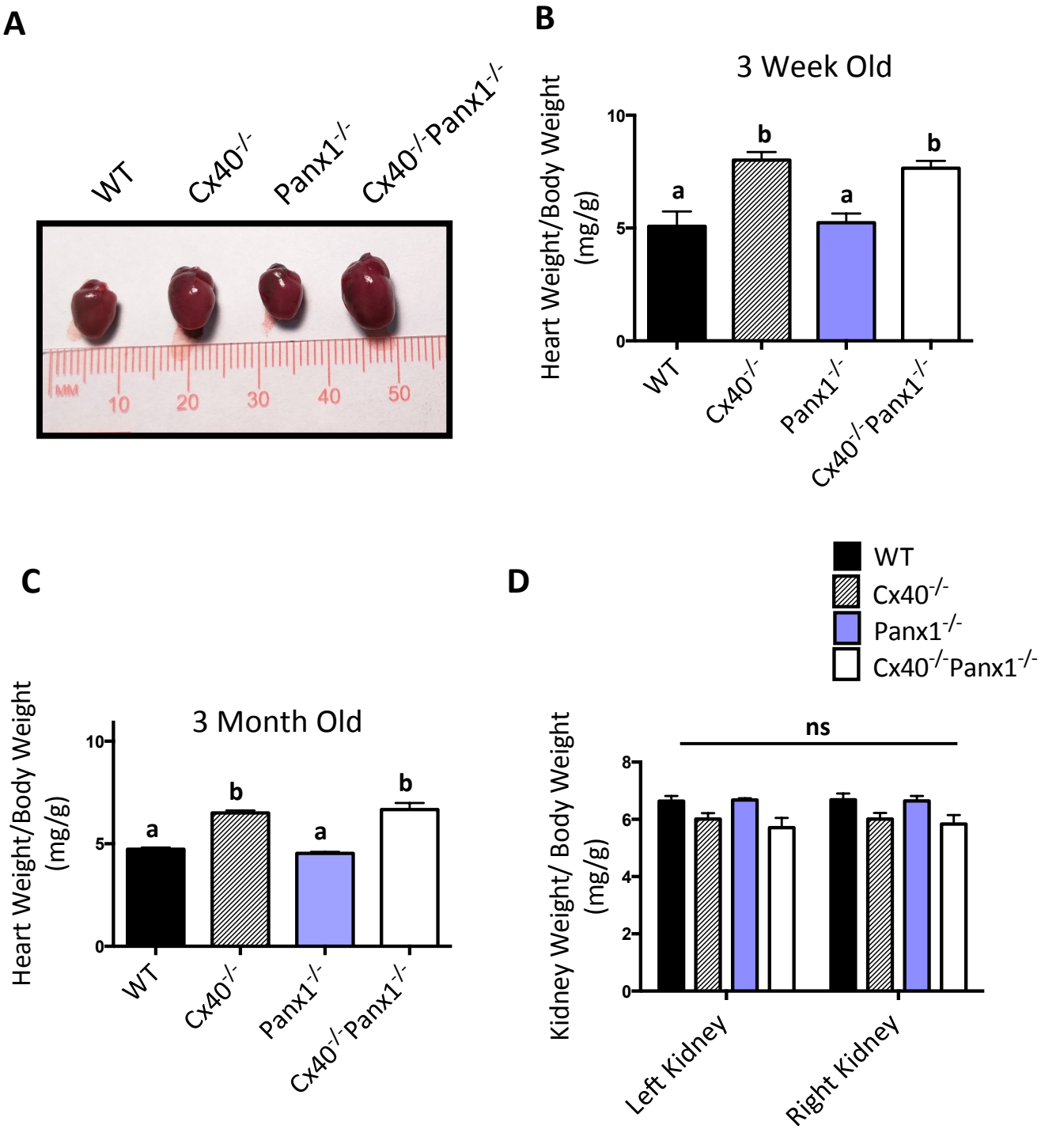
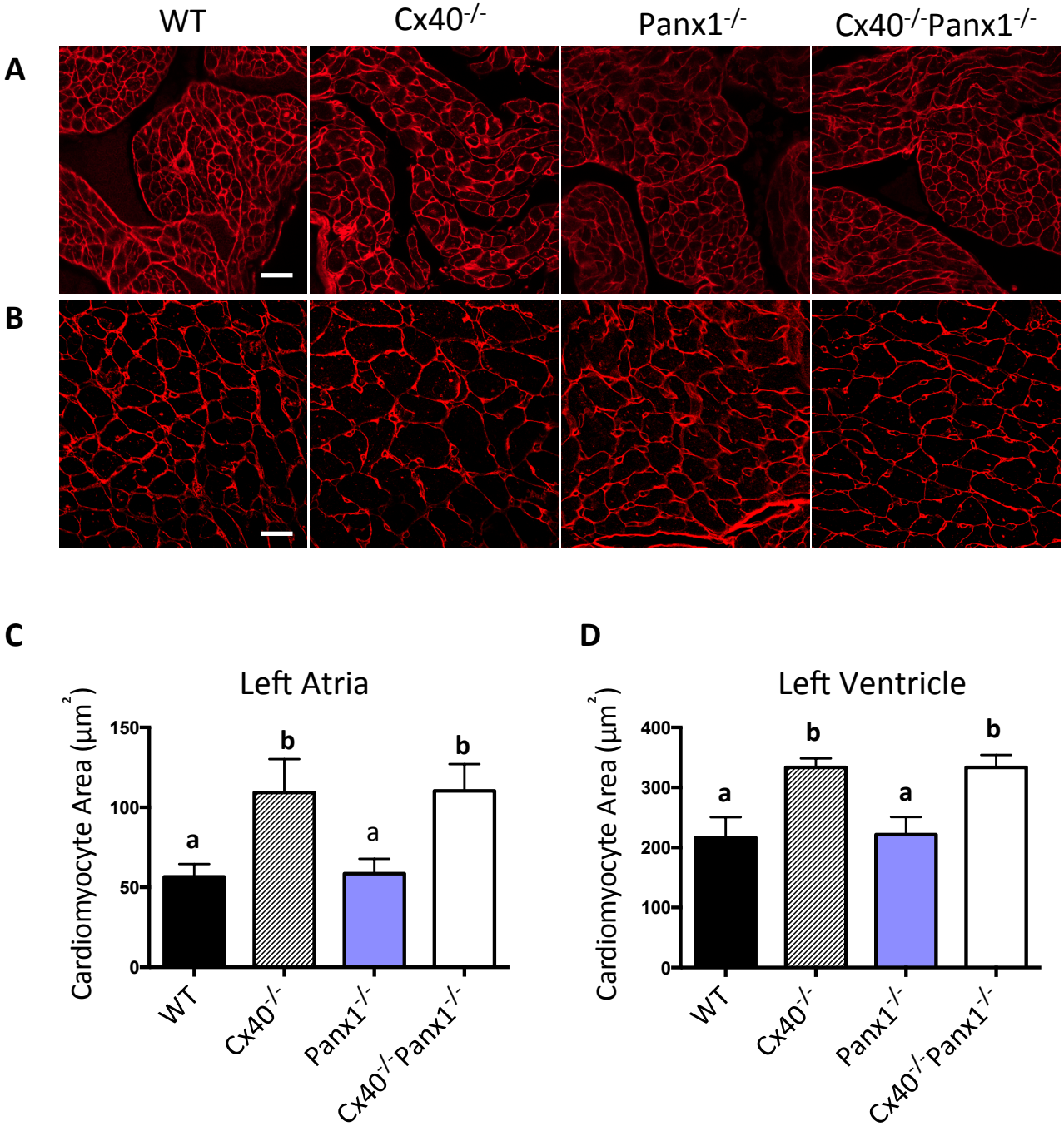

A
WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-
MM
10
20
30
40
50
B
3 Week Old
Heart Weight/Body Weight (mg/g)
10
5
0
a
b
a
b
WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-
C
3 Month Old
Heart Weight/Body Weight (mg/g)
10
5
0
a
b
a
b
WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-
D
WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-
Kidney Weight/ Body Weight (mg/g)
ns
8
6
4
2
0
Left Kidney
Right Kidney

Figure 2-5 Cx40 and Cx40/Panx1 mice have hypertrophic cardiomyocytes.

