CX43 AS A TRANSDUCER OF SIGNALS LINKED TO CARDIOPROTECTION AND DNA SYNTHESIS

By

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DEDICATED TO MY DEAR DAD AND MOM

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ABSTRACT

Background and Rationale: Connexin-43 (Cx43) is an integral membrane phosphoprotein and the major constituent of cardiac gap junctions, intercellular channels mediating metabolic and electrical coupling. In addition, Cx43 is involved in the regulation of cell proliferation, acting as an inhibitor of DNA synthesis, and is suggested to be essential for the ability of the heart to become resistant to injury in response to ischemic or pharmacological preconditioning. Cx43 is the target of several different signal transduction cascades culminating in its phosphorylation at several sites located at its C-terminal tail. Previous work in my mentor's laboratory provided evidence that: cardioprotection of isolated perfused rat hearts by ischemic preconditioning or fibroblast growth factor 2 (FGF-2) administration was closely correlated with above-physiological Cx43 phosphorylation at protein kinase C (PKC) target sites such as serine S262; (ii), the FGF-2-induced cardiac Cx43 phosphorylation at S262 was mediated by protein kinase Cε (PKCε), and this cancelled the ability of Cx43 to inhibit DNA synthesis in neonatal cardiomyocytes.

The overall purpose of this study was to further investigate the relationship between Cx43, its phosphorylation at PKCε-target sites such as S262, and PKCε-mediated end-points such as cytoprotection/cardioprotection, and stimulation of DNA synthesis. The main hypotheses were that (a) Cx43 phosphorylation at S262 mediates cardiomyocyte protection from ischemic injury by FGF-2, PKCε and ischemic preconditioning, and (b) Cx43-associated

inhibition of DNA synthesis is caused by activation of the transforming growth factor beta (TGFβ) pathway.

Materials and Methods: Primary cultures of neonatal rat ventricular cardiomyocytes as well as the Cx43-deficient cell line HEK293 were used as *in vitro* models. Simulated ischemia was achieved in the presence of a low pH medium and incubation in a hypoxia chamber. Cell injury or cell death was assessed using the lactate dehydrogenase or TUNEL assay kits, respectively. Protein expression and localization were examined by western blotting and immunofluorescence using well characterized antibodies. The fraction of cells synthesizing DNA was obtained by determining the BrdU labeling index. Gene transfer in cardiomyocytes was achieved by infection with various adenoviruses carrying: wild type Cx43 or the Cx43 phosphorylation mutants such as S262A-Cx43, or a Cx43 mutant containing a serine to aspartate (S262D-) substitution; dominant negative Smad2 or TGFRII; wild type PKCε. Gene transfer in HEK293 cells was achieved using the TransIT reagent.

Results: In the cytoprotection experiments, a modest overexpression of wild type Cx43, which localized predominantly to cell-cell contact sites, increased the resistance of cardiomyocytes to ischemic injury. In contrast, expression of a C-terminal-HA tagged Cx43, which showed localization to perinuclear sites, or of the channel domain-deficient C-terminal fragment of Cx43 (Cx43CT), which localized to cytosol and nucleus, rendered cardiomyocytes more vulnerable to ischemic injury. Preventing Cx43 phosphorylation at S262 by overexpressing S262A-Cx43 also rendered cardiomyocytes more vulnerable to ischemic injury,

and fully abolished the ability of either FGF2, or PKCε to confer cytoprotection. Conversely, the protective effect of ischemic preconditioning was only partially prevented by S262A-Cx43 expression.

In addressing the mechanism by which Cx43 inhibits DNA synthesis in cardiomyocytes we found that the inhibition of several components of TGFβ-associated signal transduction including TGFβRI (with SB431542), or TGFβRII (by overexpressing dominant negative TGFβRII), or Smad2 (by overexpressing dominant negative Smad2) had no effect on Cx43-mediated inhibition of DNA synthesis. Treatment of cardiomyocytes with TGFβ prevented the FGF-2-induced Cx43 phosphorylation at S262, detected by phospho-specific antibodies. In HEK293 cells, we found that expression of Cx43CT, lacking the channel-forming domain, retained the ability to inhibit DNA synthesis, and that this event was dependent on S262: expression of S262A-Cx43CT elicited maximal inhibition of DNA synthesis, while S262D-Cx43CT (simulating constitutive phosphorylation) had no inhibitory effect.

Conclusions: Phosphorylation of Cx43 at S262 is likely a mediator of FGF-2-induced and PKCε-mediated cardiomyocyte resistance to ischemic injury, while protection by ischemic preconditioning may be only partially dependent on Cx43 and its phosphorylation at S262. The mechanism of cytoprotection by Cx43 phosphorylation at S262 is linked to preservation of membrane targeting and intercellular channel formation, as it was abolished in the absence of channel-forming ability (Cx43CT) or aberrant Cx43 localization (Cx43-HA).

The mechanism by which Cx43 inhibits DNA synthesis is not dependent on downstream activation of TGFβ signal transduction. It is possible, however, that a component of TGFβ-triggered inhibition of DNA synthesis includes prevention of mitogen-induced Cx43 phosphorylation at S262. Furthermore, inhibition of DNA synthesis by Cx43, and its regulation by S262 phosphorylation are independent of channel-forming ability or subcellular localization.

Targeting Cx43 and its phosphorylation at S262 may provide a novel strategy to improve cardiac response to injury, by decreasing tissue loss through increased resistance as well as improving a regenerative response by disinhibiting cardiomyocyte proliferation.

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ABBREVIATIONS

A ……..Alanine
AAP10……Anti arrhythmic peptide 10
Ad…….……Adenovirus
ADP…….Adenosine-5'-diphosphate
ANOVA…….Analysis of variance
AT-1……..Angiotensin-1
ATP……..…Adenosine-5'- triphosphate
AV……..……Atrioventricular
BCA ……..……..Bicinchonic Acid
BrdU ……..Bromodeoxyuridine
BSA……..Bovine serum albumin
cAMP…….……...Cyclic adenosine 5'-monophosphate
CK1……...Casein kinase 1
Cx……Connexin
D…….Aspartic acid
Da……..Dalton
DMEM…… .....Dulbecco's modified eagle's medium
DMSO…….....……Dimethyl sulfoxide
DN…….....……Dominant negative
DNA……Deoxyribonucleic acid
ECL…….……Enhanced chemiluminescence
ECM……Extracellular matrix
EDTA …… Ethylenediaminetetraacetate
EGTA……...…Ethylene glycol tetra acetic acid
EGF……..……..……Epidermal growth factor
ERK……..……Extracellular signal-regulated kinase
ET.....Endothelin
FBS……... Fetal bovine serum
FGF...……..……Fibroblast growth factor
GAPDH…….....……...Glyceraldehyde-3-phosphate dehydrogenase
h……..……hours
HCL…….Hydrochloric acid
IP3……... Inositol-1,4,5-trisphosphate
kDa…….……Kilodalton
LDH……...……..Lactate dehydrogenase
MAPK……..Mitogen activated protein kinase
m.o.i……Multiplicity of infection
NCM……Neonatal cardiomyocytes
NOV……...……Neuroblastoma overexpressed
P*……..Phospho*
PAGE……..Polyacrylamide gel electrophoresis
PBS…….Phosphate buffered saline

PDGF……..…….Platelet derived growth factor
PKC……..……...…Protein kinase C
PKG…….....….Protein kinase G
PMA……..……Phorbol 12-myristate 13-acetate
PMSF……Phenylmethylsulfonylflouride
PVDF.....Polyvinylidene difluoride
ROS.....Reactive oxygen species
S…….Serine
SA……Sinoatrial
SDS……..Sodium dodecyl sulfate
TBST……..……Tris Buffered Saline with Tween
TGF……Transforming growth factor
TGFR……Transforming growth factor receptor
TUNEL…….Terminal deoxynucleotidyl transferase dUTP nick end labeling
TMR…….……Tetramethylrhodamine
VEGF……...……Vascular endothelial growth factor
µM……..Micromolar
µm……..Micrometer

CHAPTER I: LITERATURE REVIEW AND RATIONALE FOR PROPOSED STUDIES

I.A. CONNEXINS

I.A-1. Connexins and Gap Junction-Dependent Function

Gap Junctions are intercellular plasma membrane channels that facilitate direct communication between the cytoplasm of adjacent cells and contribute to electrical and metabolic coupling between cardiomyocytes {Beyer et al., 1990; Laird, 2006}. Vertebrate gap junctions are composed of connexons or hemi-channels, formed by integral membrane proteins called connexins, encoded by a 21 member multigene family {Beyer et al., 1990; Willecke et al., 1991}. These proteins are critical in the regulation of embryonic development, maintenance of the tissue homeostasis, normal growth and differentiation, and coordinated contraction of the excitable cells {Saez et al., 2003; Sohl and Willecke, 2004}. Gap junctions in mammals exist in nearly all cells and organs except blood cells, spermatocytes and mature skeletal muscle {Dermietzel et al., 1997}. Mutations in the connexin proteins have been shown to lead to various diseases such as Charcot-Marie tooth disease, occulodentodigital dysplasia, atrioseptal defects, and arrhythmias {Dobrowolski and Willecke, 2009}.

Connexins (Cx) are arranged in a hexameric fashion in the hemichannel {Manjunath and Page, 1985}. Two connexons align end to end when the plasma membrane of the two adjacent cells come close together within 2-3 nm (Diagram 1) {Severs, 1994}. The central pore of the connexon at its widest point measures less than 2.5 nm and allows the passage of small molecules less than 1000 Da {Goodenough et al., 1996} like ions, amino acids, sugars, second messengers like cAMP, IP_3 and ATP; they pass through the gap junctions from one cell to another cell, contributing to the metabolic coupling between the cells {Gilula et al., 1972}.

Connexins protein structure consist of two extracellular domains that are highly conserved, four transmembrane domains, one carboxy terminal, one amino terminal and an intracellular loop {Yancey et al., 1989} (Diagram 1 & 3). The extracellular domains dock the connexons of the adjacent myocytes to form the gap junction channel and they are linked by intramolecular disulfide bonds and have three conserved cysteine residues; the third transmembrane domain participates in pore formation {Hoh et al., 1991}. The intracellular cytoplasmic C-terminal acts as a gating particle and is responsible for the closure and opening of the channel via a ball and chain mechanism {Anumonwo et al., 2001}. The type of connexin forming the channel determines the selectivity and permeability properties of the channel {Veenstra et al., 1995}.

Permeability of the gap junction channels can be modified by a variety of factors such as intracellular Ca^{2+} {Wang and Rose, 1995}, pH {Ek-Vitorin et

al., 1996}, voltage {Bennett and Verselis, 1992}, and phosphorylation by various kinases {Lampe and Lau, 2000}.

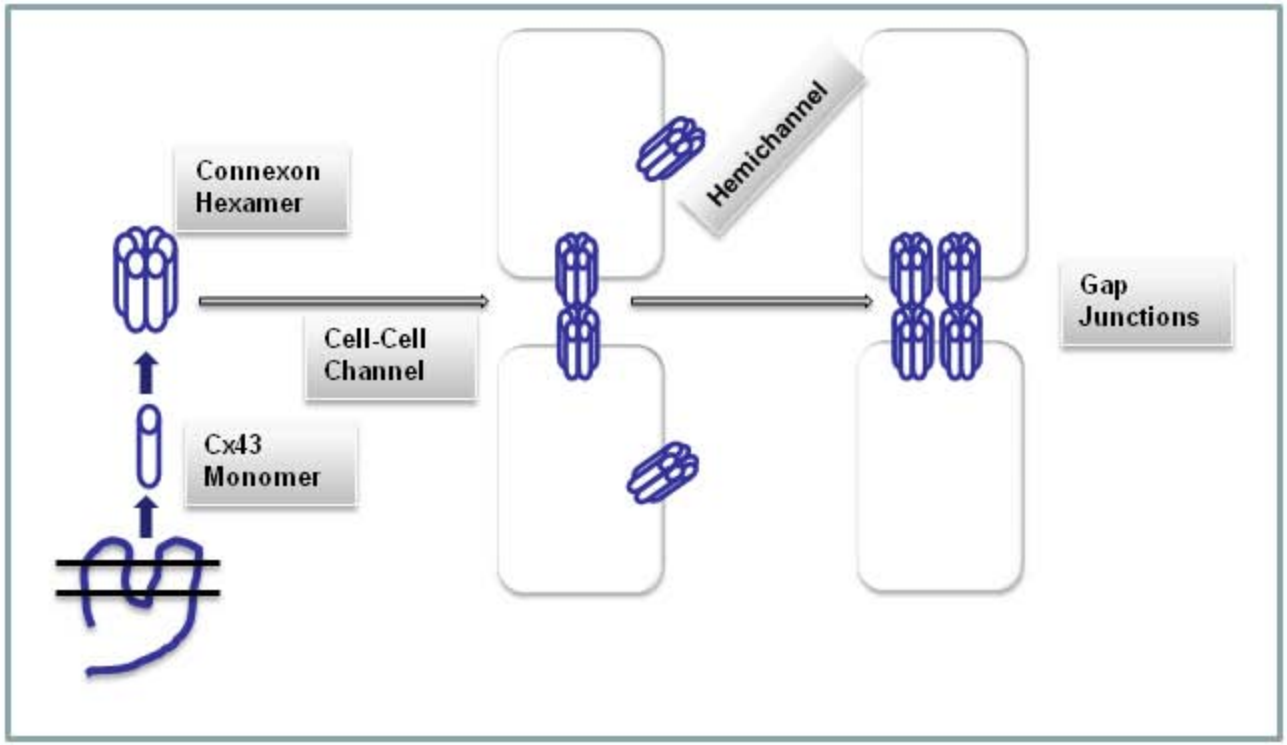


Diagram 1: Formation of connexons and Cx43 gap junction channels between cardiomyocytes

Cardiac Function: In the heart, gap junctions are responsible for uniform anisotropic impulse propagation from one working cardiomyocyte to another. There are two types of gap junctions, end-to-end and side-to-side. End-to-end gap junctions are prevalent in the normal adult cardiac ventricles and are located in the intercalated disk region, organized together with adhesion junctions (adherens junctions and desmosomes) thus assisting in the electromechanical coupling between adjacent cells {Severs et al., 2008}. The lateral or side-to-side gap junctions occur in atrial more than ventricular tissue {Spach and Heidlage, 1995}.

Effective pumping of the heart requires electrical activation in a specific spatial and temporal pattern and this process depends largely on the function and the distribution of the gap junctions. The electrical conduction properties of the heart are determined by the size, number and distribution of the gap junctions in the myocardium {Peters et al., 1993}. In the normal heart the impulse propagation is faster along the longitudinal axis compared to transverse axis {Delmar et al., 1987; Dillon et al., 1988}. The propagation of the impulse sequentially from the atria to the ventricle is due to the conduction delay in the atrioventricular (AV) node due to sparse gap junctions and fast conduction in the ventricular myocytes and purkinje fibers {Davis et al., 1995} (see Diagram 2).

Cardiac gap junctions are composed of various connexin isoforms that differ in their conductance properties. Regardless, Cx43 is by far the most abundant isoform in adult ventricular myocardium {Tribulova et al., 2008}.

Three principal connexins can be expressed in cardiomyocytes: Cx43, Cx40 and Cx45 were identified in neonatal cardiomyocytes {Darrow et al., 1995}. Cx43 is co-expressed with other connexins in various proportions in a chamber and myocyte specific manner {Severs et al., 2008}. In ventricular myocytes, Cx43 is the major connexin but it can also be coexpressed with trace amounts of Cx45. In atrial myocytes, Cx43 is expressed is similar quantities as in the ventricular myocytes but, in addition, Cx40 is also expressed in approximately equal quantities along with Cx45 {Desplantez et al., 2007}. Myocytes in the sinoatrial (SA) and the AV nodes have smaller gap junctions composed of Cx45 (and Cx30.2 in mouse) {Kreuzberg et al., 2006}.

The type of connexins in gap junctions, and their size and low conductance properties was suggested to contribute to the slowing of the impulse propagation in the AV node, ensuring a sequential contraction in the cardiac chambers {Desplantez et al., 2007}. Cx45 and Cx43 are expressed in the His bundle, whereas further downstream the myocytes in the conduction system in the bundle branches and purkinje fibres are composed of high levels of Cx40 and Cx45 (rodents). In the distal parts of the conductions system there is also expression of Cx43, especially in the conduction system myocytes {Desplantez et al., 2007}. Connexins (Cx37, Cx40 and Cx43) are also expressed in the vascular wall blood vessels in the heart. Cx37 and Cx40 are coexpressed in the endothelial cells whereas smooth muscle cells predominantly express Cx43 {Kwak et al., 2002}.

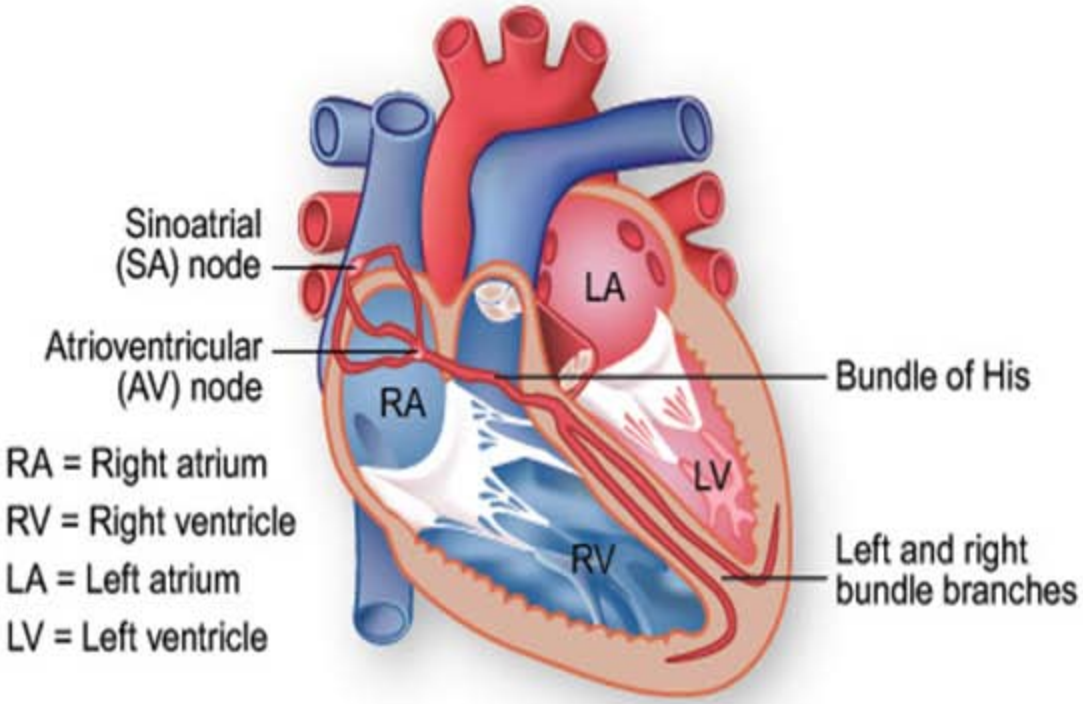


Diagram 2: Depicts the different chambers of the heart and the conduction system which includes the SA node, AV node, Bundle of His, Left and right bundle branches.

{http://www.texasheartinstitute.org/HIC/Topics/Cond/bbblock.cfm}

I.A-2. Non-Junctional Connexin Function

Hemichannels: Non-junctional channels (hemi-channels, or connexons) have been described as large nonselective ion channels residing in the nonjunctional areas of the plasma membrane before their assembly into the gap junction channels {Ebihara, 2003}. Several connexins are capable of hemi-channel formation, including Cx43 {Boengler et al., 2006b}. Cx43 hemichannels reside at the lateral sides of the cardiomyocytes as well {Boengler et al., 2006b}. Hemichannels are implicated in cell volume regulation {Quist et al., 2000} and activation of cell survival pathways {Plotkin et al., 2002}. They are predominantly in the closed state under resting conditions but open under conditions of stress such as ischemia and ischemia-reperfusion {Miura et al., 2010}. Hemichannel gating had been shown to be regulated by various factors like phosphorylation of Cx43 {Saez et al., 2005}. The phosphorylation of Cx43 at S368 by protein kinase C (PKC) was shown to close the channel whereas inhibition of PKC has been shown to open the hemichannels {Bao et al., 2004}.

Mitochondrial Cx43: While most cardiomyocyte Cx43 is located in the plasma membrane junctional and non-junctional areas, it has also been shown to be present at mitochondrial sites. Cx43 was localized in mitochondria of human umbilical vein endothelial cells and the levels were shown to increase in cellular stress induced by homocysteine {Li et al., 2002b}. Later on, Cx43 was detected in mitochondria from the mouse, pig, rat and human left

ventricular tissue. Mitochondrial Cx43 content was shown to be reduced in conditional knock-out mice {Boengler et al., 2005}. Ischemic preconditioning increases mitochondrial Cx43 {Boengler et al., 2005}. There is some evidence that cardiac mitochondria require Cx43 to be able to contribute to development of cardioprotection by ischemic preconditioning or diazoxide {Rodriguez-Sinovas et al., 2006a}. Mitochondrial Cx43 could also play a role in the mitochondrial respiration, as mitochondria from Cx43 deficient mice showed attenuated Adenosine-5'-diphosphate (ADP)-stimulated respiration and reduced respiratory control ratio compared to controls {Boengler et al., 2006a}.

Cx43 was localized to the inner mitochondrial membrane imported there via the TOM/TIM transport system {Rehling et al., 2004}. Mitochondrial Cx43 was reported to be present exclusively in the phosphorylated form indicating that phosphorylation of Cx43 could play an important role in the regulation of mitochondrial Cx43 content {Rodriguez-Sinovas et al., 2006a}. Genetically modified mice in which Cx43 was replaced with Cx32 showed that Cx43 was required for mitochondrial volume regulation {Rodriguez-Sinovas et al., 2010}. Overall, the role of mitochondrial Cx43 and its mechanism of action are not well understood.

I.A-3 Cx43 Regulation by Phosphorylation

Post-translational modifications such as phosphorylation play a central role in Cx43 function and regulate all of its properties such as intracellular

trafficking and assembly, electrical and chemical coupling, protein-protein interactions, as well as turnover {Solan and Lampe, 2009}. Before a more extensive overview on Cx43 phosphorylation is presented it is important to point out that cardiac Cx43 shows a remarkably rapid turnover (half-life of <1.5 hours) indicating that it has a dynamic role usually assigned to signaling molecules {Beardslee et al., 1998; Laird et al., 1991}. It is degraded by the proteasomal and lysosomal pathways in both adult and neonatal myocytes {Laing et al., 1997}. Since Cx43 turns over rapidly, Saffitz and others suggested that, degradation of Cx43 can serve as one of the mechanisms that control the levels of electrical coupling between cardiac myocytes by adjusting the number of channels between them, in physiological and pathological conditions {Saffitz et al., 2000}.

Phosphorylation: The C-terminal domain of connexins carries multiple phosphorylation sites, including, in the case of Cx43, 6 tyrosines and 21 serines {Axelsen et al., 2006; Lampe and Lau, 2004} (Diagram 3). Connexins continue to undergo post-translational modification throughout their life cycle which includes transport to plasma membrane, assembly in gap junction channels, gating and degradation {Solan and Lampe, 2007}. Several kinases such as members of the protein kinase C (PKC) family {Doble et al., 2004; Doble et al., 2000; Lampe and Lau, 2000}, ERK1/2 (extracellular signal-regulated kinase1/2) {Warn-Cramer et al., 1996}, the tyrosine kinase src {Kanemitsu et al., 1997}, casein kinase 1 {Cooper and Lampe, 2002}, PKA

(protein kinase A) {Darrow et al., 1996} and p34cdc2 {Solan and Lampe, 2009} have been implicated in Cx43 phosphorylation (Diagram 3).

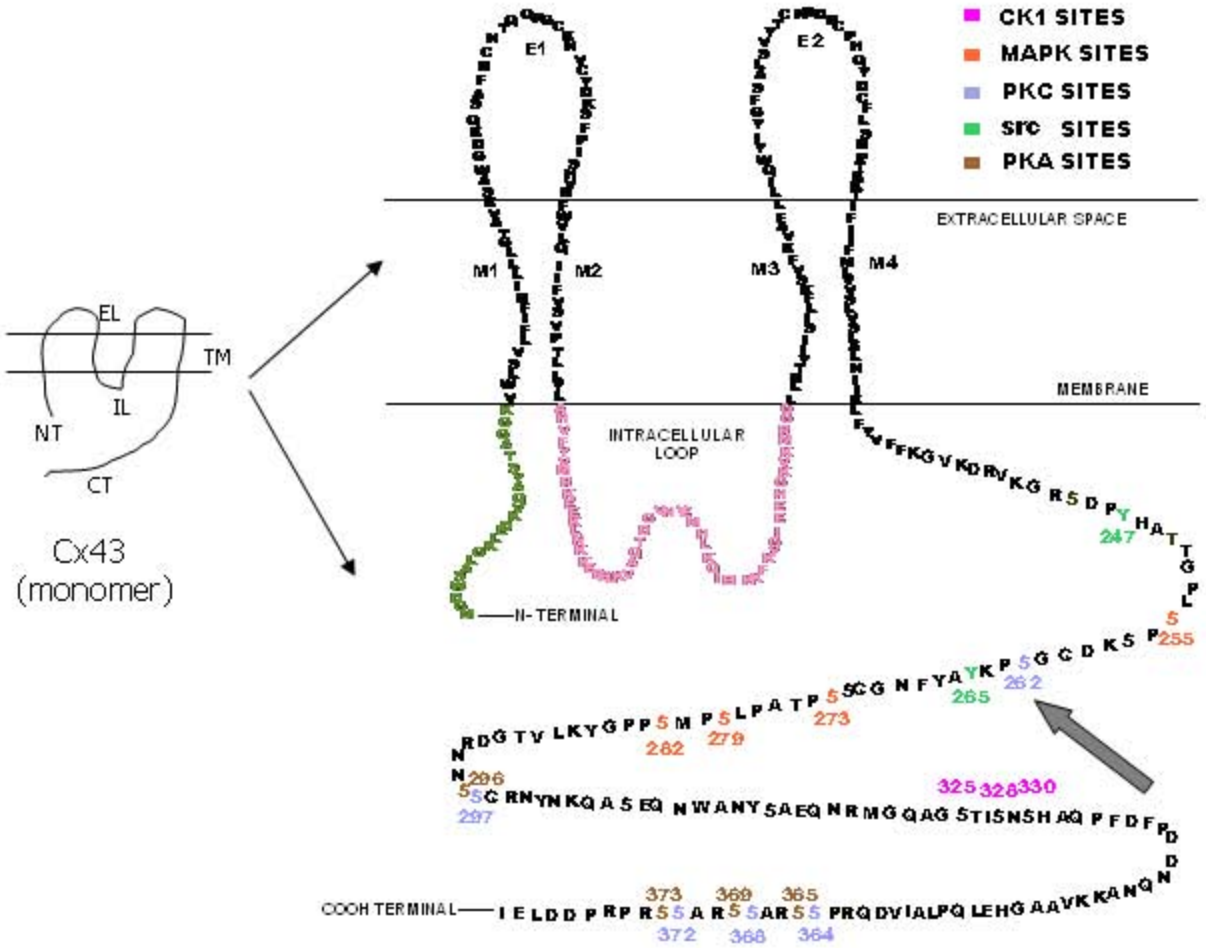


Diagram 3: Depicts the structure of Cx43 and the various kinases that phosphorylate Cx43 at various sites at its C-terminal tail including the PKC site S262

Cx43 exhibits multiple electrophoretic forms when analyzed by SDS-PAGE. Early studies using cultured cells defined the non-phosphorylated Cx43 as P0 (migrating at 41 kDa), and assigned terms such as P1 and P2 for Cx43 migrating at 43-45 kDa, presenting increased levels of phosphorylation {Musil and Goodenough, 1991}. The phosphorylation events have been shown to correlate with changes in the localization and assembly of Cx43 in the gap junctions which results in Triton X-100 insolubility {Solan and Lampe, 2007}. Studies using NRK (Normal rat kidney) cells indicated that Cx43 became resistant to Triton X-100 and located at the gap junction plaques when phosphorylated to the slowest migrating P2 isoform {Musil and Goodenough, 1991}. Later it was shown that phosphorylation at the Serine (S) 325, S328 and/or S330 sites causes Cx43 to present an electrophoretic motility similar to P2 {Solan and Lampe, 2007}. On the other hand phosphorylation at S262 was also associated with P2-like electrophoretic migration {Doble et al., 2004; Srisakuldee et al., 2009}, indicating that different Cx43 phosphorylation patterns can result in similar electrophoretic motility {Lampe et al., 2006}. Phosphorylation of Cx43 at S365 residue was shown to occur in culture cells and heart tissue and this phosphorylation event was demonstrated as a requirement for the formation of a P1 (~43 kDa) isoform {Solan and Lampe, 2007}.

Several serine sites on Cx43 are targets of PKC. These include S368 {Lampe and Lau, 2000} and S372 {Saez et al., 1997}, in addition to S262 {Doble et al., 2004; Solan and Lampe, 2009}. Doble et al showed that FGF-2

stimulated cardiac Cx43 phosphorylation at S262 by a mechanism requiring PKCε {Doble et al., 1996; 2001; 2000}. The following tables (Table 1 & 2) summarize how various agents and kinases modify Cx43 properties:

Table 1: List of kinases that phosphorylate Cx43 at various sites

Kinases that phosphorylate Cx43	Residues phosphorylated	References
$pp60^{src}$	Y247	Lin et al 2001
$pp60^{src}$	Y265	Lin et al 2001
MAPK	S255	Warn-Cramer et al 1996
MAPK	S279	Warn-Cramer et al 1996
MAPK	S282	Warn-Cramer et al 1996
CK1	S325	Cooper and Lampe 2002
CK1	S328	Cooper and Lampe 2002
CK1	S330	Cooper and Lampe 2002
PKC	S372	Saez et al 2007
PKC	S262	Doble et al 2000
PKC	S368	Lampe et al 2000
PKA	S364	Shah et al 2002

Table 2: List of factors and downstream kinases that affect gap Junctional communication

Factors affecting Cx43 phosphorylation	Activated kinases	Effect on gap junction communication
EGF (Epidermal growth factor)	MAPK	Reduction in gap junctional communication
FGF-2 (Fibroblast growth factor-2)	PKCε	Reduction in gap junctional communication
VEGF (Vascular endothelial growth factor)	c-src and MAPK	Reduction in gap junctional communication
PDGF (Platelet derived growth factor)	PKC and MAPK	Reduction in gap junctional communication
ET1 (Endothelin1)	ERK1/2 and p38	Increased gap junctional communication
AngII (Angiotensin II)	ERK1/2	Increased gap junctional communication

Regulation of the gap junctional conductance by phosphorylation of Cx43 was shown clearly by Moreno and colleagues {Moreno et al., 1994}. The PKC mediated Cx43 phosphorylation at S368 reduces both electrical and metabolic coupling {Lampe and Lau, 2000}. Tyrosine phosphorylation of Cx43 by Src kinase (at tyrosines 265 and 247) also reduces coupling {Giepmans et al., 2001; Lau et al., 1996}. In cardiomyocytes the activation of the PKA (Protein kinase A) was shown to increase gap junction mediated intercellular communication {Darrow et al., 1996}. Kwak and others have shown that in neonatal cardiac myocytes single channel conductances increase by activation of PKC and decrease by the activation of PKG {Kwak et al., 1995a}. While activation of PKC increased electrical coupling, it decreased metabolic coupling between cells {Kwak et al., 1995b}.

AAP10, an anti-arrhythmic peptide was shown to increase gap junctional conductance in adult guinea pig cardiomyocytes by a mechanism involving phosphorylation via PKC-α {Weng et al., 2002}. Incubation of neonatal cardiac myocytes with angiotensin-II (AT-II) and endothelin-1 (ET-1) increased the expression and phosphorylation of Cx43; AT-II and ET-1 were suggested to likely act via ERK1/2 and ERK1/2 and p38 respectively and increase gap junctional communication {Polontchouk et al., 2002}.

Growth factors activate various kinases and alter the function of the channel. Epidermal growth factor (EGF) inhibits junctional communication in some cell types {Maldonado et al., 1988} and was shown to activate serine phosphorylation of Cx43, mediated by ERK1/2 {Kanemitsu and Lau, 1993}.

Cx43 was shown to be phosphorylated by ERK1/2 on S255, S279, and S282 and that the EGF-induced cell uncoupling was shown to be mediated by ERK1/2-dependant phosphorylation of Cx43 {Warn-Cramer et al., 1996}. ERK5 was shown to cause EGF-induced gap junctional uncoupling by exclusively phosphorylating the serine 255 of Cx43, unlike the ERK1/2, that phosphorylates serine residues 279 and 282, in addition to 255 {Cameron et al., 2003}. Activation of PDGF receptors was shown to decrease the gap junctional communication via activation of PKC and ERK {Hossain et al., 1998}.

I.B. HEART DISEASE AND CX43

I.B-1. Ischemic Injury

The heart is one of the most energy demanding tissues in the body and contains large amounts of mitochondria to meet these needs {Halestrap et al., 2007}. The myocardium is dependent on oxidative phosphorylation for its supply of ATP in order to meet the demands for the beat to beat contraction and relaxation. During an acute ischemic episode, when, for example, a blood clot occludes a coronary artery, the blood supply to the heart is interrupted and the source of oxygen and nutrients to the myocardial tissue is removed leading to cessation of oxidative phosphorylation accompanied by decrease in the levels of ATP and creatine phosphate {Halestrap et al., 2004; 2007}. The

myocardium has the ability to survive a short period of ischemia and recovers completely if blood flow is re-established (reperfusion) within 20 min. Contractile performance is initially impaired; there is, however, complete recovery {Depre and Vatner, 2007}. Thus reversible ischemia is characterized by mild pathological changes and contractile dysfunction or 'stunning' {Pomblum et al., 2010}.