Wheat germ agglutinin staining (red) of the cardiomyocyte cell surface in left (A) atrial and (B) ventricular heart cross sections taken from 3-month old wild-type and mutant mice. Quantification of the average cardiomyocyte cell circumference revealed increased (C) atrial (N=6) and (D) ventricular (N=5) cardiomyocyte area in $Cx40^{-/-}$ and $Cx40^{-/-}Panx1^{-/-}$ mouse hearts, $p< 0.05$. Scale bar = 20 μm.


WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-
A
B
C
Left Atria
Cardiomyocyte Area (µm2)
150
100
50
0
a
b
a
b
WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-
D
Left Ventricle
Cardiomyocyte Area (µm2)
400
300
200
100
0
a
b
a
b
WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-

was significantly increased in the left atria (Figure 2.5C) and left ventricle (Figure 2.5D) of Cx40 and Cx40/Panx1 knockout cohorts of mice.

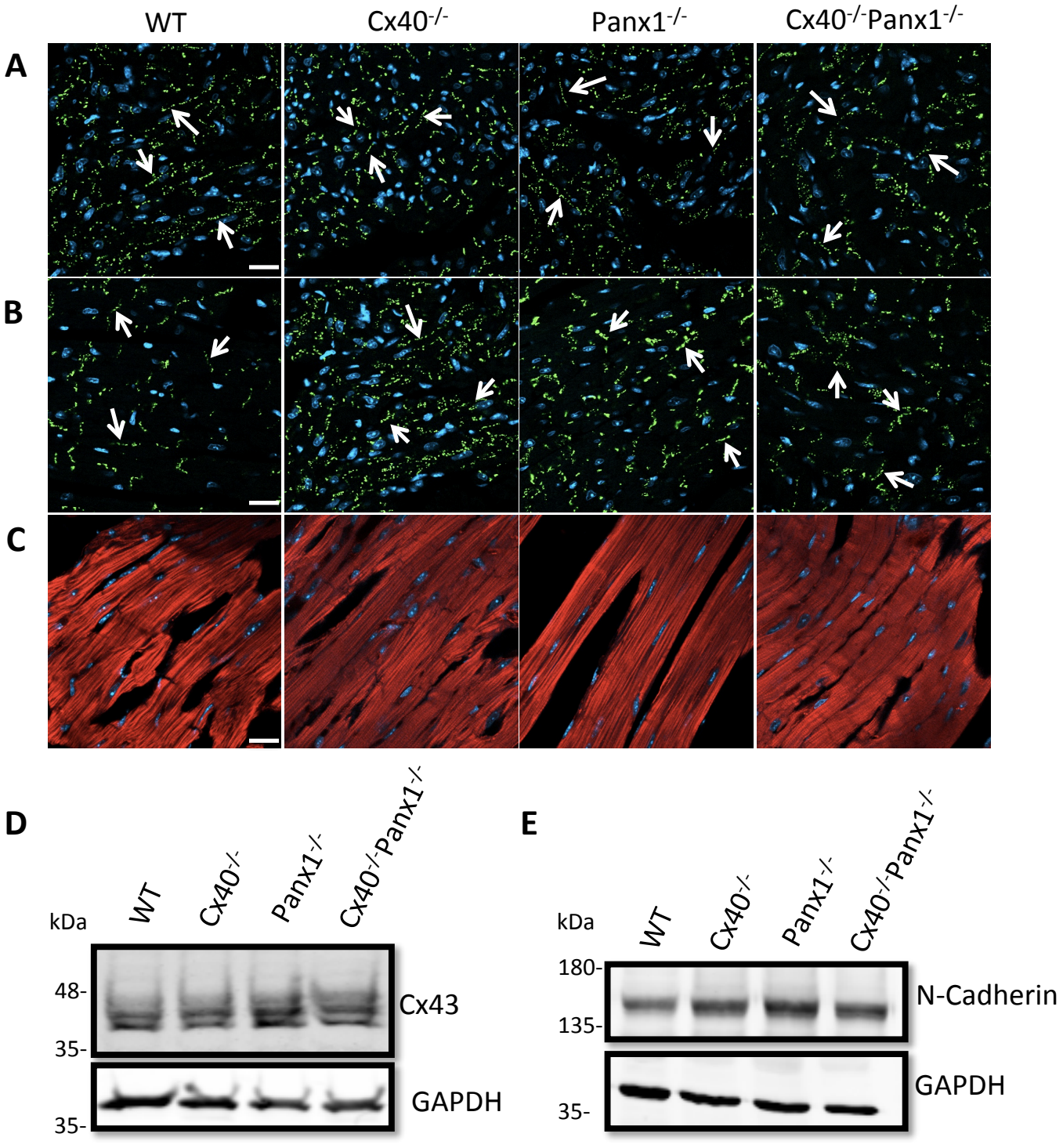
2.3.5 *Fibrotic and structural analysis of mutant mouse hearts*

Cardiac fibrosis in 3-month-old wild-type and mutant mouse hearts was assessed via Masson's Trichrome staining and Western blotting for various extracellular matrix (ECM) proteins. Masson's Trichrome staining of the left atria (Supplementary Figure 2.1A) and ventricle (Supplementary Figure 2.1B) suggested that there was minimal interstitial fibrosis in the left ventricle of both single knockouts, and double knockout mice. Immunoblotting indicated similar levels of collagen 1 and fibronectin present in the atria and entire heart among all four genotypes (Supplementary Figure 2.1 C, D). Overall findings are similar among all four genotypes with little to no fibrosis present.

Since heart hypertrophy and fibrosis are indicators of cardiovascular disease wild-type, $Cx40^{-/-}$, $Panx1^{-/-}$, and $Cx40^{-/-}Panx1^{-/-}$ hearts were examined for structural anomalies. Localization of Cx43 plaques to the intercalated disc in the atria (Figure 2.6A) and ventricles (Figure 2.6B) in mutant mice were comparable to wild-type. To assess for compensatory effects by the upregulation of other connexins, immunoblotting revealed that the levels of Cx43 were similar in the atrial tissue of knockouts and wild-type mice (Figure 2.6D). Western blotting for N-cadherin (Figure 2.6E) revealed similar levels of this junction protein among all four genotypes. These results indicate that the loss of Cx40, Panx1 or both had no detrimental effects on the structure of the intercalated disc. Finally, myofibril arrangement was evaluated by phalloidin staining (Figure 2.6C) and it was found that F-actin structure was comparable among all four genotypes.

Figure 2-6 Junctional proteins and F-actin are unaltered in the hearts of mutant mice.

In 3-month-old wild-type, $Cx40^{-/-}$, $Panx1^{-/-}$, and $Cx40^{-/-}Panx1^{-/-}$ mice Cx43 (green) localization was assessed by immunofluorescence in the (A) atria and (B) ventricles. (C) Phalloidin (red) was used to visualize F-actin in the ventricle of WT and mutant mice. (D) Western blots of Cx43 and (E) N-cadherin in atrial lysates revealed no difference in the abundance of these junctional proteins (N=3). Scale bar = 20 μm.


WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-
A
B
C
D
kDa
WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-
48-
35-
Cx43
35-
GAPDH
E
kDa
WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-
180-
135-
N-Cadherin
35-
GAPDH