Longer periods of ischemia will cause more serious cellular damage which becomes irreversible. Dephosphorylation and degradation of cellular proteins due to activation of phosphatases and proteases respectively and progressive damage to the sarcomere, cytoskeleton and gap junctions accompany this process which leads to cell death {Halestrap et al., 2007}. Ischemia elicits a shift from aerobic to anaerobic glycolysis accompanied by accumulation of inorganic phosphates, H^+ ions, and CO_2 and improper functioning of ion channels {Halestrap et al., 2007}. The lowering of the intracellular pH activates the Na^+/H^+ exchanger to restore the pH and this coupled with inhibition of Na^+/K^+ ATPase leads to rise in intracellular Na^+ ion. The Na^+/Ca^{2+} exchanger that usually pumps Ca^{2+} out of the cell is inhibited or reversed leading to accumulation of Ca^{2+} ion inside the cell {Ruiz-Meana and Garcia-Dorado, 2009}. The action potential duration is also shortened during ischemia {Opie, 1996}. Production of reactive oxygen species (ROS) increases dramatically and this is thought to play a major role in ischemic injury {Kevin et al., 2003}. Thus the ATP depletion, accumulation of intracellular calcium and increased production of ROS all contribute to cellular damage and cell death. If blood

supply is restored within the critical period when mitochondria are still intact and capable of generating ATP the damage is little and cellular functions are restored, however if the ischemia is long enough that it crosses this critical time period then the recovery is not possible and the cell dies {Halestrap et al., 2004; Ruiz-Meana and Garcia-Dorado, 2009}.

Restoration of blood flow while essential for the heart to be salvaged can also exacerbate the extent of cardiomyocyte damage leading to reperfusion-related injury {Halestrap et al., 2007}. Reperfusion injury is accompanied by necrotic injury and release of cytosolic proteins; the quantification of the extent of injury can be assessed by measuring the amount of intracellular enzymes like LDH and Troponin I released in to the bloodstream {Downey and Cohen, 2006}. Reperfusion injury is also characterized by apoptotic cell death, more so than during ischemia. In myocardial infarction myocytes around the periphery of the infarct have been shown to die by apoptosis {Anversa, 1998; Halestrap et al., 2007}.

Arrhythmia: One of the major complications of myocardial ischemia is the disruption of electrical conduction (arrhythmia) and it is recognized now that gap junctions and connexins play a very important role in the genesis of arrhythmias {Severs et al., 2008}. Acute ischemia triggers alterations in impulse propagation and impulse formation in the heart leading to a localized conduction block and creation of re-entrant arrhythmias. Early studies demonstrated presence of gap junctional alterations during ischemia such as reduction of gap junctions and loss of electrical communication or uncoupling

{Ashraf and Halverson, 1978; Hoyt et al., 1990}. Heterogenous degradation of Cx43, decreased anti-Cx43 immuno-staining at the intercalated disc region as well as dephosphorylation of Cx43 that preceded degradation were shown to occur in the canine myocardium after ischemia in vivo {Huang et al., 1999}.

Several transgenic mouse models have been developed to analyze the role of gap junction proteins in impulse propagation and arrhythmogenesis in the heart. In the Cx43 knock out mouse model it was not possible to measure conduction parameters because homozygotes died right after birth due to pulmonary outflow obstruction {Reaume et al., 1995}. Direct evidence for the link between Cx43, gap junctions and arrhythmias came from studies by Gutstein et al in which cardiac-specific inactivation of Cx43 in mice resulted in sudden death due to arrhythmias {Gutstein et al., 2001; Liu and Gutstein, 2002}.

Cx40 knock out mouse models are viable but developed arrhythmias and atrioventricular block, suggesting that Cx40 plays an important role in the genesis of arrhythmias {Kirchhoff et al., 1998}. The Cx43/Cx40 double deficient ($Cx43^{+/-}/Cx40^{+/-}$) mice showed slowed conduction in the conduction system and the ventricular myocytes {Kirchhoff et al., 2000}. The Cx45 knock-out mice did not survive beyond the 10^{th} embryonic day exhibiting atrioventricular conduction block and these findings were consistent with the studies showing expression of Cx45 in the atrioventricular tissue {Alcolea et al., 1999; Delorme et al., 1997}. All of these transgenic studies indicated the

important role of Cx43 and other connexins in maintenance of proper cardiac rhythm.

Ischemia-Reperfusion: Reperfusion injury, characterized by calcium overload and hypercontracture, was proposed to be mediated by passage of Na+ ions through gap junctions from the adjacent dying cells followed by influx of calcium ions through Na^{+}/Ca^{2+} exchange {Garcia-Dorado et al., 1997a}. Cardioprotection by the gap junction blocker heptanol given during reperfusion supported this idea {Garcia-Dorado et al., 1997b; Piper et al., 2003; Ruiz-Meana et al., 1999}. In the brain, pretreatment with octanol, a compound similar to heptanol, limited infarct size after middle cerebral artery occlusion {Rawanduzy et al., 1997}. In an isolated rat heart perfusion model, heptanol was shown to reduce infarct size and also the occurrence of arrhythmias when administered before ischemia or at reperfusion; heptanol was also shown to delay the onset of uncoupling in the prolonged ischemia model {Chen et al., 2005}. The protective effects of heptanol and octanol have been mimicked by chemically unrelated, more specific, gap junctional uncouplers such as halothane and palmatoleic acid {Rodriguez-Sinovas et al., 2006b}.

Cell survival: Gap junction mediated cell-cell communication, depending on the circumstances could also favor cell survival. In neonatal ventricular myocytes gap junctional communication prevented spontaneous apoptosis, and this was called the “Good Samaritan” effect {Yasui et al., 2000}. Thus spread of cell injury through gap junctions and this may mean that, connection

of injured tissue with the healthy tissue may save the dying cell or kill the rescuing cell {Rodriguez-Sinovas et al., 2007}.

In addition to gap junctions, Cx43 hemichannels may also play an important role in ischemic injury {Rodriguez-Sinovas et al., 2007}. Cx43 hemichannels are predominantly closed under normal conditions. Metabolic inhibition (as would occur during ischemia) was shown to cause opening of Cx43 hemichannels in rat and mouse cortical astrocytes, as indicated by increased permeability to Lucifer Yellow and ethidium bromide, an effect that was reduced by gap junction blockers or targeted deletion of Cx43 {Contreras et al., 2002}. Indeed, ischemia caused opening of Cx43 hemichannels leading to loss of membrane potential, cellular swelling and ATP release {Evans et al., 2006; Li et al., 2001}. Presence of Cx43 hemichannels has been reported for cardiomyocytes: metabolic inhibition of isolated rabbit ventricular myocytes caused a marked permeability to a fluorescent dye (calcein) as well as development of large non-selective currents {John et al., 2003; John et al., 1999; Kondo et al., 2000}. More recently, opening of Cx43 hemichannels was reported to contribute to ischemia-reperfusion injury in cardiomyocytes {Shintani-Ishida et al., 2007}.

Three different mechanisms have been suggested to be contributing to the effects of Cx43 hemichannels on cell death and cell survival. According to the first, hemichannel-mediated release of ATP or glutamate to the extracellular space may exert toxic effects, involving increases in cytosolic calcium. This was shown by studies where direct application of ATP or glutamate caused

necrosis or apoptosis in different cell types {Tsukimoto et al., 2005; Zipfel et al., 2000}. The second mechanism relates to the ability of hemi-channels to affect cell volume regulation, a key aspect in cell injury. Increasing the osmotic strength in the extracellular buffer with Ficoll was shown to prevent cell death by inhibiting water uptake through Cx46 hemichannels {Paul et al., 1991}. In later studies, single cells expressing Cx43 showed significant increases in cell volume in response to decreased extracellular calcium and this effect was not possible in cells lacking Cx43 {Quist et al., 2000}. In the third mechanism hemi-channels are proposed to regulate signal transduction cascades affecting cell death or cell survival. Forced expression of Cx43, Cx32 and Cx40 in C6 glioma cells, N2A neuroblastoma cells or HeLa (Henrietta lacks) cells was protective against various paradigms of injury; this effect was independent of gap junction formation and intercellular communication as these cells were physically isolated or exposed to pharmacological inhibitors of gap junctions {Lin et al., 2003}.

Cx43 dephosphorylation during ischemia: In an isolated perfused heart model of ischemia-reperfusion, global ischemia for 20 min caused Cx43 dephosphorylation and Cx43 lateralization away from intercalated discs {Beardslee et al., 2000}. These authors suggested a causative effect between Cx43 dephosphorylation and electrical uncoupling which was detectable after 15 min of ischemia, although direct evidence was not provided. In contrast, other studies showed persistence of gap junctional communication, as assessed by dye coupling, even after 30 min of ischemia, at a time when Cx43

is dephosphorylated {Ruiz-Meana et al., 2001}. These apparently different responses to ischemia could be explained by differential regulation of electrical versus metabolic coupling mediated by gap junctions. It is thus possible for gap junctions to maintain metabolic/chemical coupling while becoming electrically uncoupled {Ruiz-Meana et al., 2008}.

Studies by several laboratories including our own {Jeyaraman et al., 2003; Srisakuldee et al., 2009; Tanguy et al., 2000} have confirmed that ischemia causes acute changes in gap junctions including Cx43 dephosphorylation and Cx43 lateralization (distribution to the sides of myocytes instead of intercalated disk region) {Garcia-Dorado et al., 2004; Jain et al., 2003; Lampe et al., 2006; Ruiz-Meana et al., 2008}. Dephosphorylated Cx43 was present in the lateralized gap junctions whereas the phosphorylated forms remained at the intercalated disk region {Severs et al., 2008}.

I.B-2. Chronic Heart Disease and Cx43 Remodeling

Several chronic cardiac pathologies, including **hypertrophic cardiomyopathy, ischemic heart disease, and heart failure,** resulting from various etiologies are characterized by Cx43 gap junction changes or 'remodeling'. These include decreases in overall Cx43 protein and gap junctions; loss of phosphorylated Cx43 with concomitant increases in dephosphorylated Cx43; and redistribution of Cx43 to lateral cardiomyocyte surfaces {Severs et al., 2008}. Reduction in Cx43 protein together with

heterogeneity of gap junction distribution appear to act together to form an arrhythmogenic substrate.

The recognition of gap junction remodeling as a key contributor to ventricular arrhythmias stimulated various studies on its role in atrial arrhythmias and in particular **atrial fibrillation**, a common arrhythmia in which electrical activity propagates in various directions. These studies have provided contradictory results. In a dog model of atrial fibrillation an increased Cx43 expression along with gap junctional lateralization was reported {Sakabe et al., 2004}. In other studies on a goat model of atrial fibrillation there were no changes in Cx43 levels, but Cx40 was found to be heterogeneously distributed {Ausma et al., 2003}. The results in human studies were further confusing as reports showed increased Cx43, decreased Cx43, increased Cx40 or decreased Cx40 as being associated with atrial fibrillation, with some predicting lateralization of gap junctions while some others claiming heterogeneity {Nattel et al., 2007}. Atrial myocytes are mostly composed of Cx40 and irrespective of the models used, an increased heterogeneity of Cx40 distribution along with lateralization has been a common finding in atrial fibrillation {Duffy, 2009}. But none of these studies included the quantification of Cx45 in atrial fibrillation, except one study on patients where it was showed that there was no change {Dupont et al., 2001}. These discrepancies were thought to be due to differences in the species and the age of the animals used and also deficiencies in technical approach and interpretive complications {Severs et al., 2008}

Atria consists of Cx43, Cx40 and Cx45 in various amounts and the gap junction channels may be composed of Cx43 alone, Cx40 alone, Cx45 alone or they might exist as heterotypic or homomeric channels consisting of mixture of these connexins, thus impacting on the conduction of these channels, as different connexins have been shown to have different conduction properties {Duffy and Wit, 2008}. Atrial fibrillation is also associated with fibrosis, inflammation and hypoxia that could potentially lead to activation of signaling molecules that lead to structural and functional remodeling of connexins {Boos et al., 2006; Everett and Olgin, 2007; Thijssen et al., 2002}. Whereas most studies have focused on regulators of Cx43, studies on regulators of Cx40 have not been well studied. Thus the functional significance on the findings on alterations in connexins during atrial fibrillation in different models makes it difficult to interpret as, along with changes in content and distribution, there could also be changes in the functional properties of the channels where most studies have not focused on.

All of the connexin isoforms expressed in the atria are phosphoproteins; phosphorylation of Cx43 has been extensively studied whereas alteration in the phosphorylation status of Cx40 under pathological states has not been studied yet {Duffy and Wit, 2008}. Cx40 has been shown to exist in the phosphorylated form and has been shown to be phosphorylated by PKC and ERK {van Veen et al., 2006} but the specific sites of phosphorylation are still unknown. Most studies on atrial fibrillation that have quantified connexins have not looked at the phosphorylation status of connexins and their functional

significance in the pathogenesis of atrial fibrillation. Thus alterations in the phosphorylation status of these connexins in addition to changes in the content and distribution could play a role in the pathogenesis of atrial fibrillation.

There is some debate as to whether reduced Cx43 in the diseased human ventricle can give rise to arrhythmic substrate and **ventricular fibrillation**. Computer modeling predicts that in mammalian heart even a substantial reduction of Cx43 has little impact on propagation velocity {van Rijen et al., 2006}, and thus the 50% reduction in Cx43 seen in the failing human ventricle may not have an impact on conduction. A much greater reduction in Cx43 (by 90%) observed in mouse models may be required for arrhythmogenesis {Gutstein et al., 2001; Jongsma and Wilders, 2000; van Rijen et al., 2006}. The reason for this apparent discrepancy may lie on the fact that just measuring average Cx43 levels disguises the importance of spatial heterogeneity in Cx43 reduction. This was demonstrated in a chimeric mouse model with only patches of myocardium lacking Cx43 (while surrounding myocardium was normal) which indicated that heterogeneity of Cx43 expression is critical to abnormal impulse propagation and contractile dysfunction {Gutstein et al., 2001}. Thus reduction in Cx43 coupled with heterogeneity of gap junction distribution appears to act together to form an arrhythmogenic substrate.

The molecular mechanisms underlying gap junctional remodeling are the subject of many current investigations. Cx43 expression is regulated at the transcriptional and post-transcriptional levels. A recent study identified a role

of microRNA-1 in silencing GJA1 (Cx43 encoding gene) in ischemic heart disease; microRNA-1 expression has also been shown to be elevated in the diseased human ventricle and antisense blocking of this elevation prevents arrhythmia in a similar animal model {Yang et al., 2007}. Extracellular signaling pathways that lead to altered expression of connexins in disease could be triggered by mechanical forces, and include a mixture of extracellular and intracellular mediators like cAMP (cyclic adenosine mono phosphate), Angiotensin II, VEGF (vascular endothelial growth factor), JNK (c-Jun N-terminal kinase) and FAK (focal adhesion kinase) {Saffitz and Kleber, 2004; Yamada et al., 2005}. In a transgenic mouse model, activation of JNK lead to downregulation of Cx43, slowing of conduction, contractile dysfunction and heart failure {Petrich et al., 2004}.

In **cardiac hypertrophy,** Cx43 content increases in the early stages {Saffitz et al., 1994} but decreases in the chronically hypertrophied hearts {Peters et al., 1993}. In hypertrophic cardiomyocytes, gap junctions were shown to redistribute over the whole surface accompanied by a decrease in the density of the gap junctions at the intercalated disks {Emdad et al., 2001}. In idiopathic dilated cardiomyopathy there is a significant reduction in the Cx43 content of the left ventricle, accompanied by an increase in Cx40 in the endocardial surface {Dupont et al., 2001}. In humans with **end stage heart failure** due to idiopathic dilated cardiomyopathy and myocarditis and in the ventricles of patients with decompensated hypertrophy smaller areas of gap

junctions disarray was found compared to what is seen in the infarct border zones {Kostin et al., 2004; Kostin et al., 2003}.

Marked reductions in Cx43 transcript and protein levels occur in addition to alterations in gap junction organization in hearts of transplant patients with end-stage congestive heart failure. This reduction of Cx43 occurs irrespective of the nature of origin of heart failure whether it is due to aortic stenosis, idiopathic dilated cardiomyopathy or ischemic heart disease {Severs et al., 2008}. The Cx43 reduction is spatially heterogenous and during the course of the disease develops progressively as observed in pressure overloaded hearts caused by aortic stenosis {Kostin et al., 2004} and in non-failing ventricles of patients with ischemic heart disease {Peters et al., 1993}. These findings show that in cardiac disease even in the absence of infarction, Cx43 gap junction distribution becomes heterogenous. In **hypertrophic cardiomyopathy** which is the most common cause of arrhythmias and sudden death in young adults, Cx43 gap junction arrangement is disordered {Sepp et al., 1996}.

A different form of gap junction remodeling occurs **in hibernating myocardium** in patients with ischemic heart disease. Hibernating myocardium refers to regions of the ventricle, which contract improperly but recover when the normal blood flow is restored. In hibernating myocardium gap junctions at the periphery of the intercalated disk region are smaller in size and the amount of Cx43 per intercalated disc is reduced in the affected regions compared to the normal regions of the same heart {Kaprielian et al., 1998}. These early studies were the first to indicate that remodeling of Cx43 gap junctions likely

contributes to impairment in the contraction of the ventricles and arrhythmias in humans {Severs et al., 2008}.