2.3.6 *Physiology of the cardiovascular system in mutant mice*

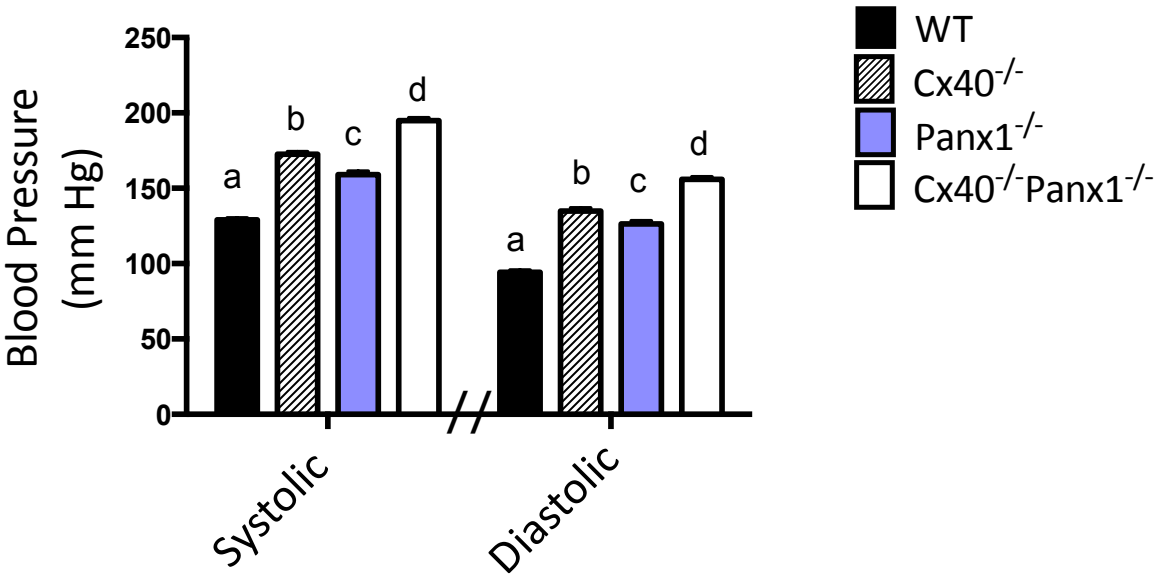
To begin to characterize the cardiovascular physiology of the mutant mice, blood pressure testing via the Coda tail-cuff system was performed on all four genotypes at 3 months of age. Somewhat to our surprise, we discovered that $Cx40^{-/-}$ and $Panx1^{-/-}$ mice were both hypertensive, and mice lacking both large-pore channels were more severely hypertensive (Figure 2.7A). To confirm that the hypertension observed was not due to experimentally-induced anxiety, blood pressure measurements of anesthetized mice were performed and it was found that the three knockout strains remained hypertensive while the wild-type mice were normotensive (Supplementary Figure 2.2A). Furthermore, heart rates (HR) were measured and found to be comparable among all four genotypes ranging from a mean HR of 598 beats per minute (BPM) in wild-type mice to 630 BPM in the $Cx40^{-/-}Panx1^{-/-}$ mouse (Figure 2.7B). Interestingly, heart rates in anesthetized mice were variable, and it is speculated that this was due to individual differences in drug metabolism (Supplementary Figure 2.2B).

To determine if the aortic vasomotor signaling properties were affected by the ablation of Cx40 and/or Panx1 from endothelial cells, or if the hypertension found in the mutant mice caused endothelial damage, isometric tension experiments were performed on aortic rings using a wire myograph. Four parameters were assessed; vasocontractile responses to phenylephrine (PE) and to potassium chloride (KCl) agonists, and vasodilation to methacholine (MCh) and sodium nitroprusside (SNP) agonists. It was found that contractile responses to PE and KCl were similar amongst mutant and wild-type mice (Figure 2.8A, B). Contrastingly, MCh mediated vasodilation was significantly decreased in $Cx40^{-/-}$, $Panx1^{-/-}$, and $Cx40^{-/-}Panx1^{-/-}$ mice after the addition of 100 nM or more of agonist. Nevertheless, receptor independent vasodilation as assessed by SNP was unaltered among all four genotypes (Figure 2.8C, D).

Figure 2-7 Panx1 and Cx40/Panx1 mice are hypertensive.

(A) Tail-cuff hypertension testing revealed that mutant mice have significantly higher systolic and diastolic blood pressure than wild-type mice, with the double knockout of Cx40 and Panx1 having an additive effect (N=9). (B) Mean heart rate is similar among all four genotypes (N=9). One-way ANOVA, a, b, c, d = $p<0.001$.

A


Blood Pressure (mm Hg)
250
200
150
100
50
0
a
b
c
d
a
b
c
d
Systolic
Diastolic
WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-

B

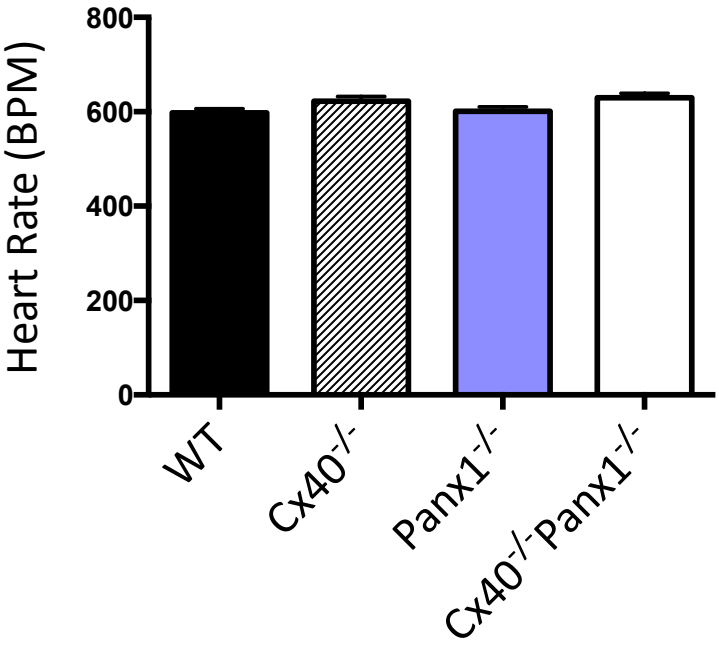
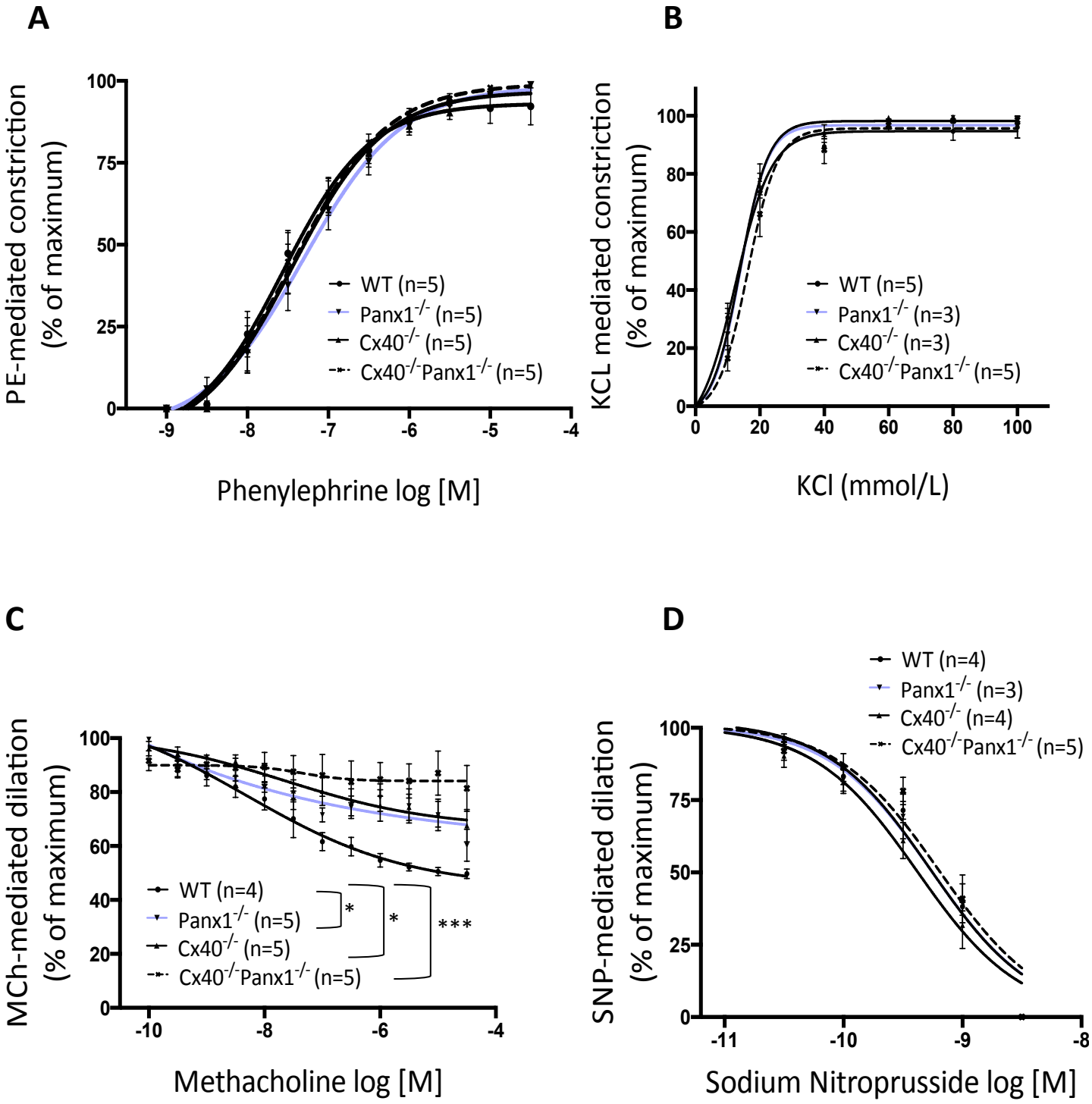

Heart Rate (BPM)
800
600
400
200
0
WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-

Figure 2-8 Endothelium-dependent vasodilatory responses are blunted in mutant mice.