Cx45 is abundant in embryonic cardiomyocytes but only expressed in limited quantities in adult cardiomyocytes {Alcolea et al., 1999; Kanter et al., 1992}. Yamada and colleagues reported an increase in Cx45 expression in failing hearts accompanied by a significant alteration of Cx43:Cx45 ratio {Yamada et al., 2003}. It was suggested that Cx43 and Cx45 form hybrid channels in the human ventricular myocytes leading to alterations in the conduction properties of gap junctions. Cx43 and Cx45 form heteromeric channels and were shown to exhibit reduced single channel conductance compared to Cx43 homomeric channels {Martinez et al., 2002}.

Because Cx45 can interact with Cx43 to form gap junctions these data suggested that Cx45 expression played a role in reducing cell-cell coupling and promoting arrhythmogenesis in the failing heart. Overexpression of Cx45 resulting in experimentally induced increases in the Cx45:Cx43 ratio in transgenic mice was shown to reduce gap junctional communication and cause an increased vulnerability to ventricular tachycardia {Betsuyaku et al., 2006}. Thus although Cx45 may have a limited role in cell-cell coupling in the normal heart it appears to play a vital role in arrhythmogenesis in heart failure.

I.C. DEVELOPMENT OF CARDIOPROTECTION AND CX43

I.C-1. Ischemic and Pharmacological Preconditioning

Subjecting the heart to brief periods of ischemia, a procedure termed ischemic preconditioning, renders it more resistant to a subsequent severe insult such as relatively prolonged ischemia followed by reperfusion. Ischemic preconditioning (or acute cardioprotection) was initially demonstrated in dog hearts {Murry et al., 1986} and since then has been confirmed in all species including humans. Its effectiveness is dependent on the length of ischemic insult, being lost if ischemia is maintained for too long a period {Kloner, 2006; Yellon and Downey, 2003}. In addition, the acute protective effect lasts for up to about two hours, and is mediated, to a large extent, by post-translational protein modifications and subcellular translocations. The protected, injury-resistant phenotype does however re-emerge after 24 h and can last up to 3 days. This phenomenon is termed as **second window of preconditioning,** and is dependent on activation of gene expression {Schwarz et al., 1997}. Preconditioned hearts exhibit reduced infarct size, reduced cytosolic enzyme release, preservation of contractile function and fewer incidences of arrhythmias following ischemia-reperfusion {Halestrap et al., 2007}.

Since the early studies, it became evident that several conditions and substances can raise cellular resistance to injury. Activators of G-protein

coupled receptors (such as adenosine), volatile anesthetics (such as halothane), growth factors such as FGF-2, or low doses of noxious stimuli (such as reactive oxygen species), to name but a few, can activate signal transduction pathways similar to those of ischemic preconditioning, and are collectively referred to as pharmacological preconditioning {Huffmyer and Raphael, 2009; Murphy and Steenbergen, 2007}.

The preconditioning response occurs not only in intact hearts but also in isolated cardiomyocytes indicating that preconditioning can directly protect cardiomyocytes from ischemia-reperfusion damage and cell death {Miura et al., 2007}. In fact, ischemic and/or pharmacological preconditioning is encountered in many cell types and tissues, such as, liver and brain {de Rougemont et al., 2009; DeFazio et al., 2009}.

Recent studies at both the basic research and clinical levels have expanded our understanding of the potential of preconditioning by demonstrating the existence of **systemic or remote preconditioning** as a mechanism of multi-organ protection. The basic concept is that transient episodes of ischemia-reperfusion in one part of the body, such as a limb, can afford systemic protection to organs such as the heart {Kharbanda et al., 2009}. For example, patients undergoing coronary bypass graft surgery subjected to brief episodes of ischemia-reperfusion of the right upper limb displayed remarkably less (43%) myocardial injury {Hausenloy et al., 2007; Kin et al., 2004}.

The ability to mount a cardioprotective response is not limited to non-ischemic tissue, as it would appear that there is potential to activate protective signal transduction even after the onset of ischemia. Ischemic hearts can be subjected to **post-conditioning,** namely brief cycles of ischemia-reperfusion during the early phase of reperfusion, a manipulation which reduces infarct size and improves recovery of heart function {Kin et al., 2004}. This protocol has significant clinical potential since it can be induced during angioplasty prior to full restoration of flow {Halestrap et al., 2007}. This protective effect was shown to cause improved endothelial function, a reduction in apoptosis, microvascular injury and tissue superoxide generation {Halkos et al., 2004; Zhao et al., 2003}.

Mechanism of preconditioning: For at least the last 20 years, a considerable amount of time and effort has been dedicated to resolving the molecular mechanisms underlying the induction of increased resistance to injury by preconditioning. Signal transduction pathways and key targets capable of (a) triggering, (b), mediating, (c) acting as effectors of the preconditioning response have been identified. In general, ischemic preconditioning enhances release of agonists of G-protein coupled receptor (GPCR) families (opioid, adenosine, bradykinin) leading to enhanced activation of cell survival pathways (downstream activation of PKC isoforms, MAPKs and PI3/Akt kinases) and inhibition of cell death pathways. These pathways converge upon cellular effectors of the protective response which are found in the plasma membrane as well as the mitochondria, and likely

other subcellular sites. Effectors of cardioprotection include the sarcolemmal as well as mitochondrial K_{ATP} channels; and the mitochondrial permeability transition pore {Downey et al., 2007; Garcia-Dorado et al., 2006; Halestrap et al., 2007; Hausenloy and Yellon, 2006}. Several mitochondrial and/or mitochondria-associated proteins have been implicated in the orchestration of the mitochondrial response to preconditioning and injury. Mitochondria-located Cx43 may play a role in this context {Boengler et al., 2005}, in addition to its role as the main constituent of gap junctions in working cardiomyocytes. For a comprehensive review of signals associated with preconditioning the reader is referred to recent reviews: {Huffmyer and Raphael, 2009; Steenbergen et al., 2009}.

The PKC family of serine/threonine kinase has several members {Churchill et al., 2008}. Of these, the PKCε isoform is thought to play a key role in preconditioning, and cytoprotection in general. Hearts from mice lacking PKCε were incapable of preconditioning, while modest PKCε overexpression mimicked the preconditioning response {Cross et al., 2002; Inagaki et al., 2006; Saurin et al., 2002}. PKCε activity was also shown to prevent reperfusion induced arrhythmias {Inagaki et al., 2005; Yue et al., 2006}. Non selective inhibition of PKC by chelerythrine and calphostin C was shown to block the ability of preconditioning to delay the uncoupling and prevent redistribution of Cx43, pointing to the key role of PKC in mediating the effects of preconditioning on gap junctions {Jain et al., 2003}.

I.C-2. Pre- and Post- Conditioning-like cardioprotection by FGF-2

Our laboratory has provided extensive evidence for the acute cardioprotective effect of FGF-2 as reviewed in {Kardami et al., 2007b}. Briefly, FGF-2 was shown to protect neonatal cardiomyocytes from hydrogen peroxide or serum starvation-induced damage {Padua et al., 1998}. FGF-2 administration to the isolated adult rat heart by perfusion prior to ischemia resulted in a 4-fold increase in tissue associated FGF-2, and significant protection from subsequent ischemia-reperfusion induced myocardial damage and contractile dysfunction {Padua et al., 1998; 1995}. Post-ischemic administration of FGF-2 to the isolated heart was similarly beneficial {Jiang et al., 2002; 2004; 2009}, diminishing ischemic-reperfusion induced cell death and loss of function.

Similar observations were made in the mouse model, where exogenous FGF-2 administration or chronic endogenous overexpression (in transgenic mice) increased cardiac resistance to ischemia-reperfusion injury {Sheikh et al., 2001}. Intramyocardial administration of FGF-2 during an evolving myocardial infarction (induced surgically by irreversible coronary ligation) in vivo, provided acute, as well as long-term, protection from tissue loss and functional deterioration {Jiang et al., 2002; 2004}. The beneficial effects of FGF-2 included prevention of ischemia-induced dephosphorylation of Cx43, and preservation of its intercalated disk localization, indicating an ability to

preserve the normal patterns of intercellular communication and rhythm {Srisakuldee et al., 2009; 2006}.

The acute protection afforded by FGF-2 was shown to require FGFR1 and activation of the PKC signaling cascade {Jiang et al., 2002; 2004; Padua et al., 1998}. Chronic overexpression of FGF-2 resulted in increased levels of membrane associated (activated) PKCα and PKCε consistent with the protective effect associated with these PKC isoforms {Sheikh et al., 2001}. Additional downstream signals activated by FGF-2 include the so-called 'survival kinases' such as ERK and Akt which could also be contributing to its protective response {Kardami et al., 2007b; Matsunaga et al., 2009}. Even though it was shown that in the FGF-2 overexpressing mice cardioprotection was dependent on ERK signaling (House et al 2005}, work in my supervisor's laboratory has shown that inhibition of PKC completely blocked cardioprotection by FGF-2, while retaining elevated levels of ERK activation, indicating that ERK activation may occur upstream of PKC and may not by itself be sufficient to mediate FGF-2 induced cardioprotection {Kardami et al., 2007b}. Nitric oxide and nitric oxide synthases have also been implicated in mediating both ischemic preconditioning as well as FGF-2 induced cardioprotection {Detillieux et al., 2004; 2003}.

Studies using added FGF-2 have suggested that endogenous FGF-2, released on a beat to beat basis to the extracellular environment by cardiac cells is likely to contribute to a healthy myocardium and confer resistance to injury {Sheikh et al., 2001}. This seems to be the case as FGF-2 deficient

hearts which are more vulnerable to injury compared to wild type hearts, while over-expression of FGF-2 in the FGF-2 deficient hearts was cardioprotective {House et al., 2003}.

Is Cx43 involved in FGF-2-induced cardioprotection? FGF-2 has been shown to increased the interaction of activated PKCε with Cx43 at the intercalated disks {Doble et al., 2000}, and result in extra phosphorylation of Cx43 at the PKC-target sites S262 and S368 *in vitro* and in the perfused adult rat heart {Doble et al., 2004; Srisakuldee et al., 2009; 2006}. It is of interest that the FGF-2-induced extra Cx43 phosphorylation at S262 and S368 occurred irrespectively of whether FGF-2 was given prior to or after ischemia, and suggested a possible contribution to an injury-resistant phenotype {Srisakuldee et al., 2009}, perhaps by decreasing gap junction mediated intercellular coupling. The protective effect of anesthetics on ischemia-reperfusion has been attributed to their ability to decrease metabolic coupling between cardiomyocytes, and event which prevents the spread of injurious metabolites {Hu and Liu, 2009}. A similar situation can be envisaged for FGF-2 which decreases cardiomyocyte dye-coupling in concordance with activating PKCε and increasing Cx43 phosphorylation {Doble et al., 1996; 2004; 2000}. Overall, Cx43 and gap junctions may act as mediators/effectors of cardioprotection by FGF-2 and ischemic preconditioning.

I.C-3. Role for Cx43 in Cardioprotection

There is increasing evidence to support an important role for Cx43 in the development of an injury-resistant phenotype in the heart and also other organs. In general, a positive relationship exists between Cx43 and the ability for preconditioning {Schulz et al., 2007}. For example, hearts from Cx43 (+/-) mice, expressing 50% less Cx43, as well as aging and/or failing hearts which have decreased Cx43 levels, are incapable or deficient in mounting an ischemic preconditioning response {Heinzel et al., 2005}. Similar findings have been obtained in non-muscle systems. The Cx43-mediated cytoprotection is not fully understood, and there is some controversy regarding the role of intercellular communication (gap junctions) in this context. In isolated mouse hearts, infarct size reduction by ischemic preconditioning was abolished by the gap junction uncoupler heptanol suggesting that some intercellular communication was required {Li et al., 2002a}. On the other hand, isolated cardiomyocytes which do not form gap junctions were reported to retain their ability for preconditioning {Li et al., 2004}. Because the magnitude of protection in isolated myocytes was not as strong as that in whole hearts it is possible that a different mechanism operates in isolated versus connected cells. It is also possible that Cx43 can regulate the preconditioning response by both channel-dependent and independent mechanisms.

Co-localization of Cx43 with protein kinases that are known mediators of preconditioning (PKCε,α) have also pointed to the possibility that Cx43 and its

phosphorylation are participating in cytoprotection {Doble et al., 2000; Ping et al., 2001; Schulz et al., 2003}. Phosphorylation of Cx43 was shown to be preserved in the preconditioned compared to non-preconditioned pig myocardium subjected to ischemia {Schulz et al., 2003}. These studies were confirmed in isolated rabbit {Miura, 2003} and rat hearts {Jain et al., 2003}.

Improved cell volume regulation has been suggested to be one potential end effector of protection by ischemic preconditioning. Cx43 hemichannels are implicated in this process as they improved cell volume homeostasis {Li et al., 2004}. In addition to a role for sarcolemmal Cx43 (gap junctions, hemi-channels), there is increasing evidence from several laboratories that mitochondrial Cx43 is important in cardioprotection by ischemic preconditioning {Boengler et al., 2009}. The exact role of mitochondrial Cx43 is not as yet well characterized. It is possible that the ability of Cx43 to form hemichannels may play a role in this context {Boengler et al., 2009}. There is some evidence {Heinzel et al., 2005} that mitochondrial Cx43 is required for a modest amount of mitochondrial reactive oxygen species (ROS) generation which is required to activate/propagate protective signal transduction {Das et al., 1999; Forbes et al., 2001; Pain et al., 2000}. Cx43 deficient mice were shown to have a functional deficit in the ROS formation in response to diazoxide, and this led to inability to be protected {Heinzel et al., 2005}.

Cx43 phosphorylation at PKC sites and cardioprotection: Srisakuldee and colleagues identified a close correlation between development of an injury-resistant state and phosphorylation of Cx43 at PKC target sites

{Srisakuldee et al., 2009; 2006}. Isolated perfused adult rat hearts were subjected to the following (cardioprotective) treatments: ischemic preconditioning; diazoxide perfusion; FGF-2 pre-treatment followed by 30 min global ischemia; 30 min global ischemia followed by 60 min reperfusion in the presence or absence of FGF-2. Cx43 phosphorylation was assessed by western blotting with phospho-specific antibodies. Ischemic preconditioning, diazoxide, and FGF-2 pre-ischemic or post-ischemic treatments elicited a P*Cx43 state, defined as above-physiological levels of phospho-S262-Cx43 and phospho-S368-Cx43. Phospho*(P*)Cx43 was sustained during global ischemia and was accompanied by attenuation of ischemia-induced Cx43 dephosphorylation and prevention of Cx43 lateralization. Post-ischemic FGF-2 treatment also diminished dephosphorylated Cx43. These studies suggested that increased Cx43 phosphorylation at PKC sites such as S262 may be required to mediate development of a cardioprotected state, a question explored further in this thesis.

I.D.REGULATION OF CELL PROLIFERATION AND CONNEXIN-43

I.D-1. General Overview

(portions of this section have been reprinted, with permission, from our review {Kardami et al., 2007a})

A role of connexin and gap junctions in proliferative cell growth and tumour suppression was proposed by early studies documenting an inverse relationship between gap junction intercellular communication and tumour growth {Loewenstein and Kanno, 1966; Loewenstein and Rose, 1992}. These studies identified decreased connexin expression and/or decreased intercellular communication in many tumour cells and tumourigenic cell lines. Later, overexpression of connexins in cancer cell lines was found to reduce tumorigenicity of these cells to varying degrees indicating a tumour suppressor role for connexins {Jiang and Gu, 2005}. Several studies since then were done to confirm these findings and to dissect the role of connexins on cell growth using different cell types/tumors and also various connexins such as Cx43, Cx32 and Cx26 {Jiang and Gu, 2005; Mesnil et al., 1995}. In HeLa cells transfection of Cx26 but not Cx40 reduced tumorigenicity {Mesnil et al., 1995} whereas in C6 glioma cells Cx43 but not Cx32 transfection normalized growth {Bond et al., 1994; Zhu et al., 1991}. Thus, different connexins can have different functions and cannot be assumed to impart similar results on cells.

Findings from connexin gene knock-down approaches and knock-out mouse models further strengthened these findings. Genetically engineered mice lacking Cx32 or Cx43 were shown to be more susceptible to liver and/or lung tumorigenesis {King and Bertram, 2005}. In human breast cancer cell lines Cx43 gene knockdown using siRNA produced a more aggressive phenotype {Shao et al., 2005}. Consistent with the above findings, retinoids and carotinoids that have an ability to suppress carcinogen-induced neoplastic

transformation were shown to up-regulate Cx43 expression {King and Bertram, 2005}.

Although a majority of studies supported a tumour/growth suppressive role for connexins, some reported the opposite. In early placental formation, Cx40 gap junctions and gap junctional intercellular communication were shown to be required for the proliferation of extravillous trophoblasts {Nishimura et al., 2004}. In models of atherosclerosis and restenosis Cx43 promoted smooth muscle cell proliferation {Chadjichristos et al., 2006; Kwak et al., 2003}. In another study, Cx43 expression and gap junctional intercellular communication were required for the FGF-2 induced proliferation of neural progenitor cells {Cheng et al., 2004}. These apparent contradictions could reflect the different responses to connexin expression in different scenarios, for example in normal versus cancer-related cell proliferation. They may also reflect molecular (post-translational) changes within the same connexin molecule dictating a differential response. Hence the precise mechanisms by which connexins regulate cell growth under physiological or pathophysiological conditions needs to be understood. It is clear that this would be a reflection of not only cell- and tissue-type and/or the connexin isoform that is being studied but also context-specific regulation of connexin function. Compounding this complexity is a lack of clear understanding as to whether connexin 43 can regulate growth by channel-dependent or independent pathways, or both.

Intercellular communication and growth regulation: Work from several laboratories identified an inverse relationship between magnitude of inhibition

of proliferative growth and degree of gap junctional intercellular communication. The original hypothesis proposed was that junctionaly diffusible molecules limit cell proliferation when certain cell densities are reached {Mehta et al., 1986; 1999}. Several reports have pointed to the link between loss of growth control and loss of gap junctional intercellular communication, both induced by tumour promoters {Trosko and Ruch, 1998}. In contrast, several other studies have provided evidence that connexins inhibit growth in the absence (and irrespectively) of gap junctional intercellular communication. Expression of communication-incompetent Cx43 was shown to be as effective as the wild type in suppressing tumorigenicity in rat bladder carcinoma cell line {Krutovskikh et al., 2000}. Connexins were shown to inhibit growth in the presence of uncoupling agents, in isolated cells or even if they were not located at the junctional areas {King and Bertram, 2005; Moorby and Patel, 2001}. The notion of intercellular communication independent growth inhibition has been further strengthened by several reports showing that the C-terminal tail domain (lacking channel forming ability) is capable of growth inhibitory activity {Dang et al., 2003; Fu et al., 2004; Zhang et al., 2003}.

I.D-2. Regulation of cardiomyocyte DNA synthesis and Cx43 phosphorylation

As observed for many (although not all) other cell types Cx43 expression in cardiomyocytes acts to inhibit DNA synthesis {Kardami et al., 2007a}. On

the other hand, FGF-2 (and other mitogens) stimulates cardiomyocyte DNA synthesis {Kardami, 1990; Pasumarthi et al., 1996}. As already mentioned, Cx43 (a tumour-suppressor) is a downstream target of FGF-2 (mitotic) signal transduction: FGF-2 activates PKCε which then phosphorylates Cx43 at PKC target sites such as S262 {Doble et al., 1996; 2004; 2000}. Doble and colleagues went on to show that this phosphorylation event on Cx43 blocked its ability to suppress DNA synthesis {Doble et al., 2004}. Briefly: increased expression of wild type Cx43 decreased cardiomyocyte DNA synthesis, but this decrease was maximized upon over-expression of mutant Cx43 which was incapable of becoming phosphorylated at S262 due to a conversion of S262 to alanine (S262A-Cx43). On the other hand, expression of a Cx43 mutant simulating the charge effect of constitutive phosphorylation by converting S262 to glutamate (S262D-Cx43) allowed full mitotic stimulation of cardiomyocytes {Doble et al., 2004}. Overall this work suggested that phosphorylation of cardiomyocyte Cx43 at S262 is required to allow cell cycle progression in response to mitogens.

The ability of S262D-Cx43 to ‘neutralize’ growth inhibition by Cx43 was found to be dependent on cardiomyocyte cell-cell contact (and perhaps cell-cell communication via gap junctions), as it was not evident in sparsely seeded isolated cardiomyocytes {Doble et al., 2004}. Similar observations have been made for other cell types {Moorby and Patel, 2001}. It is possible that that Cx43-related growth inhibition is modulated by different pathways in isolated cells versus inter-connected cells.

I.E. REGULATION OF CARDIOMYOCYTE PROLIFERATIVE GROWTH

The ability to create new functional cardiomyocytes (regeneration) after myocardial infarction, by stimulating the proliferation of myocytes (or myocytes precursors) capable of entering and traversing the cell cycle would provide a means to prevent the inevitable transition to heart failure, a major cause of death world-wide. Cardiomyocytes are proliferative during the embryonic and early neonatal stages. Subsequently cardiac myocytes increase in mass and size mainly by hypertrophy (increased size of individual myocytes) rather than hyperplasia (increase in numbers via mitotic division) {Ahuja et al., 2007; Laflamme and Murry, 2005}. Nevertheless, numerous studies by Anversa and colleagues have provided evidence that the heart is a dynamic organ that contains stem or progenitor cells that renew cells in the myocardium including cardiomyocytes {Srinivas et al., 2009}. Resident stem/progenitor cells differentiate to “amplifying” cells that have characteristics of immature cardiomyocytes, are capable of proliferation, and eventually form adult cardiomyocytes {Boni et al., 2008}. A recent report indicated that although most adult, binucleated cardiomyocytes are terminally differentiated cells, mononucleated adult cardiomyocytes are capable of re-entering the cell cycle if stimulated by neuregulin, a member of the EGF family of growth factors, acting via the ErbB2/B4 receptors {Bersell et al., 2009}.

Whether these populations are sufficient or can be mobilized/manipulated to provide effective cardiac regeneration after myocardial infarction remains the subject of intense research effort as well as debate {Yi et al., 2010}. Regardless, however, of whether the adult heart possesses some innate ability for regenerative activity (by resident progenitor cells, and/or mononucleated adult myocytes) or whether cell transplantation (of cardiomyocyte precursors, for example) is required to promote regeneration, it is important to know factors and mechanisms modulating (stimulating or inhibiting) cardiomyocyte proliferation. This understanding can provide strategies for stimulating or dis-inhibiting cardiomyocyte proliferation as may be needed during cardiac repair after myocardial infarction. In this context, Cx43, a protein essential for cardiomyocyte function which can nevertheless inhibit DNA synthesis, may act as a downstream integrator of pro- as well as anti-mitotic stimuli. The following sections will provide a brief overview of factors affecting cardiac myocytes proliferation and their relationship to Cx43.

I.E-1. Factors promoting cardiomyocyte proliferation

Several growth factors acting on plasma membrane tyrosine kinase receptors are capable of stimulating cardiomyocyte DNA synthesis and cell division. These include members of the FGF family (FGF-2 and FGF-1), epidermal growth factor (EGF), platelet derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), and neuregulin {Bersell et al., 2009; Goldman et

al., 1996; Kardami et al., 2007a; Kubin et al., 2003}. These growth factors stimulate entry and progress through the various steps of the cell cycle, by promoting expression of genes and various kinases/phosphatases involved in cell cycle regulation. For recent reviews on the subject the reader is referred to {Ahuja et al., 2007; Bicknell and Brooks, 2008}.

FGF-2: FGF-2 is an extensively characterized growth factor that belongs to a large family of structurally related heparin binding growth factors consisting of 23 members {Auguste et al., 2003}. FGF-2 displays a broad spectrum of biological functions including stimulation of cell proliferation, cytoprotection, angiogenesis, stem cell differentiation, tissue repair and osteogenesis {Grose and Dickson, 2005}. FGF-2 expression increases during cardiac injury and is expected to play an important role in the repair process; see reviews by {Detillieux et al., 2004; Kardami et al., 2007b}.

The biological effects of FGF-2 are mediated by binding to its cell membrane receptors (FGFR1-4) and activation of several signal transduction pathways {Detillieux et al., 2003}. Ligand binding leads to receptor dimerization and activation and intrinsic tyrosine kinase activity causing phosphorylation of tyrosine residues on the receptors. These serve as docking sites for the recruitment of signaling enzymes with SH2/PTB domains {Dailey et al., 2005}. FGF-2 activates the RAS-MAP kinase pathways (ERK1/2, p38, and JNK), PI3K/AKT pathway and PLCγ-PKC pathway eliciting various biological effects {Schlessinger, 2000}. Stimulation of cardiomyocyte proliferation by FGF-2 requires the activity of PKCε {Kardami et al., 2003},

casein kinase 2 {Jiang et al., 2004}, and as, discussed in a previous section (I.D-2), is dependent on Cx43 phosphorylation at S262 (by PKCε) {Doble et al., 2004} (Diagram 4).

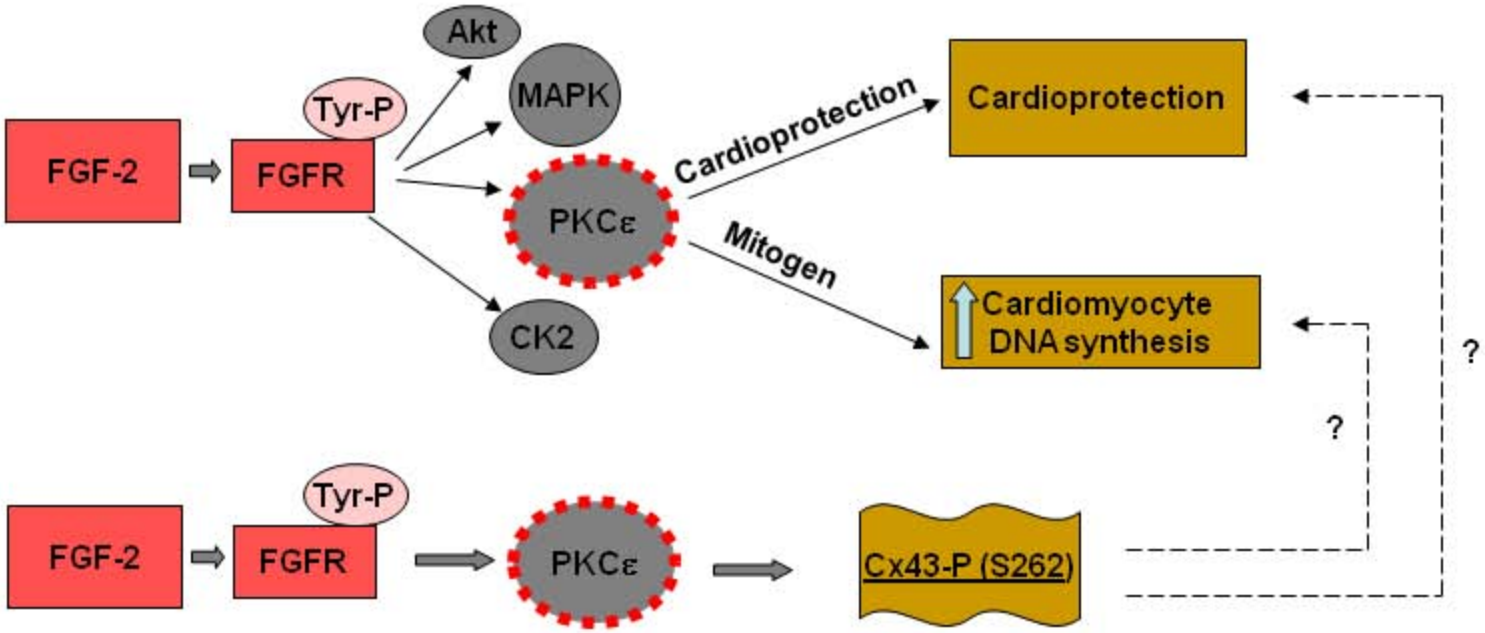


Diagram 4: Depicts the signaling pathways of FGF-2 involved in cardioprotection and cardiomyocyte DNA synthesis

I.E-2. Factors inhibiting cardiomyocyte proliferation

Transforming growth factor β1 (TGF β1) is a potent inhibitor of cardiomyocyte DNA synthesis and proliferation: it has been shown to prevent serum- as well as FGF-2-induced stimulation of cardiomyocyte DNA synthesis {Kardami, 1990; Sheikh et al., 2004}. As mentioned earlier, Cx43 expression has also been found to inhibit cardiomyocyte DNA synthesis {Doble et al., 2004}.

TGFβ: The TGFβ superfamily is composed of structurally related members categorized in two major groups (i) TGFβ/Activin group and (ii) BMP /GDF (bone morphogenic protein/growth and differentiation factors) group {Xiao and Zhang, 2008}. TGFβ consists of 3 different isoforms, TGFβ1, TGFβ2 and TGFβ3 {Derynck and Feng, 1997}. These cytokines play an important role not only during development but also postnatally in various physiological and pathological states by regulating a wide range of cellular processes such as cell growth, migration, differentiation, extracellular matrix synthesis, and apoptosis {Derynck and Akhurst, 2007; Massague et al., 2000}. Most of the knock-out mouse strains targeting TGFβ signaling molecules turned out to be embryonically lethal, pointing to the importance of TGFβ signaling in embryonic development {Xiao and Zhang, 2008}. TGFβ is a potent inhibitor of cell proliferation in most primary cells including epithelial cells {Heldin et al., 2009} and cardiomyocytes {Kardami, 1990; Sheikh et al., 2004}. The mechanism by which TGFβ inhibits cell growth may vary with each cell but has

been shown to involve the inhibition of c-myc expression, and induction of inhibitors of cell cycle such as p21 and p15 {Feng and Derynck, 2005; Massague et al., 2005}.

All three isoforms of TGFβ (TGFβ 1,2,and 3) play an important role during cardiac development {Ramos-Mondragon et al., 2008}. Using Cre/loxP system, cardiac targeted knock-out strains of many TGFβ signaling genes have been developed {Xiao and Zhang, 2008}. These studies have revealed the specific roles of various TGF ligands and signaling molecules in physiological as well as in pathological states.

In the adult heart TGFβ1 plays a vital role in cardiac hypertrophy, fibrosis and cardiac failure {Azhar et al., 2003; Ruiz-Ortega et al., 2007}. TGFβ has been implicated in the initiation of fibrosis by causing alterations in the normal balance between biosynthesis and degradation of extracellular matrix, i.e by increasing the synthesis and decreasing the proteolytic activity in the extracellular matrix (ECM) simultaneously {Agrotis et al., 2005}. TGFβ1 upregulates, (a) extracellular matrix proteins such as collagens, fibronectin, tenascin, membrane proteoglycans, and, (b), protease inhibitors such as tissue inhibitors of metalloproteinases and plasminogen activator inhibitor-1 that inhibit the degradation of ECM. These actions are mediated through downstream Smad signaling {Wang et al., 2002}. The profibrotic effect of TGFβ was shown to include its ability to stimulate differentiation of fibroblasts to myofibroblasts and its ability to induce epithelial-myofibroblast transdifferentiation {Agrotis et al., 2005}. Myofibroblasts are hypersecretory for

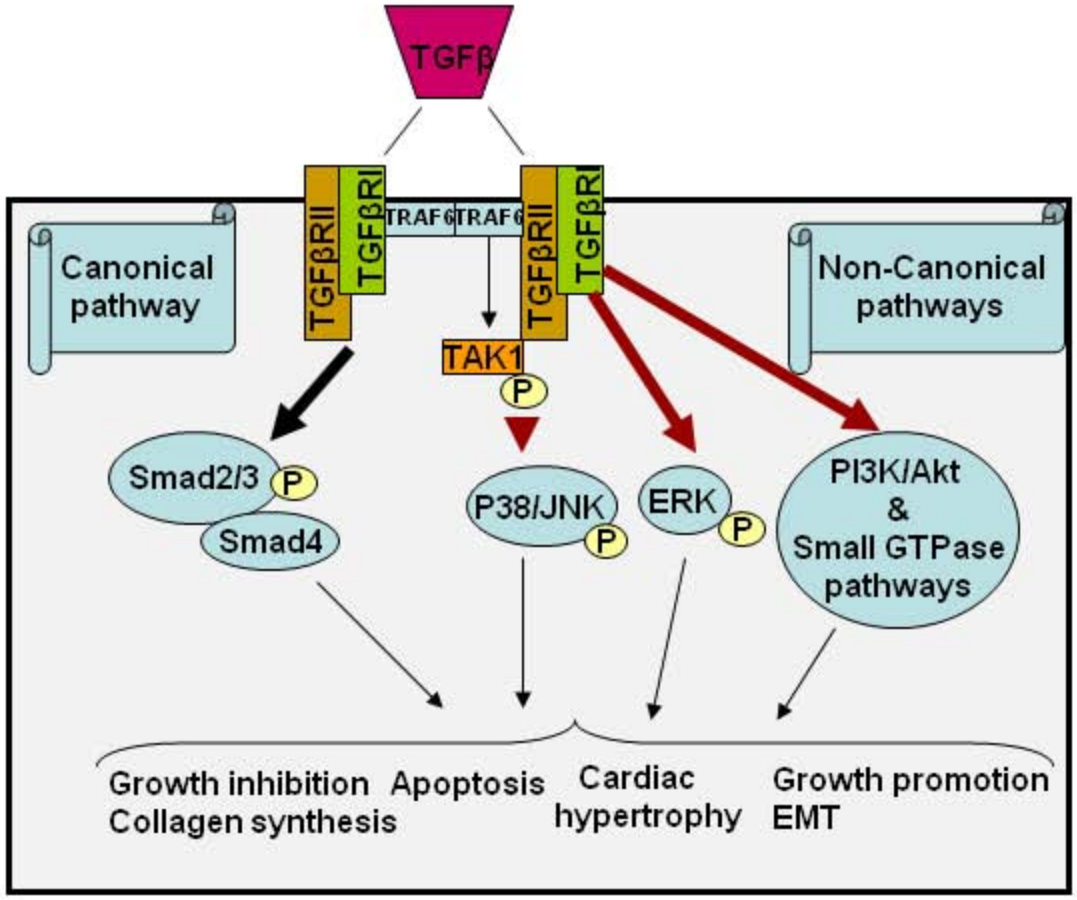
matrix proteins and play an important role in wound healing; proliferation of these myofibroblasts occurs in pathological states of the heart such as myocardial infarction and heart failure {Dugina et al., 2001}. The levels of TGFβ increase after myocardial infarction and myofibroblast differentiation was shown to occur within days of myocardial infarction {Drobic et al., 2007}.