Time course dose response curves to (A) phenylephrine (PE) and (B) potassium chloride (KCl) were generated to assess thoracic aorta contractility and to (C) methacholine (MCh) and (D) sodium nitroprusside (SNP) to assess aorta vasodilation among 2-4 month old wild-type, $Cx40^{-/-}$, $Panx1^{-/-}$, and $Cx40^{-/-}Panx1^{-/-}$ mice. *$p<0.05$.


A
PE-mediated constriction (% of maximum)
100
75
50
25
0
-9
-8
-7
-6
-5
-4
Phenylephrine log [M]
WT (n=5)
Panx1-/- (n=5)
Cx40-/- (n=5)
Cx40-/-Panx1-/- (n=5)
B
KCL mediated constriction (% of maximum)
100
80
60
40
20
0
0
20
40
60
80
100
KCl (mmol/L)
WT (n=5)
Panx1-/- (n=3)
Cx40-/- (n=3)
Cx40-/-Panx1-/- (n=5)
C
MCh-mediated dilation (% of maximum)
100
80
60
40
20
0
-10
-8
-6
-4
Methacholine log [M]
WT (n=4)
Panx1-/- (n=5)
Cx40-/- (n=5)
Cx40-/-Panx1-/- (n=5)
*
*
***
D
SNP-mediated dilation (% of maximum)
100
75
50
25
0
-11
-10
-9
-8
Sodium Nitroprusside log [M]
WT (n=4)
Panx1-/- (n=3)
Cx40-/- (n=4)
Cx40-/-Panx1-/- (n=5)

2.4 Discussion

Both Panx1 tissue-specific and global knockout mice have been engineered in recent years to assess the roles of these large-pore channels in normal physiology and disease[34, 35]. Through these studies Panx1 has emerged as an important player in cardiovascular physiology. This point was emphasized when Petric *et al*. reported high levels of Panx1 within murine atria, and that Panx1 ablation led to increased susceptibility to atrial fibrillation[23]. As well, Panx1 has been implicated in α1-adrenoreceptor mediated vasoconstriction[19], blood pressure regulation[36], and renal function[37]. Interestingly, the gap junction protein Cx40 shares many of these characteristics including its abundance in the atria, and involvement in cardiac, vascular, and renal function[38, 39]. Therefore in the current study we addressed the consequences of ablating both Panx1 and Cx40 channels in the same mouse model as we hypothesized this would lead to an increasingly severe cardiovascular phenotype.

2.4.1 *Engineering and phenotyping of mice lacking both Cx40/Panx1*

Our novel $Cx40^{-/-}Panx1^{-/-}$ mouse is the first global knockout mouse model in which a connexin and pannexin channel type have been co-ablated, and complements one other similar strategy where Cx43 and Panx3 were conditionally ablated in bone tissue[24]. Since both Cx40 and Panx1 have been reported to release overlapping small members of the metabolome from intracellular stores to the extracellular milieu[3,4], we anticipated that the double knockout mouse might have a reduced ability to survive. Intriguingly, while double-knockout mice were fertile and viable, the combined ablation of Cx40/Panx1 caused a severe reduction in prenatal and newborn pup survival. A previous report describes mild pre/postnatal death in the $Cx40^{-/-}$ mouse. Those authors hypothesized that prenatal embryo death was due to inadequate propagation within the cardiac conduction system, while postnatal pup death was likely a combination of improper

septation and increased cardiac workload during the establishment of pulmonary circulation[12]. Similarly, in our study, although not statistically significant, Cx40 null mice had 22% fewer offspring and 10% newborn death compared to wild-type. Intriguingly, this phenotype although minor in the single Cx40 knockout mouse became detrimental when Panx1 and Cx40 were co-ablated as over half of the $Cx40^{-/-}Panx1^{-/-}$ litter was lost either to pre or postnatal mortality. These findings point towards a concomitant role for Cx40 and Panx1 in the development of the maturing embryo. Qualitative evaluation of the dead $Cx40^{-/-}Panx1^{-/-}$ embryos revealed that in utero death might be due to insufficient cephalic circulation and hemorrhaging in the thorax. This observation points towards a cardiovascular cause for embryonic lethality, and potentially to the dynamic regulation of Panx1 along with Cx40 in fetal heart development[8]. The developmental regulation of Panx1 has been reported in skin[40] further suggesting that Panx1 may play critical roles in the development of multiple organs.

2.4.2 *$Cx40^{-/-}Panx1^{-/-}$ mice have cardiac hypertrophy due to Cx40 ablation*

Arterial hypertension is the most common cause of pressure overload within the heart and often leads to pronounced cardiac hypertrophy, with the left ventricle being the most severely affected heart chamber. Within this organ, hypertrophic growth is due to biomechanical stress, which causes cardiomyocyte enlargement by potentiating changes in cardiac gene expression and reactivation of the fetal gene program[41]. This phenomenon has been previously reported in Cx40 null mice wherein hypertrophic characterization relied exclusively on the quantification of heart weight[13]. Here, we observed cardiac hypertrophy in young and adult $Cx40^{-/-}$ mice and confirmed that it was due to the enlargement of left ventricular cardiomyocytes. Contrary to our expectations, the phenotype observed in mice lacking both Cx40 and Panx1 was not exacerbated.

This indicates that the cardiac hypertrophy found in the $Cx40^{-/-}Panx1^{-/-}$ mouse is unilaterally due to the knockout of Cx40 channels. Additionally, both $Cx40^{-/-}$ and $Cx40^{-/-}Panx1^{-/-}$ mice had pronounced left atrial cardiomyocyte enlargement. This is intriguing because unlike left ventricular cardiomyocyte hypertrophy, which is often associated with a hypertensive phenotype, left atrial dilatation may be due to pathologies that arise from defects in the cardiac conduction system like atrial fibrillation[42] and both the $Cx40^{-/-}$ and $Panx1^{-/-}$ mice are increasingly susceptible to atrial arrhythmias[13, 23].

2.4.3 *Mutant mouse hearts lack fibrosis & display normal GJ/myofibril arrangement*

Hypertrophy-induced myocardial remodeling is characterized by augmented interstitial fibrosis, gap junction lateralization, and cytoskeletal remodeling which are alterations that often lead to a detrimental arrhythmogenic phenotype and heart failure[43]. Interstitial fibrosis was only minimally identified by Masson's trichrome staining and there were no significant changes in collagen 1 or fibronectin in either the left atria or ventricle of any mice suggesting that mutant mouse hearts were not particularly fibrotic. Likewise, the intercalated disc distribution of Cx43 and N-cadherin, as well as the myofibril arrangement was unaltered in mutant mice. Taken together these findings indicate that the cardiac hypertrophy found in single Cx40 and double Cx40/Panx1 knockout mice may lead to myocardial remodeling however mutant hearts do not yet possess detrimental ECM protein depositions or alterations in intercalated disc integrity that would lead to heart failure. Because hypertrophy is often an adaptive physiological process in response to increases in cardiac workload, and is dynamically regulated, we speculate that in later life, markedly increased fibrosis, gap junction and cytoskeletal remodeling may occur in our mutant mice[44].

2.4.4 *Mutant mice are hypertensive and possess altered vasomotor responses*

Because the development of cardiac hypertrophy is often secondary to hypertension, tail-cuff blood pressure measurements were performed on all mutant mice. The hypertensive phenotype of Cx40 knockout has already been reported to be mediated by the renin-angiotensin-aldosterone system[45]. Previous findings of hypotension in an inducible model of selective Panx1 ablation from smooth muscle cells led to speculation that the combined ablation of Cx40/Panx1 would lead to a more normalized blood pressure[36]. Surprisingly, it was found that, along with the $Cx40^{-/-}$ mice, Panx1 global knockout mice had increased systolic and diastolic blood pressures whether mice were awake or anesthetized. Furthermore, hypertension in the double knockout was potentiated in awake mice by the combined ablation of Cx40 and Panx1. These results were intriguing and unexpected because not only do they point towards a role for Panx1 in blood pressure regulation, but also imply that Panx1 may protect the heart from hypertension-induced growth. However, since hypertension is a multifactorial disease and all mice used in this study were global knockouts, this raised questions as to where Panx1 was contributing to blood pressure regulation.

Hypertension is due to many different factors, which cause alterations in peripheral resistance and cardiac output. Peripheral resistance is mediated by small arteries, whereas cardiac output is influenced by blood volume, and thus under renal control[46]. As of yet the cause of hypertension in Panx1 global knockout mouse remains unknown but several possibilities exist. Extensive research previously performed on resistance arteries in a mouse model in which Panx1 was selectively ablated from smooth muscle cells indicates that the hypertension observed in our

study is not due to increased vasoconstriction of the small arteries themselves but rather a consequence of system wide ablation of Panx1 [19,36].

The first possibility involves the participation of Panx1 in endothelium derived hyperpolarization factor relaxation. In fact, global Panx1$^{-/-}$ mice display increased resistance due to the blunted response of Panx1$^{-/-}$ mouse arterioles to muscarinic receptor agonist acetylcholine[47]. The second possibility involves Panx1 channels on erythrocytes releasing ATP during low blood pO_2 to stimulate vasodilation. Without this feedback mechanism it is possible that peripheral resistance could be increased[48]. A third regulatory mechanism that could be absent in Panx1$^{-/-}$ mice and govern its hypertension involves altered feedback between the carotid body and brainstem. In short, it has been shown that type II cells in the carotid body propagate ATP released from Panx1 channels onto afferent nerve terminals that synapse with cardiorespiratory centers in the brainstem for the regulation of blood pressure and respiratory rate[49]. In addition, Panx1 channels have been localized in both cortical and medullary tubule segments of the kidney and in the renal vasculature. Functionally little is known about the role of Panx1 in the renal system, however based on its similar localization profile to Cx30 within the apical collecting duct system, and its role in purinergic signaling, it has been previously hypothesized that Panx1 may play a role in pressure natriuresis, and that Panx1$^{-/-}$ mice might possess a salt retention phenotype[50] and consequently hypertension. All in all because there is evidence that Panx1 plays such a diverse role in blood pressure regulation it is hard to speculate if one or all of the aforementioned factors as a conglomerate are producing the hypertensive phenotype found in the global knockout mouse, however selective ablation of Panx1 in these various organ systems using Cre-lox technology could help predict causation. We therefore postulate that Cx40 and Panx1 act either via similar or different mechanisms to induce hypertension in the double knockout mouse.

Another important consideration to address is why the hypertension found in Panx1$^{-/-}$ mice did not cause cardiac hypertrophy. More recently it has been suggested that hypertension associated left ventricular hypertrophy may not be due to pressure overload alone as large cohort studies show that only 50% of the variability in left ventricular mass can be explained by increased systolic pressure[51]. Recently neurohormonal factors that can exert trophic effects via cytokine and growth factor production, the NO-cGMP pathway, and genetic factors have come into the limelight for their suggested roles in the induction of hypertrophy[52, 53]. Therefore, these data suggest the presence of an unknown, unmeasured risk factor and implicate Panx1 in the regulation of cardiac hypertrophy.

The endothelium also plays a critical role in maintaining normal physiology and is involved in the modulation of vascular tone through the balanced release of relaxing and contracting factors. In certain cardiovascular pathophysiologies such as hypertension this balance may be disrupted which leads to impairments in endothelium –mediated vasodilation and endothelial dysfunction[54]. Both Cx40 and Panx1 are primarily localized to endothelial cells in elastic arteries, are implicated in the regulation of arterial tone by acting as facilitators of vasodilation, and both Cx40 and Panx1 mutant mice have been found to possess a decreased vasodilatory response in conduit arteries[55, 56, 57]. Our study demonstrated that the combined ablation of Cx40 and Panx1 had an additive effect on the impairment of vasodilation but did not produce alterations in vasoconstriction. In fact, Cx40$^{-/-}$Panx1$^{-/-}$ mouse aorta was found to possess severe endothelial dysfunction, characteristic of its minimal relaxation response to muscarinic receptor agonist MCh but unaltered response to endothelium independent vasodilator SNP. We postulate that the drastic decrease in vasodilation seen in the Cx40/Panx1 double knockout mouse is a combination of hypertension induced endothelial damage and the combined alteration of multiple

endothelium derived vasodilatory pathways most notably nitric oxide mediated signaling in the case of Cx40[57] and endothelium-derived hyperpolarizing factor in regards to Panx1 ablation[47].

In summary we have engineered and characterized the first Cx/Panx global knockout mouse that was bred from mice lacking Cx40 or Panx1 that exhibited distinct cardiovascular phenotypes. Consistent with earlier reports, Cx40 knockout mice produced approximately normal litter sizes but exhibited pronounced cardiac hypertrophy accompanied by minimal fibrosis, yet no GJ or myofibril remodeling. These mice were hypertensive and displayed decreased vasodilatory responses to methacholine. Here, we present the first evidence that Panx1 mutant mice are hypertensive, and confirm that they have normal heart mass and decreased vasodilatory responses to muscarinic receptor agonists. Importantly, the combined ablation of Cx40 and Panx1 led to decreased pre- and postnatal litter sizes, increased hypertension, and severe endothelial dysfunction. However, in other cases the phenotype of the Cx40 null mouse closely mirrored the $Cx40^{-/-}Panx1^{-/-}$ mouse including the cardiac hypertrophy. This suggests that even though Panx1 and Cx40 large-pore channels may act via differential mechanisms, they have a co-regulatory role in controlling vascular response.

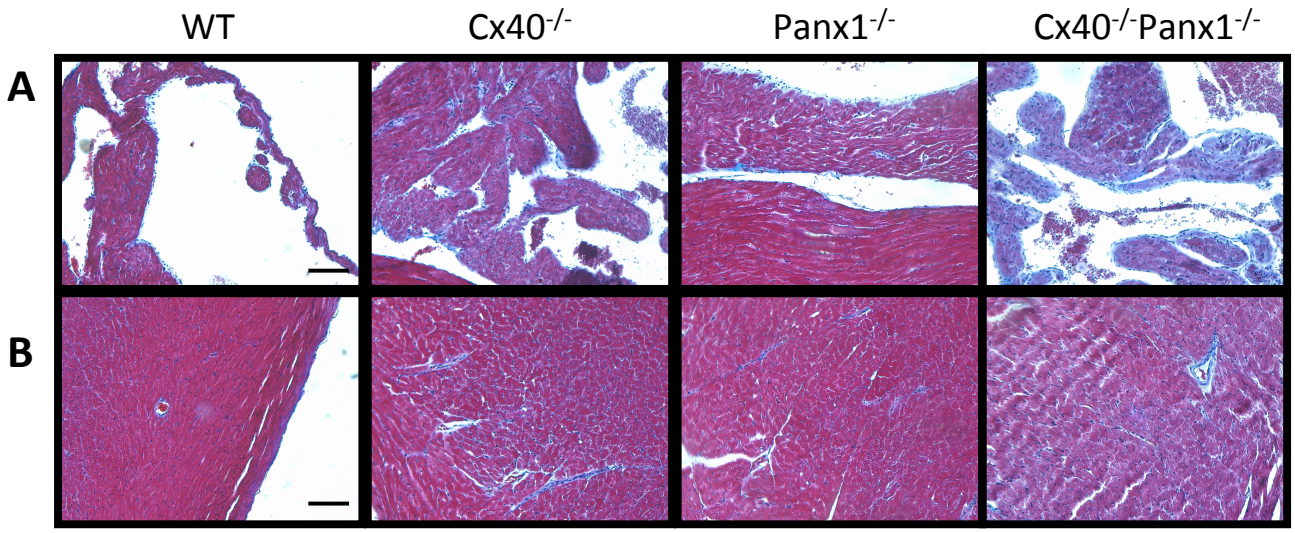
2.5 Acknowledgements

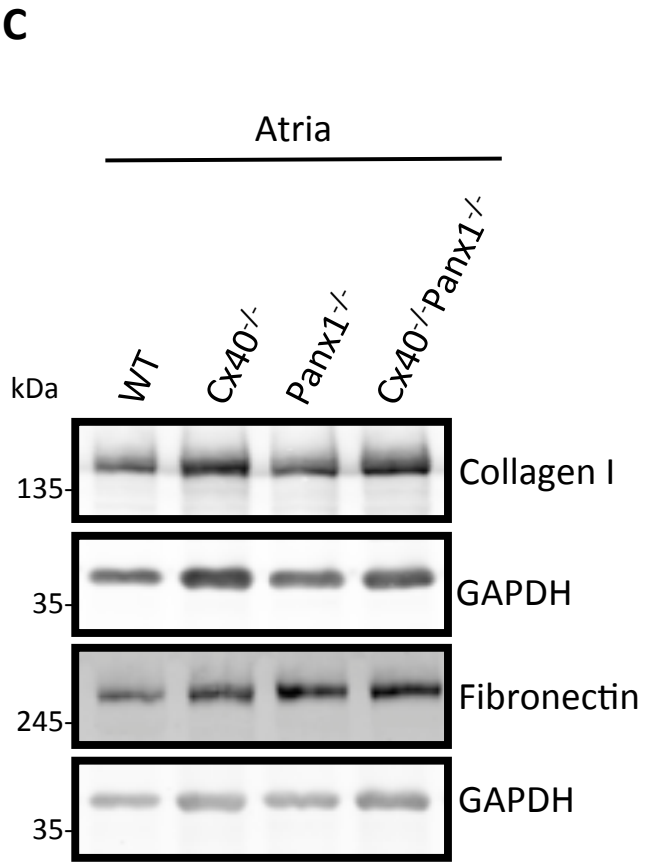
This work was supported by the Canadian Institutes of Health Research (Grant 130530) to DWL. Thank you to the Flynn lab for the use of Masson's Trichrome reagents and Collagen 1 antibody aliquots.