TGFβ signaling pathway (canonical and non-canonical): The TGFβ ligand exerts its cellular effects by binding to its serine/threonine kinase cell membrane receptors TGFβRI and TGFβRII; a third receptor, a betaglycan, has also been shown to assist in the ligand uptake by the membrane receptors and participate indirectly in the signal transduction {Derynck and Feng, 1997}. TGFβRI is a group of receptor types of which ALK5 and ALK1 play an important role in TGFβ signaling. Once activated, TGFβ dimers bind to TGFβRII, which then transphosphorylates and activates TGFβRI forming a heterotetrameric complex {Heldin et al., 1997}. Upon activation the TGFβRI phosphorylates its downstream signaling molecules called Smads {Massague, 1998}. Since the activation could occur either via ALK-5 or ALK-1 the further downstream signaling of TGFβ and its intracellular effects depends on which one of these receptors is activated. TGFβ, signals through the combination of TGFβRII and ALK5 in most cells {Piek et al., 1999} (Diagram 5).

The Smad proteins are downstream signaling molecules in the TGFβ signaling pathway. The Smad family of proteins consists of 8 members and is further grouped in to three subfamilies; Receptor activated-, R-Smads, common mediator-, co-Smads, and inhibitory-Smads, I-Smads). Of the five R-

Smads, Smad2 and 3 are specifically activated by TGFβRI {Chang et al., 2002; Ruiz-Ortega et al., 2007}. Once the R-Smads are phosphorylated at the two serine residues in the SSXS motif at their c-termini, they bind to co-Smad (Smad4) and translocate to the nucleus to activate gene transcription with the help of other nuclear factors {Massague and Wotton, 2000}. The I-Smads, Smad6 and Smad7 are the major negative regulators of TGFβ signaling and they inhibit R-Smads by competing for the receptors or co-Smads and also by targeting the receptors for degradation {Javelaud and Mauviel, 2004; Lebrin et al., 2005}.

The TGFβ signaling through the Smad molecules constitutes the canonical pathway. In addition, TGFβ has also been shown to activate other signaling molecules such as JNK, p38 MAPK by phosphorylation and activation of TAK1 (TGFβ activated kinase 1), ERK pathway via activation of Ras through tyrosine phosphorylation and PI3K/Akt pathway, all of which constitute non-canonical TGFβ signaling pathways {Zhang, 2009}.



EMT-Epithelial Mesenchymal Transition

Diagram 5: Depicts the canonical and non-canonical signaling pathways of TGFβ

In addition to gene knock-out studies, small molecule inhibitors have been used in understanding the signal transduction mechanisms and also dissecting the role of specific signaling pathways involved in various biological processes. Compounds that selectively inhibit TGFβR have a potential to be used for therapeutic applications in the treatment of various diseases like fibrosis, late stage cancer, atherosclerosis and other pathologies linked to TGFβ pathways. SB431542 is a potent inhibitor of ALK5 (TGFβR1) and it has been shown to inhibit the *in vitro* phosphorylation of Smad2 by acting as a competitive ATP binding site kinase inhibitor {Callahan et al., 2002}.

TGFβ and Cx43: There is some evidence in the literature to suggest a possible link between growth inhibition by TGFβ1 and Cx43, and *vice versa*. Cx43 was reported to potentiate TGFβ signaling in the atrial-derived cardiomyocyte cell line HL-1. Dai and colleagues showed that Cx43 strengthened TGFβ signaling by releasing Smad2 from microtubules via competitive binding, thus making it available for phosphorylation by TGFβRI and nuclear translocation {Dai et al., 2007}. TGFβ family members have been implicated in controlling Cx43 gene expression. TGFβ upregulated Cx43 expression in primary cells and transformed cell lines, such as vascular smooth muscle cells {Rama et al., 2006}, osteosarcoma cells {Chatterjee et al., 2003}, bovine aortic endothelial cells {Larson et al., 2001}, and in human osteoblastic cells {Chiba et al., 1994}. In mammary gland epithelial cells, the antiproliferative effect of TGFβ was shown to be partly mediated by increasing Cx43 gene expression via the PI3K/Akt pathway {Tacheau et al., 2008}.

TGFβ can also alter the phosphorylation status of Cx43: Exposure of MC3T3-E1 cells to TGFβ decreased relative levels of the phosphorylated 46 kDa form of Cx43 {Wyatt et al., 2001}. Thus Cx43, its phosphorylation status, and/or gap junction mediated intercellular communication are downstream targets of TGFβ signaling and potentially mediate some of its effects such as inhibition of cell proliferation. This is explored further in this thesis.

I.F. RATIONALE AND HYPOTHESES

As outlined in the preceding literature review, in addition to its traditional role as a major component of cardiac gap junctions and therefore electrochemical coupling of cardiomyocytes, Cx43, and its phosphorylation at PKC target sites, have been linked to two major events. These are, (a) the ability of cardiomyocytes to develop an injury-resistant state, and (b) the ability of cells to synthesize DNA. Both of these events can be triggered by FGF-2 and both are dependent on the FGF-2-induced stimulation of PKCε.

One major aim of these studies is to better understand the role of Cx43 and its phosphorylation at S262 in mediating FGF-2-PKC cardioprotection**.** Specific hypotheses to be examined under this aim are:

Specific Hypothesis 1: Lack of phosphorylation at S262 renders cardiomyocytes more vulnerable to ischemic injury

Specific Hypothesis 2: Phosphorylation of Cx43 at S262 is required for FGF-2, PKCε and ischemic preconditioning induced cardiomyocyte protection from ischemic injury.

The second major aim is to better understand the mechanism by which Cx43 and its phosphorylation modulates DNA synthesis, the role of its C-terminal domain, and the role of TGFβ1-related signal transduction in this process. Specific hypotheses to be examined under this aim are:

Specific Hypothesis 3: Phosphorylation at S262 site cancels growth inhibition by the C-terminal tail of Cx43 irrespectively of the presence of the channel-forming domain.

Specific Hypothesis 4: Cx43-mediated inhibition of DNA synthesis includes downstream activation of $TGF\beta_1$ triggered signal transduction

Specific Hypothesis 5: TGF β_1 prevents the FGF-2-induced Cx43 phosphorylation at S262.

CHAPTER II: MATERIALS AND METHODS

II.A. MATERIALS

II.A-1. Animals

Animals were obtained from the Central Animal Care Facility at the University of Manitoba. Animal use was in accordance with Canadian Council on Animal Care regulations.

II.A-2. Antibodies

Rabbit polyclonal antibody, recognizing both phosphorylated and dephosphorylated Cx43 and raised against res.367-382 (P.AB), has been described and characterized {Doble et al., 2000}. Mouse monoclonal antibody recognizing dephosphorylated Cx43 (#13-800) was purchased from ZYMED laboratories (CA). Rabbit polyclonal anti-P-262-Cx43 antibody recognizing Cx43 phosphorylated at S262 and rabbit polyclonal anti-TGFRII antibodies were purchased from Santa Cruz Biotechnology (CA). Rabbit monoclonal P-Smad2 antibody was purchased from Upstate Cell Signaling Solutions (NY). Mouse monoclonal Smad2 antibody was purchased from Cell Signaling Technology (MA). Rabbit polyclonal antibody recognizing GAPDH was purchased from abcam (MA). Mouse monoclonal α-actinin antibody and rabbit anti-actin antibody were purchased from SIGMA (Missouri, USA). Goat anti

mouse and anti rabbit HRP (horse radish peroxidase) secondary antibodies, were obtained from Bio-Rad (CA). The concentrations of various antibodies used for western blotting and immunofluorescence are shown in Tables 3 and 4.

Table 3: Antibody concentrations for western blotting

Antibody	Blocking solution	Primary AB concentration	Secondary AB concentration
TGFβRII AB	10% Milk	1:1000/ 1% Milk Overnight at 4^{o}C	1:10000/ 1% Milk
Smad2	10% Milk	1:1000/ 5% Milk Overnight at 4^{o}C	1:10000/ 1% Milk
P-Smad2	10% Milk	1:1000/ 5% Milk Overnight at 4^{o}C	1:10000/ 5% Milk
Cx43 P.AB	10% Milk	1:10000/ 1% Milk	1:10000/ 1% Milk
#13-800	10% Milk	1:1000/ 1% Milk	1:10000/ 1% Milk
Cx43 P-262 AB	3% BSA	1:5000/ 1% BSA Overnight at 4^{o}C	1:10000/ 1% BSA
GAPDH	10% Milk	1:5000/ 1% Milk	1:10000/ 1% Milk
Actin AB	10% Milk	1:5000/ 1% Milk	1:10000/ 1% Milk

Table 4: Antibody concentrations for immunocytochemistry

Antibody	Primary AB concentration	Secondary AB concentration
Smad2 AB	1:100/ 1%BSA-PBS	AntiMouse Texas Red/ 1:100
PKCε AB	1:100/ 1%BSA-PBS	Alexafluor488/2.5 µl/ml
TGFβRII AB	1:200/ 1%BSA-PBS	Alexafluor488/2.5 µl/ml
Cx43 P.AB	1:2000/ 1%BSA-PBS	Alexafluor488/2.5 µl/ml

II.A-3. Chemicals

The lactate dehydrogenase (LDH) assay kit and In Situ cell death detection kit (TUNEL-TMRred assay kit) were purchased from Roche Diagnostics (Mannheim, Germany). BrdU assay kit was purchased from Amersham (NJ). SB431542 was purchased from TOCRIS Bioscience (Bristol, UK). SB431542 was dissolved in DMSO just before use to get a stock concentration of 100 mM. It was further dissolved in the culture media to obtain a final concentration of 10 or 20 μM. TGFβ1 was purchased from R&D Systems (USA). TGFβ1 powder was dissolved in a solution containing 4 mM HCL and 0.5% bovine serum albumin (BSA) to give a stock concentration of 10 μg/ml. The final concentration of 5 ng/ml was achieved by dissolving it in the culture media. Recombinant 18 KDa FGF-2 was made in our own laboratory as described {Jiang et al., 2007; 2004}. The final concentration of 10 ng/ml was obtained by dissolving it in the culture media.

II.B. METHODS

II.B-1. Western Blot Analysis

This procedure was performed exactly as described previously {Doble et al., 1996}. Plates with cardiomyocytes were immediately frozen with liquid nitrogen and stored at –80^{o}C. The cells were scraped with 1x SDS/PAGE sample buffer containing protease and phosphatase inhibitor cocktails (SIGMA

-P2850, P5726, P8340). The samples were sonicated and centrifuged (14,000 rpm for 10 min, Eppendorf centrifuge 5417C) to remove any undissolved residue and obtain the final sample. Protein concentration was determined by the BCA protein assay reagent (Pierce) followed by spectrophotometry (SpectramaxPlus384 (Molecular Devices)). Lysates were analyzed on 10% polyacrylamide gels {Laemmli, 1970} at 10 µg protein/lane using 60 mA constant current. To determine the molecular mass, broad range (6.5-200 KDa) standards (Bio-Rad) were used. The proteins on the gel were electrophoretically transferred to PVDF (polyvinylidene difluoride) membranes. The membranes were incubated with a blocking solution containing 10% milk in Tris-buffered saline with TWEEN-20 ((v/v) (SIGMA) (TBST)), for 1 hr at room temperature. Subsequently membranes were treated with primary antibody (antibody concentrations are listed in Table1) for 1 hr at room temperature or overnight at $4^{o}C$ with shaking. Antibodies were dissolved in 1% milk in TBST or 1% or 5% BSA in TBST. The membranes were then rinsed with 1% milk in TBST (4 X 10min) or 0.1% BSA and incubated with anti-rabbit (1:10000) or anti-mouse HRP secondary antibodies for 1 hr at room temperature. Membranes were rinsed with plain TBST (4 X 5 min). Finally membranes were incubated in ECL+plus (enhanced chemiluminescence) Western blotting detection system obtained from Amersham Pharmacia, wrapped in the sheet protectors and exposed to the imaging film (Kodak X-omat LS) for 5-15 min to detect the chemiluminescent signal. A Konica (SRX-101A) film processor was used to process the films.

II.B-2. Immunocytochemistry

Cells grown on glass coverslips were fixed with 1% paraformaldehyde and then permeabilized with 0.1% Triton-X 100 in PBS. TUNEL-TMR red staining was done and primary antibodies (concentrations are listed in Table 2) were added to the coverslips. Then the coverslips were probed with appropriate anti-rabbit or anti-mouse fluorescent secondary antibodies. Nuclei were stained with Hoechst dye 33342 (Calbiochem). The coverslips were then mounted on the slides using the Prolong Antifade Kit (Invitrogen).

II.B-3. TUNEL Assay

In Situ cell death detection kit (TUNEL assay kit) was used as per manufacturer's (Roche Diagnostics, Mannheim, Germany) instructions. TUNEL staining was done as a part of immunocytochemistry procedure. Each coverslip (n=6 coverslips/group) was divided in to 8 fields and the TUNEL positive nuclei and the total nuclei were counted in each field. TUNEL staining index was obtained by calculating the percentage of TUNEL positive nuclei with respect to the total nuclei. Previous studies in our laboratory have found that cardiomyocytes (identified by actinin staining) could also be identified by their very strong punctate anti-Cx43 staining and their propensity to exist in well connected clusters. The later method was thus used to identify cardiomyocytes in our cultures. Non-myocytes were clearly identified by their

weak anti-Cx43 staining and distinct nuclear shape. Overall non-myocyte contamination was at ~5% total.

II.B-4. Lactate Dehydrogenase (LDH) Assay

Supernatant from different experimental groups was collected after 6 h of hypoxia and frozen immediately. The degree of cardiomyocyte injury was assessed by measuring the amount of LDH released in to the media during hypoxia using the Roche LDH assay kit. Absorbance values were used to calculate the relative cytotoxicity.

II.B-5. Adenoviral Infections

The cardiomyocytes were infected with various adenoviruses for the hypoxic experiments as well as BrdU labeling experiments. Recombinant adenoviruses expressing no cDNA insert (Ad-Vector) or recombinant adenoviruses expressing wild-type-Cx43 (Ad-WT-Cx43), S262A-Cx43 (Ad-S262A-Cx43), S262D-Cx43 (Ad-S262D-Cx43) were used at 2 m.o.i (multiplicity of infection) {Doble et al., 2004}. Ad-PKCε {Ping et al., 1999} and Ad-DNTGFR {Sheikh et al., 2004} were used at 50 m.o.i. Ad-DNSmad2 was a generous gift from Dr. Wells (University of Pennsylvania School of Medicine, PA) and was used at 100 m.o.i {Uemura et al., 2005}.

II.B-6. Neonatal Rat Ventricular Myocyte Cultures (NCM)

Cardiomyocytes were isolated from the ventricles of 1 day old rat pups according to standard procedures {Doble et al., 1996; Doble et al., 2000}. For the hypoxia experiments freshly isolated myocytes were plated at a density of 800,000 cells per 35 mm collagen coated plate in the presence of 10 % bovine calf serum. For the BrdU labeling experiments the freshly isolated myocytes were plated in collagen coated 6 well plates at a density of 400,000 cells per well in the presence of 10% bovine calf serum with or without FGF-2. To analyze the effect of TGFβ_1 and FGF-2 on S262 phosphorylation of Cx43, myocytes were maintained in collagen coated 6-well plates at a density of 400,000 cells per well in the presence of 0.5% bovine calf serum with supplements (0.5% BSA, 1% penicillin/streptomycin, 0.04% vitamin C, 0.1% Insulin, 0.1% Transferrin/Selenium) for 48 h.

II.B-7. Simulated Ischemia in Cultures of Neonatal Cardiomyocytes

Cardiomyocytes were infected with various adenoviruses and after overnight incubation were subjected to simulated ischemia. To simulate ischemia, myocytes were placed in 'ischemic medium' (118 mM NaCl, 24 mM $NaHCO_3$, 1 mM $NaH_2PO_4 \cdot H_2O$, 2.5 mM $CaCl_2.2H_2O$, 1.2 mM $MgCl_2$, 0.5 mM sodium$\cdot$EDTA$\cdot 2H_2O$, 20 mM sodium lactate, and 16 mM KCl, pH 6.2) {Zhao et al., 1998} and transferred to a hypoxia chamber maintained at 37°C, in an atmosphere of 95 % N_2 and 5 % CO_2, for 6 h. Myocytes incubated in the

normal atmosphere of the culture incubator (not in hypoxia chamber) served as controls. A simplified representation of the hypoxia set-up is shown in Diagram 6.