2.6 Supplementary Figures

Figure 2-9 (Supplementary Figure 2.1) Masson's Trichrome staining and assessment of ECM proteins in the hearts of mutant mice.

Light micrographs of Masson's Trichrome staining in the left (A) atria and (B) ventricle of 3 month old mice revealed minimal fibrosis in all mutant mice. Western blotting revealed that the levels of collagen I and fibronectin were similar in mouse lysates of (C) atria and (D) whole hearts (N=3). Bar = 40 μm.


WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-
A
B


C
Atria
kDa
WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-
135-
Collagen I
35-
GAPDH
245-
Fibronectin
35-
GAPDH

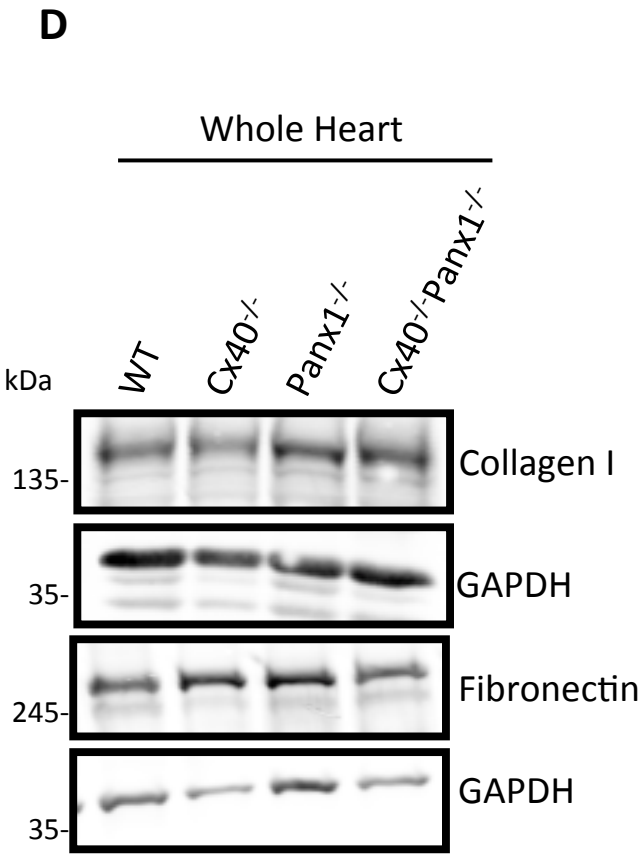
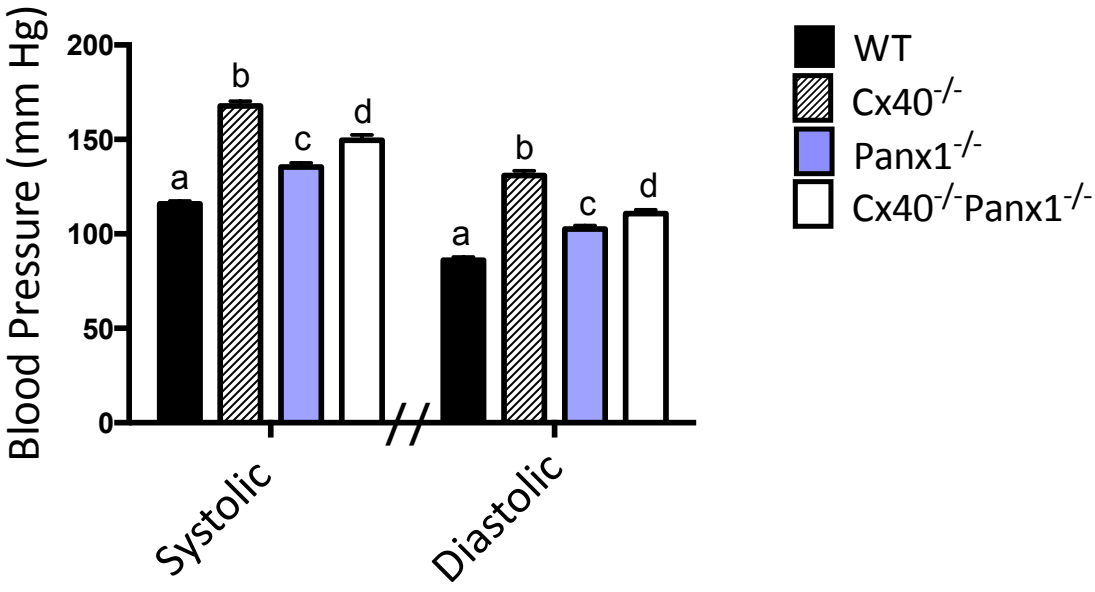

D
Whole Heart
kDa
WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-
135-
Collagen I
35-
GAPDH
245-
Fibronectin
35-
GAPDH

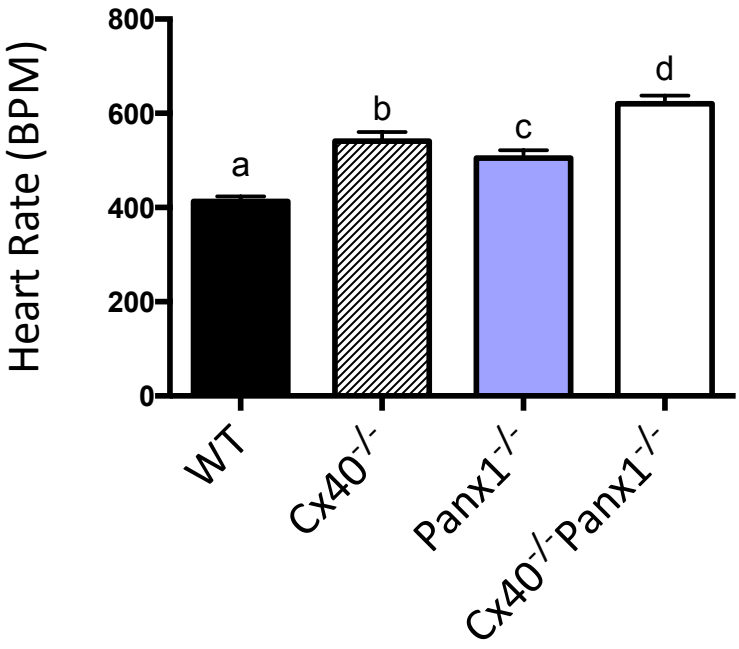
Figure 2-10 (Supplementary Figure 2.2) Mutant mice remain hypertensive under anesthesia.

(A) Tail-cuff hypertension testing revealed that anesthetized mutant mice have significantly higher systolic and diastolic blood pressure than wild-type mice, showing that anxiety effects did not cause previously reported hypertensive phenotypes (N=4). (B) Mean heart rate is variable among all four genotypes due to differential metabolism (N=4). $p<0.001$.

A


Blood Pressure (mm Hg)
200
150
100
50
0
a
b
c
d
a
b
c
d
Systolic
Diastolic
WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-

B


Heart Rate (BPM)
800
600
400
200
0
a
b
c
d
WT
Cx40-/-
Panx1-/-
Cx40-/-Panx1-/-

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Chapter 3

3 General Overview

3.1 Discussion and Conclusions

The gap junction protein Cx40 has long been categorized as an integral large-pore channel in cardiovascular physiology, participating in cardiac[1], vascular, and renal system function[2]. More recently however, the single membrane channel forming protein, Panx1, has also emerged as a participant in the maintenance of cardiovascular system physiology. In fact, recent reports highlight the overlapping roles of Cx40 and Panx1 within the heart as the ablation of both of these genes leads to an increased susceptibility to atrial fibrillation[3, 14]. Within the vasculature Panx1 has also emerged as an important player and is implicated in α1-adrenoreceptor mediated vasoconstriction[4], EDHF mediated vasodilation[5], blood pressure regulation[6], and renal function[7]. As well both Cx40 gap junction channels and Panx1 large pore channels are similarly localized, and abundant in the atria of the heart and within the arteries[3, 8, 9, 10]. Therefore due to the overlapping roles of these channels in cardiovascular function and their similar localization profiles we hypothesize that the global ablation of these two large-pore channels would lead to an increasingly severe cardiovascular phenotype.

To this end, we engineered and phenotyped the first global knockout mouse model lacking both a connexin and a pannexin channel, specifically Cx40 and Panx1. Since both of these channel proteins have been reported to function in similar cellular signaling events and system physiology we predicted that the double knockout mouse would have a reduced ability to survive[3, 11, 12, 13]. This prediction was found to be accurate as the combined ablation of Panx1/Cx40 caused increased prenatal and neonatal mortality irrespective of fertility. Double knockout mice that survived 3 days post-birth were found to be viable and live normal lifespans.

Mutant mice lacking Cx40 and Panx1 were also similar in weight to single knockout and wild-type mice up to a year of age indicating a normal metabolism. Interestingly, there is conflicting evidence on $Cx40^{-/-}$ mouse survival and a previous report describes mild increases in pre/postnatal death in this mutant mouse line[14]. Comparably, although our findings were not statistically significant Cx40 null mice had 22% fewer births and 10% newborn pup deaths compared to wild-type mice. Moreover, although this phenotype presented as minor in the single Cx40 knockout mouse its severity was increased when Cx40 and Panx1 channels were co-ablated as less than half of the $Cx40^{-/-}Panx1^{-/-}$ litter survived to 3 days post birth. These results may indicate a co-regulatory role for Cx40 and Panx1 in the development of the maturing embryo. They may also point towards the dynamic regulation of Panx1 channels along with Cx40 in embryonic heart development[15] as previous authors hypothesized that single $Cx40^{-/-}$ pre/postnatal mortality was due to inadequate propagation within the cardiac conduction system and improper septation during the establishment of pulmonary circulation[14]. The developmental regulation of Panx1 throughout various organ systems is not a new idea and has been previously reported in skin[16] in keeping with our findings that Panx1 may be developmentally regulated within the heart. Future studies to be performed should include a more in depth time point analysis of the in utero death of both $Cx40^{-/-}Panx1^{-/-}$ and single Cx40 and Panx1 knockout strains in order to ascertain if embryo death is occurring before or after cardiac development. As well q-PCR analysis of Panx1 transcript within the murine heart throughout embryonic development would allow us to conclude whether or not Panx1 is up or down regulated during embryo maturation and whether this dynamic regulation might be similar to that of the Cx40 large-pore channel.

Cardiac hypertrophy is often a secondary risk factor associated with hypertension induced pressure overload of the heart[17]. Within this organ, the left ventricle is the region primarily affected and may be characterized by reactivation of the fetal gene program and subsequent cardiomyocyte enlargement[18]. Pathologic alterations associated with cardiac hypertrophy such as increased organ mass have been previously characterized in the Cx40 knockout mouse[14]. Here, we observed increased heart mass in young and adult $Cx40^{-/-}$ mice and confirmed that it was true cardiac hypertrophy by showing that left ventricular cardiomyocytes were enlarged. Somewhat surprisingly, the $Cx40^{-/-}Panx1^{-/-}$ mouse possessed similar increases in cardiomyocyte area and the phenotype was not found to be increasingly severe. These findings are consistent with the conclusion that the ablation of Cx40 leads to the hypertrophic phenotype found in the $Cx40^{-/-}$ $Panx1^{-/-}$ mouse irrespective of Panx1 ablation. Additionally, increased left atrial cardiomyocyte area was observed in Cx40 and Cx40/Panx1 null mice. Intriguingly, unlike in the ventricle increases in left atrial cell size have been associated with cardiac conduction system abnormalities and the increased prevalence of atrial fibrillation[19], which is curious since both $Cx40^{-/-}$ and $Panx1^{-/-}$ mice are increasingly susceptible to atrial arrhythmias[3, 14].

Cardiac hypertrophy is often accompanied by myocardial remodeling which may lead to arrhythmias, and subsequent heart failure[20]. Increased interstitial fibrosis, gap junction lateralization, and actin remodeling are all characteristic of this pathological process[21]. In our study this process had yet to be observed in the 3-month-old mice used here. To expand on these findings interstitial fibrosis due to excess ECM deposition was only minimally detected by Masson's trichrome staining and there was no significant changes in collagen 1 or fibronectin in both the left atria and ventricle of all mice. This suggests that mutant mouse hearts were not particularly fibrotic. Similarly, the intercalated disc integrity and distribution of the gap junction

protein Cx43 and adherens protein N-cadherin was unaltered in mutant mice, and myofibril arrangement was not affected. Taken together these findings indicate that the cardiac hypertrophy found in single Cx40 and double Cx40/Panx1 knockout mice has not yet led to myocardial remodeling in these young adult aged mice. However because hypertrophy is often an adaptive physiological process in response to increased cardiac workload, and is plastic, we speculate that as $Cx40^{-/-}$ and $Cx40^{-/-}Panx1^{-/-}$ mice age increases in fibrosis, and gap junction and cytoskeletal remodeling are likely to occur and therefore this phenomenon may be worthy of further investigation[22].

It has been documented that the ablation of one connexin may cause the down regulation of another family member[23]. The opposite has been found to be true for pannexins in the smooth muscle[24]. Recent reports have shown that when Panx1 is ablated Panx3 displays compensatory upregulation in smooth muscle cells[24]. Thus the assessment of levels and localization of the GJ protein Cx43 acted twofold, not only to confirm intercalated disc integrity, but also to ensure that this junctional protein had not been up or down regulated by the knockout of Cx40 and/or Panx1. Similarly, the profile of the GJ protein Cx40 was unchanged in wild-type and Panx1 null mice indicating that Cx40 and Panx1 do not act in a co-regulatory fashion. An interesting follow up study would be to assess for Panx3 upregulation in the heart as this has yet to be done, and to characterize the aorta of the Cx40/Panx1 single knockouts and the double knockout mouse to ensure pannexin and connexins protein levels are unaltered.

Due to previously published research describing the RAAS mediated hypertensive phenotype of the $Cx40^{-/-}$ mouse[25] and the slightly hypotensive phenotype of smooth muscle specific $Panx1^{-/-}$ mice[5], we postulated that Cx40/Panx1 co-ablation would have a normalizing effect on blood pressure. Therefore we performed tail-cuff blood pressure measurements on wild-type and

mutant mice and intriguingly not only was the $Cx40^{-/-}$ mouse found to be hypertensive but the $Panx1^{-/-}$ mouse possessed this phenotype as well. Moreover the combined deletion of Cx40 and Panx1 created a double null mouse with a severely hypertensive phenotype. Although surprising these results were very interesting as they highlighted two key concepts; that Panx1 may play a role in blood pressure regulation irrespective of its function in smooth muscle cells, and that it may also play a protective role against the induction of cardiac hypertrophy.

Hypertension is a multifactorial disease stemming from pathologies that provoke alterations in peripheral resistance and cardiac output[26]. Because the Panx1 mutant mouse is a global knockout model it is hard to pinpoint the exact cause of its hypertensive phenotype however we can speculate here. Due to the widespread characterization of the small resistance arteries of a mouse in which Panx1 was selectively ablated from smooth muscle cells it is clear that the hypertensive phenotype found in the global $Panx1^{-/-}$ mouse is not due to increased arterial resistance but rather an effect of systemic knockout[4, 5]. Regulatory mechanisms that may be contributing to this phenomenon will be briefly delineated here, as they were previously discussed at some length in the introductory chapter. There are two mechanisms linked to increased peripheral resistance that have been associated with Panx1 channel function. The first involves the stimulation of vasodilation during low oxygen conditions by the release of ATP from Panx1 channels located on erythrocytes[27]. The second mechanism involves Panx1 channels acting in feed-back regulation between the carotid body and brainstem for the regulation of arterial pressure[28]. Another factor that could contribute to increased arterial resistance in $Panx1^{-/-}$ mice is their blunted vasodilatory response to muscarinic agonists as shown previously[29], and found in our current study. Moreover, Panx1 channels have been implicated in cardiac output changes due to their hypothesized role in pressure natriuresis and salt retention[30]. To summate Panx1 may play a

role in blood pressure regulation by modulating the aforementioned mechanisms either individually or together which is producing the hypertensive phenotype found in our global Panx1$^{-/-}$ mouse.