Hypoxia chamber:

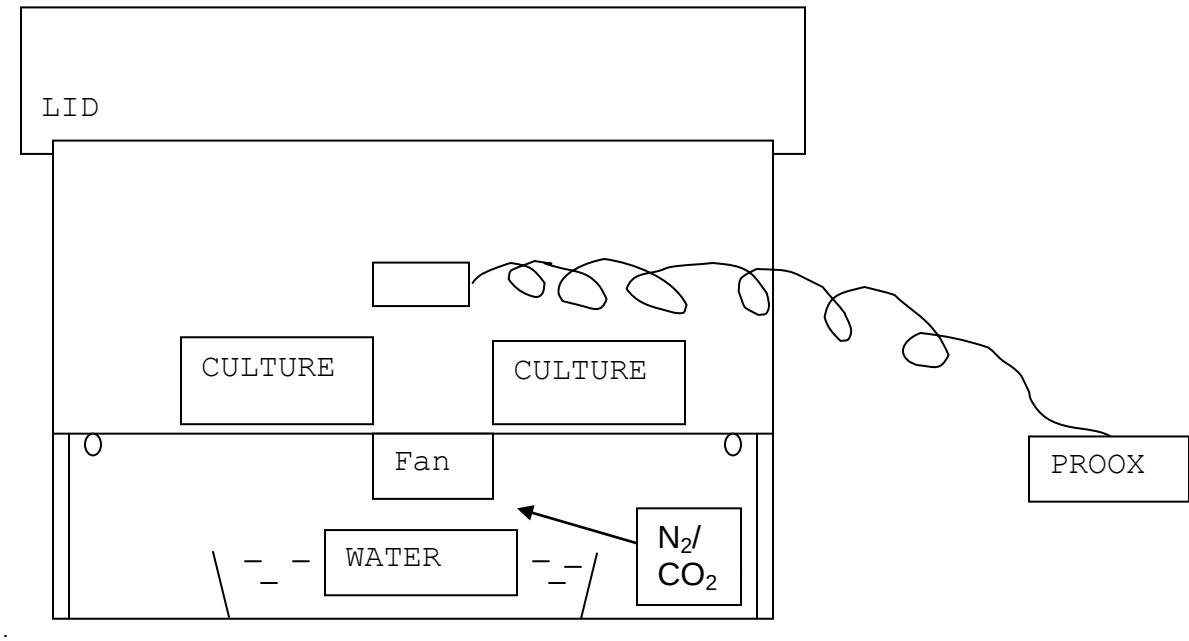


Diagram 6: The hypoxia chamber consists of a square chamber (40 cm x 40 cm x15 cm) and a lid. The chamber is made of clear plexiglass and sits inside the 37^{o}C incubator. It is connected to a gas tank (Nitrogen/CO_2) via PROOX, the oxygen controller (Reming Bioinstruments Company). When the PROOX reaches a targeted set point of oxygen (0%), the CO_2 reaches its required concentration of 5% by default. The gas tank was set to have the right concentration of CO_2 to reach this desired set point.

II.B-8. Bromodeoxyuridine (BrdU) Labeling assay

Neonatal rat ventricular cardiomyocyte cultures were incubated with BrdU (3 µg/ml) for 8 h prior to the termination of various experiments. The coverslips were then fixed with 1% paraformaldehyde for 15 min at 4^{o}C, and then permeabilized with 0.07M NaOH for 2 min at room temperature. Labeling for sarcomeric α-actinin (to identify myocytes), BrdU (nuclear) and Cx43 were done simultaneously using monoclonal antibodies against mouse α-actinin and BrdU (1:1, Amersham) and rabbit polyclonal Cx43 antibody in 1% (w/v) BSA-PBS. BrdU and α-actinin and were visualized with Texas Red-conjugated anti-mouse Ig (1:100; Jackson Laboratories) and Cx43 with Alexafluor488 (Invitrogen).

II.B-9. Determination of BrdU Labeling Index (BrdU LI)

BrdU labeling index was determined by calculating the percentage of BrdU positive myocyte nuclei with respect to the total nuclei. Not less than three coverslips per group were scored for each study. Sixteen random fields were scored per coverslip, to determine the fraction of myocytes in S-phase with respect to the total nuclei.

II.B-10. HEK 293 Cell culture

Human embryonic kidney (HEK) 293 cells (Stratagene, La Jolla, CA) were grown in Dulbecco's minimal essential hi-glucose medium supplemented

with 5% FBS and 100 µg/ml each of streptomycin and penicillin. Cells were passaged when the culture reached around 70% confluence.

II.B-11. HEK 293 cell culture and transient gene transfer

HEK 293 cells were seeded at the density of 1×10^5 per well in 6-well (35 mm/well) plates, one day before transfection. One µg from each cDNA was added per well, using the transfection reagent Trans IT 293 (Mirus, Madison, WI) according to the manufacturer's instructions. This procedure resulted consistently in over 60% transfection efficiency, assessed by immunofluorescence for expression of protein after each experiment. Cells were allowed to grow for 24–48 hours after gene transfer, and then processed for immunofluorescence or protein extraction.

II.B-12. Cx43CT gene transfer

The Cx43CT construct was obtained by PCR amplification of the C-terminal coding region of rat Cx43 {Dang et al., 2003}. The coding region from amino acids 243–382 (CT243) were amplified with sense primer 5′-CCGGAATTCAGCATGGATCTCTTCTACGTC-3′ and anti-sense primer 5′-CGCTCTAGATTAAATCTCCAG-3′ and used for construction of C-terminal Cx43 (Cx43CT). The cDNA coding for CT243 (described in {Dang et al., 2003}) was introduced in NCM using adenoviral gene transfer, at an m.o.i. of 5. The construct was fully sequenced in both directions (Cortec DNA service

Laboratories Inc., Kingston, ON, Canada). After adenoviral gene transfer, neonatal cardiomyocytes (NCM) were maintained either in normal non-ischemic conditions or subjected to mild ischemic stress by overnight incubation in 'ischemic medium' followed by immunofluorescence-based determination of the proportion of TUNEL-positive nuclei.

II.B-13. Densitometric Analysis

Densities of the western blot bands were determined using the Bio-Rad Model GS-800 densitometer with Molecular Analyst software (Bio-Rad). The bands were first normalized to the loading control and then the normalized values were used for statistical analysis.

II.B-14. Data Analysis

The GraphpadStat and SigmaStat software programs were used for data analysis. Data are presented as mean ± SEM (standard error of the mean). Statistical analysis was performed using either one-way ANOVA to compare more than two groups or two-way ANOVA to compare more than two groups with two independent variables. $P<0.05$ was considered statistically significant. $P<0.01$ was considered statistically very significant.

CHAPTER III: RESULTS

III.A. THE EFFECT OF CX43 AND ITS PHOSPHORYLATION AT S262, ON NEONATAL CARDIOMYOCYTE (NCM) RESISTANCE TO ISCHEMIC INJURY

Cx43 is phosphorylated by various kinases including PKCε and becomes dephosphorylated during ischemia, an event that precedes its eventual degradation {Lampe et al., 2006; Lampe and Lau, 2000}. Cx43 is also minimally phosphorylated at S262 in normal hearts {Srisakuldee et al., 2006}. Cardioprotective treatments like ischemic preconditioning and treatment with FGF-2, stimulates Cx43 phosphorylation on the PKC target site serine S262 {Doble et al., 1996; 2000; Srisakuldee et al., 2009}. Previously FGF-2 has been shown to be cardioprotective in a PKC dependant manner {Jiang et al., 2002} and PKC is a central mediator of protective effect by ischemic preconditioning {Ping et al., 2002}. FGF-2 levels increase locally during myocardial injury and FGF-2 also interacts with Cx43 by phosphorylating it via PKCε, Thus, a cause-and-effect relationship between the PKC-mediated Cx43 phosphorylation at S262 and the resistance of NCM to ischemic injury was sought. An examination was made of whether preventing phosphorylation of Cx43 at S262 will increase neonatal cardiomyocyte (NCM) vulnerability to ischemic injury and if it renders cardiomyocytes incapable of being protected by FGF-2 and ischemic preconditioning. Some of the results presented here

have been published in Srisakuldee et al 2009 and are reproduced here with permission (see Appendix).

III.A-1. Establishment of an *in vitro* model simulating myocardial ischemic injury

Confluent neonatal cardiomyocyte (NCM) cultures were used. To simulate aspects of ischemic exposure, NCM were placed (a) in 'ischemic medium', and (b) in 'ischemic medium' plus incubation in a hypoxia chamber in an atmosphere of 95% N_2 and 5% CO_2 (0% oxygen), for up to 24 h. Cultures maintained in standard medium (0.5% serum in DMEM medium) were used for comparisons. Cellular injury was evaluated at various time points by measuring release of cytosolic enzymes (lactic dehydrogenase, LDH), an indicator of plasma membrane damage, and cell death, by determining the proportion of myocytes nuclei staining positive for TUNEL.

A typical set of results for TUNEL-staining index and LDH release are shown in Fig.I-1A&B respectively. Incubating NCM in 'ischemic medium' alone had no significant effect on either LDH release or TUNEL-staining index, at any time point (6, 12 and 24 h), compared to corresponding control cultures. In contrast, incubation with 'ischemic medium' plus hypoxia elicited significant, time-dependent increases in both LDH release and TUNEL staining compared to control, or 'ischemic' medium-only incubated cultures, at all corresponding time points. LDH release and TUNEL-staining index of cultures kept in the

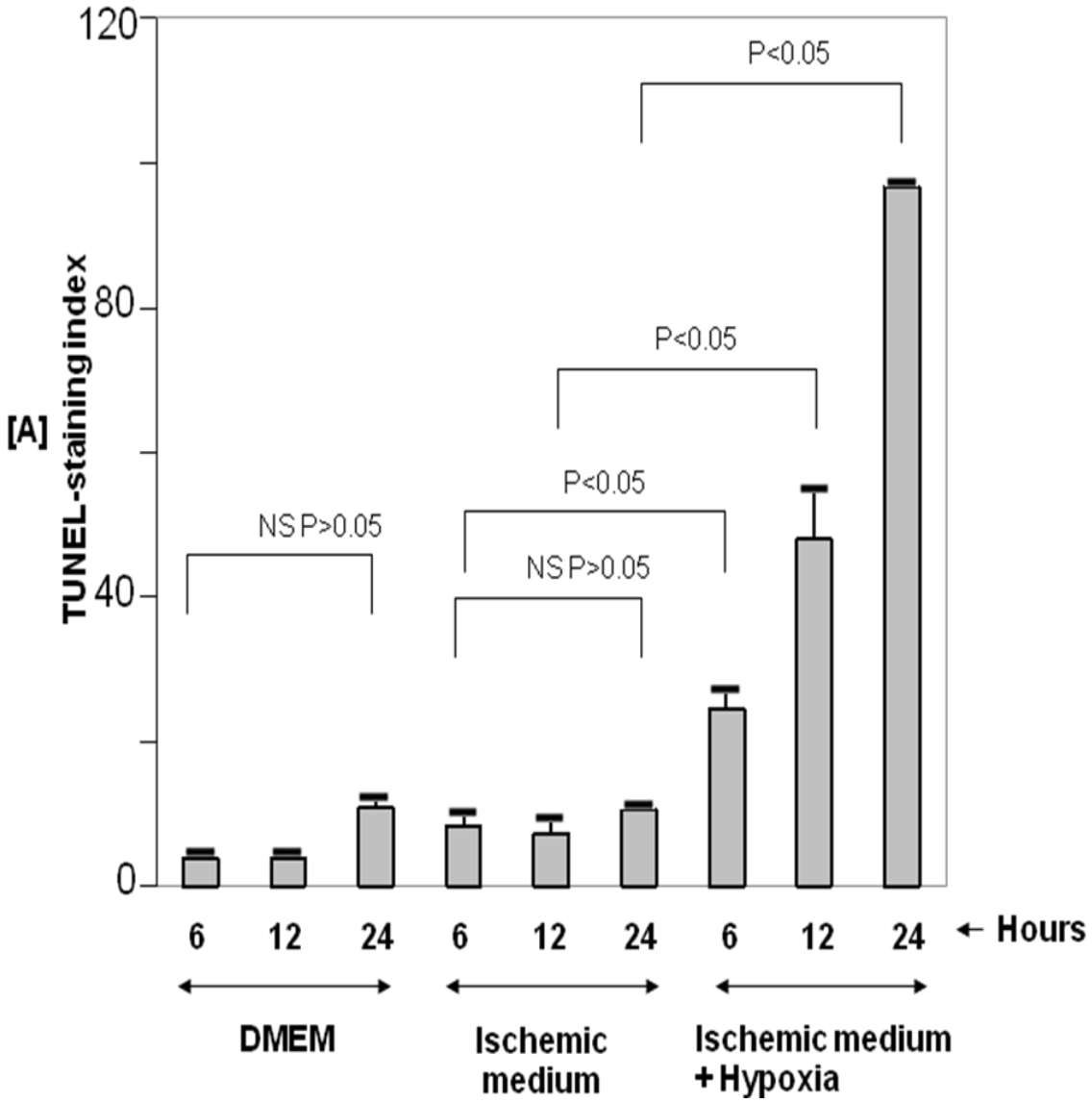
hypoxia chamber showed significant time-dependent increases in cell death and injury, nearing 100% TUNEL staining at 24 h (Fig.I-1A).

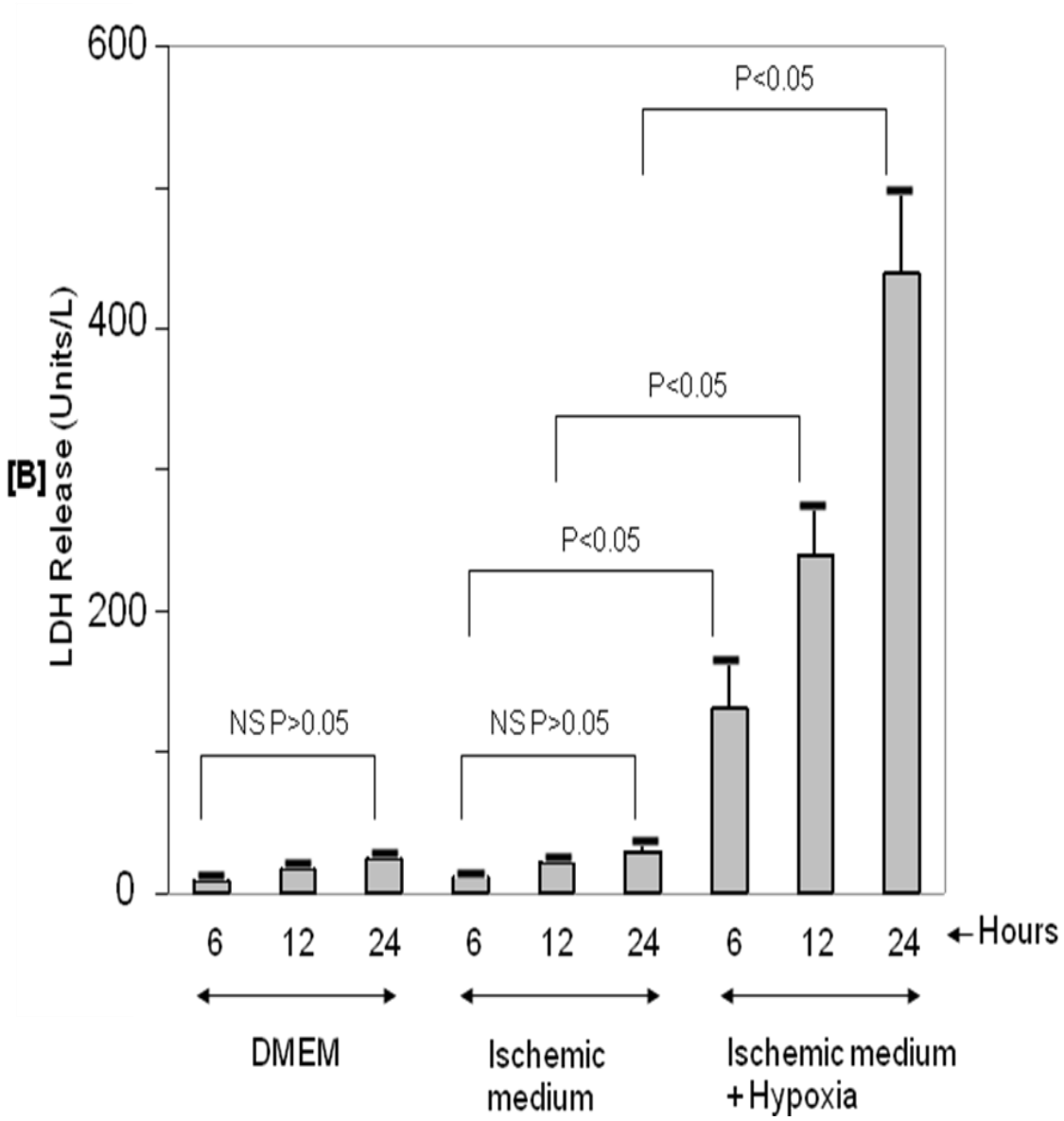
We then examined the effect of simulated ischemia (=incubation in 'ischemic medium' plus exposure to hypoxia) on relative levels of total Cx43, dephosphorylated Cx43 (41 kDa, recognized by both P.AB and #13-800) and phosphorylated Cx43 (43-45 kDa, recognized by P.AB). Results are shown in Fig.I-2: At 6 h of hypoxia we observed a relative increase in 41 kDa and a relative decrease in 43-45 kDa Cx43, consistent with its anticipated dephosphorylation. Increased levels of dephosphorylated Cx43 were also confirmed by a potent increase of immunoreactivity with #13-800 antibody at this time point. There was no significant loss of total Cx43 at 6 h in this particular experiment. In contrast, 12 and 24 h of hypoxia were accompanied by progressive loss of Cx43, indicated by decreased immunoreactivity with P.AB. Some dephosphorylated Cx43 was detectable at 12 h and this was reduced to background levels at 24 h. This experimental series was repeated once more, with comparable results. As the same amount of protein (10 µg) was loaded per lane, we do not think that the major differences detected in total Cx43 immunoreactivity between various lanes such as between lanes 3 and 4 or 5 and 6 in (Fig.I-2a) reflect differences in loading.