The troubles in ascribing a specific mechanism to the increased blood pressure found in the Panx1$^{-/-}$ mouse emphasizes the drawbacks of the global ablation of a ubiquitous protein involved in many physiological responses. With new Cre-lox technology the selective ablation of Panx1 in relevant organs, such as the kidney, could help predict causality. To add, the *Panx1* gene has yet to be selectively deleted from the kidney so this mouse model would not only be relevant to the study of hypertension in these animals, but also to help define the roles of Panx1 in renal physiology. An additional follow up study that could help shed some light on this phenomenon, without the involved process of creating a new transgenic mouse line would be to assess the levels of renin, angiotensin, and aldosterone in the plasma of the Panx1$^{-/-}$ mice. Kidneys could also be harvested, sectioned, stained and assessed for general structural characteristic such as nephron number, atrophy, and cell death.

Another important consideration when addressing the results of the tail-cuff hypertension testing is the sophistication and sensitivity of the technique itself. Tail cuff testing is an indirect blood pressure monitoring technique that uses various sensing modalities to detect changes in blood flow during cuff occlusion[31]. Advantages in using this approach include: the non-invasiveness of the technique dissuading from costly and time-consuming surgical methods, the ability to perform repeat measures over a longer time-frame, the animals remain conscious, and the ability to perform measurements on large cohorts of animals[32]. It is said to be an optimal technique when trying to detect large changes in systolic/diastolic pressures[33]. Two main disadvantages of tail cuff blood pressure monitoring are the small sampling period which is measured and that it

may act as an acute system stressor[34, 35]. Many researchers have postulated that since tail cuff only measures a minute period of the circadian cycle and does not reflect changes in both day and night cycles it is not an accurate measure to obtain an average blood pressure[34]. As well, due to the fact that rodents are nocturnal, obtaining blood pressure values when they are most active may be of vital importance to the study[34]. A second caveat to the tail cuff blood-pressure monitoring system is that restraint induces acute stress in rodents, which may affect short-term pressure measurements[35]. In fact several studies have found that restraint stress causes acutely elevated blood-pressure and increased stress hormone production[35, 36]. In our study to circumvent this issue mice were given a 2-week acclimatization period in which they were conditioned to the CODA system. As well, tail-cuff blood pressure measurements were performed on anesthetized mice to rule-out acute stress and anxiety affects and all three mutant mouse lines remained hypertensive compared to wild-type. Furthermore, in order to validate our system for measuring blood pressure, a previous study was performed in which tail cuff blood-pressure measurements taken on the CODA system were compared to radiotelemetry measurements and found to be nearly identical[37]. In the future radiotelemetric studies could be performed on our mutant mice to assess their blood pressure throughout the full length of the circadian cycle.

Interestingly, although the Panx1 null mice presented with hypertension they did not show concomitant cardiac hypertrophy as was observed in $Cx40^{-/-}$ and $Cx40^{-/-}Panx1^{-/-}$ mice. This finding although intriguing, is not entirely unique. In the human population a multitude of studies show that only half of the variability in left ventricular mass can be attributed to increased blood pressure (as reviewed by Katholi)[38]. To try and elucidate alternative causes for the induction of cardiac hypertrophy genetic factors, the NO/cGMP pathway and neurohormones have been investigated and found to play a role in hypertrophic growth[39, 40]. Therefore our findings in the

Panx1$^{-/-}$ mouse model show that this large-pore channel may play a previously unknown role in the development of cardiac hypertrophy.

Within blood vessels the endothelium is highly involved in the regulation of vascular tone by modulating contractile responses and mediating the release of vasodilators[41]. This balance is often disrupted secondary to hypertension leading to endothelial dysfunction and deficits in endothelium-mediated vasodilation[41]. As previously noted in the introductory chapter both Cx40 and Panx1 large-pore channels influence vascular tone in elastic arteries by modulating vasodilatory responses, and Cx40$^{-/-}$ and Panx1$^{-/-}$ mice have been shown to possess blunted vasodilatory responses to muscarinic receptor agonists [42, 43, 44]. As well, since both of these channel proteins are localized to endothelial cells in larger arteries, we hypothesized that the combined ablation of Cx40 and Panx1 would lead to a severe loss of endothelial function[45, 46]. Consistent with this notion our experimental results demonstrate that the concomitant ablation of Cx40 and Panx1 in our double knockout mouse model had a cumulative effect on vasodilatory responses and creating an increasingly blunted response. The minimal vasodilatory response in the aorta of double null mice to the muscarinic agonist MCh was not mimicked when an endothelium independent vasodilator was applied, indicating that these mice possess severe endothelial dysfunction. Due to its severity we speculate that the endothelial damage seen in the Cx40$^{-/-}$Panx1$^{-/-}$ mouse is due to a combination of the down regulation of vasodilatory pathways regulated by Cx40 and Panx1, namely NO and EDHF mediated vasodilation, and hypertension damage[29, 44].

In conclusion this research contributed several distinct and unique findings to the gap junction and pannexin fields. We engineered the first ever connexin/pannexin global knockout mouse from Cx40 and Panx1 single knockout animals and characterized this novel model for overt

phenotypes. This characterization revealed $Cx40^{-/-}Panx1^{-/-}$ mice that did survive past day 3, had normal weights, and were fertile. Single $Cx40^{-/-}$ mice exhibited previous reported phenotypes including cardiac hypertrophy, hypertension, and blunted vasodilatory responses. Interestingly, within the Cx40 null heart cardiac hypertrophy had not yet led to myocardial remodeling. Similarly, it was confirmed that Panx1 null mice possess normal heart size and decreased vasodilatory responses to muscarinic agonists. Remarkably global Panxl knockout mice were discovered to be hypertensive, a finding which has been previously unreported in the literature. As well, the combined ablation of Cx40 and Panx1 was found to lead to increased embryonic and neonatal mortality, severe hypertension, and augmented endothelial dysfunction. Therefore the results found in this thesis suggest that Panx1 and Cx40 large pore channels have co-regulatory roles in certain physiological processes including embryonic and neonate survival, hypertension, and vascular responses.

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4 Appendices

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1) Jelen M, Barr K, Feng Q, Isakson BE, Gros R*, Laird DW*. Decreased Survivability and Increased Hypertension in Mice Lacking Both Cx40 and Panx1. (In preparation, Cardiovascular Research)

2) Jelen M*, Barr K, Gros R, Laird DW. Cardiovascular Characterization of $Cx40^{-/-}Panx1^{-/-}$, $Cx40^{-/-}$, and $Panx1^{-/-}$ Mice. Poster session presented at: London Health Research Day; 2016 March 29; London, Ontario. (MSc. Program)

3) Jelen M*, Barr K, Gros R, Laird DW. Cardiovascular Characterization of $Cx40^{-/-}Panx1^{-/-}$, $Cx40^{-/-}$, and $Panx1^{-/-}$ Mice. Oral Platform presentation presented at: Nexin Forum for Connexin/Pannexin researchers; 2016 February 18; London, Ontario. (MSc. Program)

4) Jelen M*, Barr K, Gros R, Laird DW. Cardiovascular Characterization of $Cx40^{-/-}Panx1^{-/-}$, $Cx40^{-/-}$, and $Panx1^{-/-}$ Mice. Poster session presented at: American Society for Cell Biology Conference; 2015 December 16; San Diego, CA. (MSc. program)

5) Jelen M*, Barr K, Laird DW. Characterization of a Novel Cx40-/-Panx1-/- Double Knockout Mouse with a Focus on the Cardiovascular System. Poster session presented at: Anatomy and Cell Biology Research Day; 2015 October 23; London Ontario. (MSc. program)

6) Jelen M*, Persinger M. The effects of L-arginine Combined with Sildenafil or Icariin on Memory Formation in Male Wistar Rats. Oral platform presentation presented at: Neuroscience and Psychology Undergraduate Thesis Seminars; 2014 April 11; Sudbury, Ontario. (HBSc. program)