Overall, these pilot studies established an *in vitro* model to be used in subsequent studies. According to this model, "simulated ischemia" by incubation in ischemic medium plus hypoxia for 6 h causes intermediate levels of cellular injury and death, accompanied by dephosphorylation of Cx43.

<u>Figure I-1A&B:</u> Simulated ischemia induces increases in [A] TUNEL-staining index (cell death) and [B] LDH release in NCM

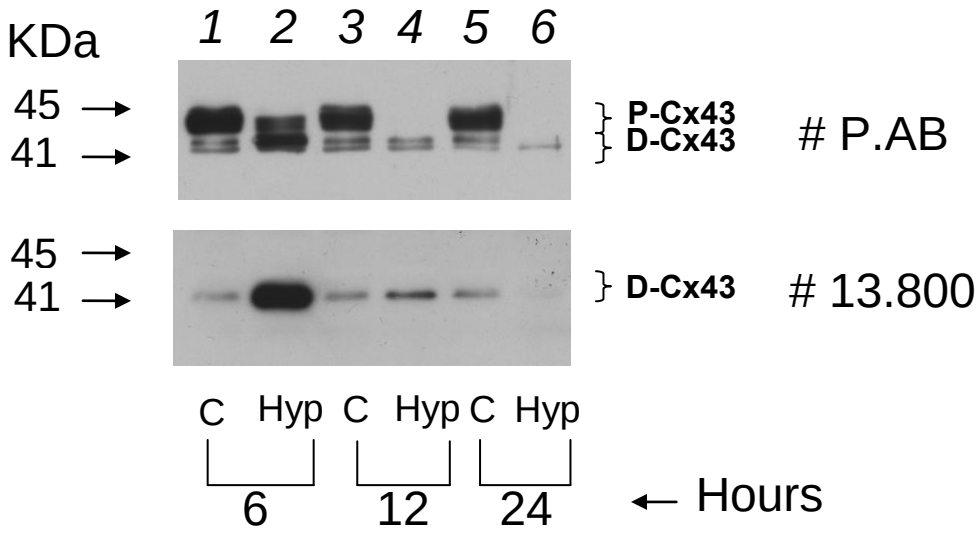
The y-axis shows either the [A] TUNEL-staining index or [B] LDH release in NCM incubated in DMEM or ischemic medium or simulated ischemia as indicated in the x-axis. NCM subjected to 6, 12 and 24 h of simulated ischemia showed significant increase in the LDH release and TUNEL-staining index compared to cardiomyocytes that were either incubated in either DMEM or ischemic medium alone. Brackets indicate comparisons between groups. (*$P<0.05$, ANOVA, n=6). Data are mean+SEM.


[A]
TUNEL-staining index
120
80
40
0
P<0.05
P<0.05
P<0.05
NS P>0.05
NS P>0.05
6
12
24
6
12
24
6
12
24
← Hours
DMEM
Ischemic medium
Ischemic medium + Hypoxia


[B]
LDH Release (Units/L)
600
400
200
0
P<0.05
P<0.05
P<0.05
NS P>0.05
NS P>0.05
6
12
24
6
12
24
6
12
24
←Hours
DMEM
Ischemic medium
Ischemic medium + Hypoxia

<u>Figure I-2:</u> In vitro simulated ischemia causes Cx43 dephosphorylation

Western blot showing the phosphorylation status of Cx43 in neonatal cardiomyocytes subjected to different time points of simulated ischemia. All lanes were loaded with 10 µg protein lysate from the indicated groups. The panel (a) was probed with (#P.AB) recognizing both phosphorylated (43-45 kDa) and dephosphorylated (41 kDa) Cx43 and the panel (b) with an antibody (#13-800) recognizing dephosphorylated (41 kDa) Cx43 as indicated. Increased relative levels of dephosphorylated (41 kDa) Cx43 were observed at 6 h followed by progressive degradation of Cx43 at 12 and 24 h of ischemia.



III.A-2. Cx43 expression and NCM vulnerability to ischemic injury

The first question asked was if a moderate increase in endogenous Cx43 levels, achieved by Ad-WT-Cx43 infection, would influence NCM vulnerability to simulated ischemia. Control cultures were infected with similar m.o.i (multiplicity of infection) of empty Ad-Vector. Cell injury and/or cell death were measured before (Fig.I-3) and after (Fig.I-4) subjecting the Ad-treated cultures to simulated ischemia.

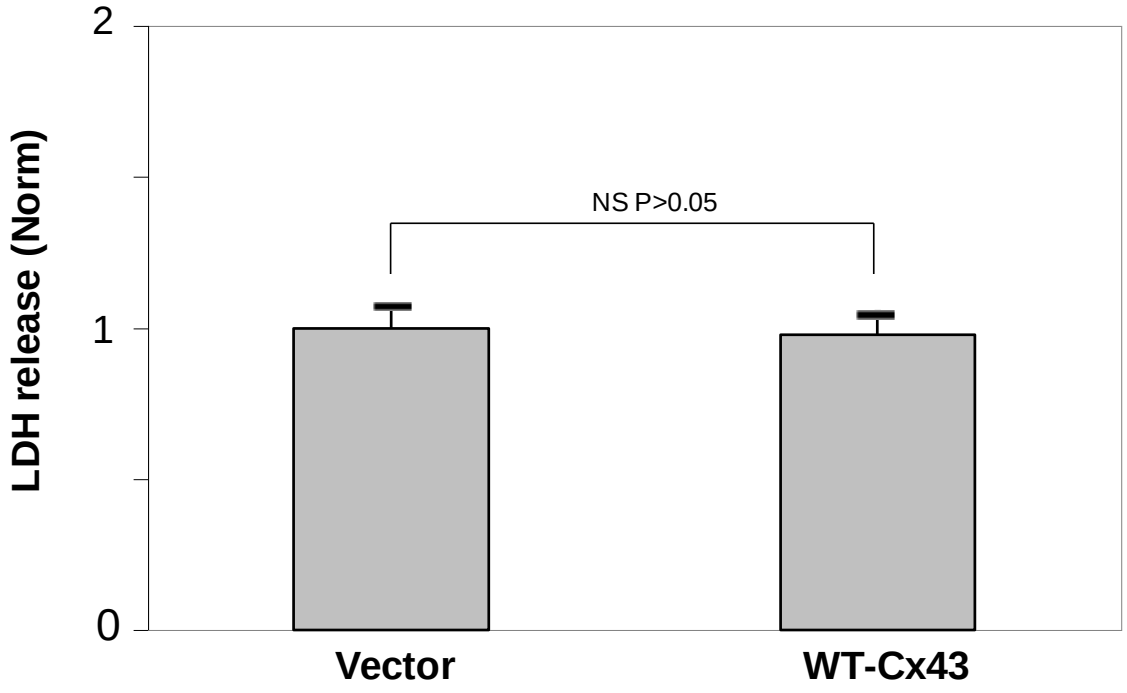
In the absence of simulated ischemia, LDH release levels were similar between Ad-Vector treated and Ad-WT-Cx43-treated NCM (Fig.I-3; $P>0.05$, $n=9$). However, in the presence of simulated ischemia (Fig.I-4A&B), significant attenuation of both TUNEL-staining (by 79%) and LDH release (by 25%) was observed in Ad-WT-Cx43 compared to Ad-Vector-treated cultures. These comparisons were 'extremely' significant, at $P<0.0001$, $n=6$.

Expression of Cx43 was assessed by immunofluorescence and western blotting. Representative data are shown in Fig.I-5. Ad-WT-Cx43-infected cells displayed increased intensity of immunostaining with P.AB, localizing appropriately to cell-cell contact sites. Western blotting before subjecting cells to simulated ischemia confirmed increased Cx43 expression in Ad-WT-Cx43 cultures: relative intensity of anti-Cx43 immunoreactive bands was significantly increased (~2.5-fold; $P<0.05$, $n=3$) (Fig.I-5C) compared to Ad-vector-infected cultures. After simulated ischemia, Ad-Cx43 cultures continued to accumulate more, predominantly phosphorylated (43-45 kDa) Cx43, while Ad-Vector cultures accumulated predominantly dephosphorylated 41 kDa Cx43 (Fig.I-

5B). Representative TUNEL-staining data (after simulated ischemia) are included in Fig.I-5(D).

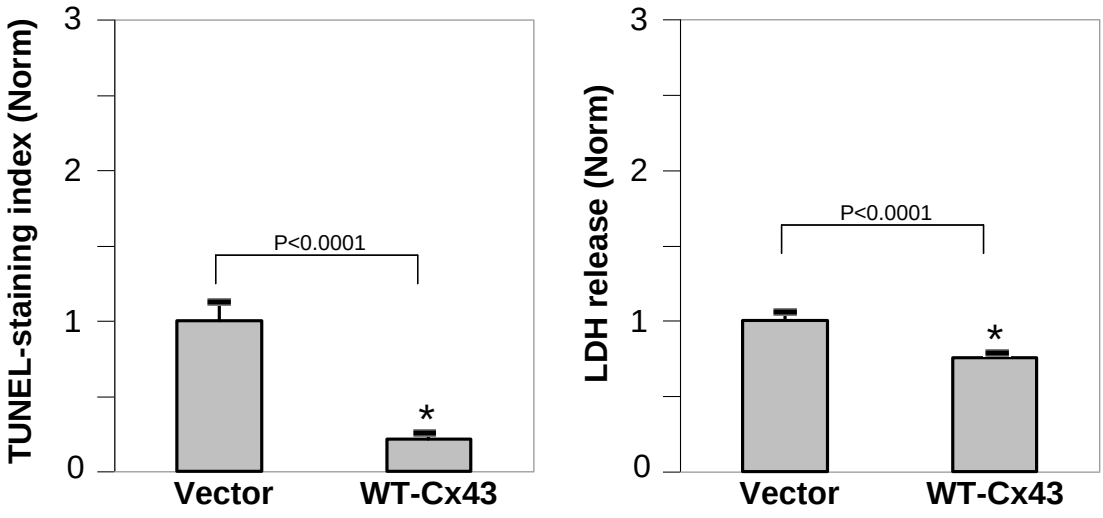
<u>Figure I-3:</u> Overexpression of WT-Cx43 has no effect on NCM LDH release in the absence of ischemia

The y-axis shows LDH release of NCM infected with Ad-Vector or Ad-WT-Cx43 and incubated in DMEM under normoxic conditions. In each set of experiments, values from control (Ad-Vector-only infected) cultures were arbitrarily set to 1-fold and all other values were normalized accordingly. The graph shows no difference in LDH release in NCM overexpressing WT-Cx43 compared to Ad-Vector infected cardiomyocytes. (P>0.05, student t test n=9). Data are mean+SEM.



<u>Figure I-4:</u> WT-Cx43 decreases the vulnerability of NCM to ischemic injury

The y-axis shows either the TUNEL-staining index [A] or LDH release [B] of NCM infected with Ad-Vector or Ad-WT-Cx43 and subjected to simulated ischemia for 6 h. In each set of experiments, LDH values from control (Ad-Vector-only infected) cultures were arbitrarily set to 1-fold and all other values were normalized accordingly. The graph shows a significant decrease in the LDH release and TUNEL-staining index in NCM overexpressing WT-Cx43 compared to Ad-Vector infected cardiomyocytes. (*P<0.0001, student t test n=6). Data are mean+SEM.



<u>Figure I-5:</u> Detection of Cx43 overexpression and TUNEL-staining by fluorescence

[A] Representative double-immunofluorescence images shows NCM before or after hypoxia, infected with Ad-Vector alone or Ad-WT-Cx43, and stained (green) with a polyclonal antibody for total Cx43 and counterstained with Hoechst (blue) to detect nuclei.

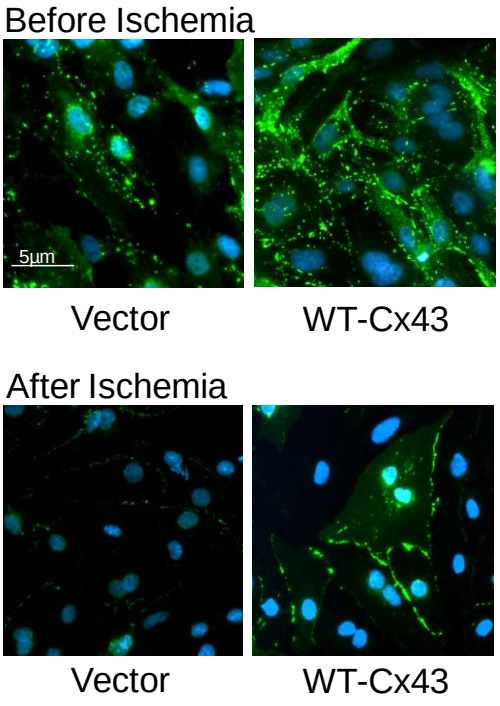
[B] Representative western blot of NCM before and after hypoxia, probed for total Cx43 in Ad-Vector and Ad-WT-Cx43 expressing cultures, as indicated. Both the panels show migration of extensively phosphorylated (43-45 kDa) and dephosphorylated (or minimally phosphorylated) Cx43. Staining for actin or GAPDH was used to demonstrate even loading.

[C] The y-axis shows the Cx43 expression in NCM infected with either Ad-Vector or Ad-WT-Cx43 as indicated in the x-axis. The graph shows significant increase in Cx43 expression in NCM infected with Ad-WT-Cx43 compared to Ad-Vector infected cardiomyocytes. ($P<0.05$, student t test $n=3$). Data are mean+SEM.

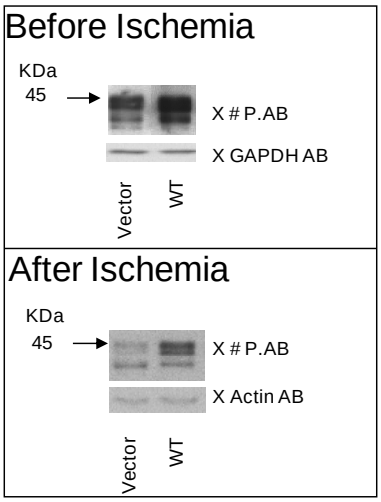
[D] Neonatal cardiomyocytes infected either with Ad-Vector (V) or Ad-Cx43CT (CT), as indicated, were incubated overnight with 'ischemic medium' under normoxic conditions, and then examined for the fraction of TUNEL-positive myocyte nuclei (TUNEL-staining Index). Shaded columns denote values from myocytes incubated in ischemic medium, labeled V^{isch} or CT^{isch}. These mild ischemic conditions did not elicit any significant increase in TUNEL staining between the Vector-infected myocyte groups. In contrast, a significant increase ($P<0.01$, $n=4$) in TUNEL staining was observed between the Cx43CT infected myocyte groups when subjected to mild ischemia. (NS denotes $P>0.05$)

[E] Representative dual-fluorescence images of NCM stained for TUNEL (red; to identify nuclei in dying cells) and Hoechst (blue; to identify all nuclei). Cultures were treated with Ad-Vector (Vector; upper panels) Ad-WT-Cx43 (lower panels) as indicated. Arrows indicate TUNEL positive nuclei.

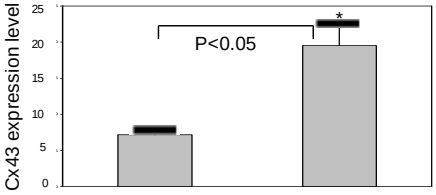
[A] Immunofluorescence images showing the expression of WT-Cx43



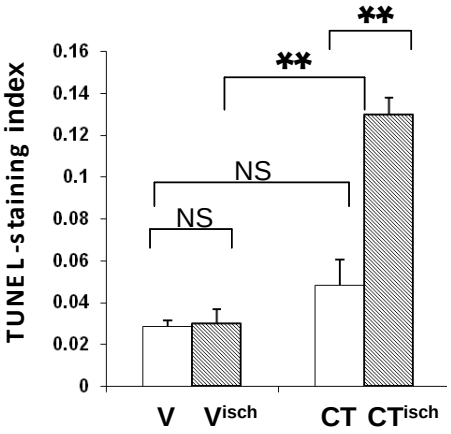
[B] Cx43 levels after gene transfer



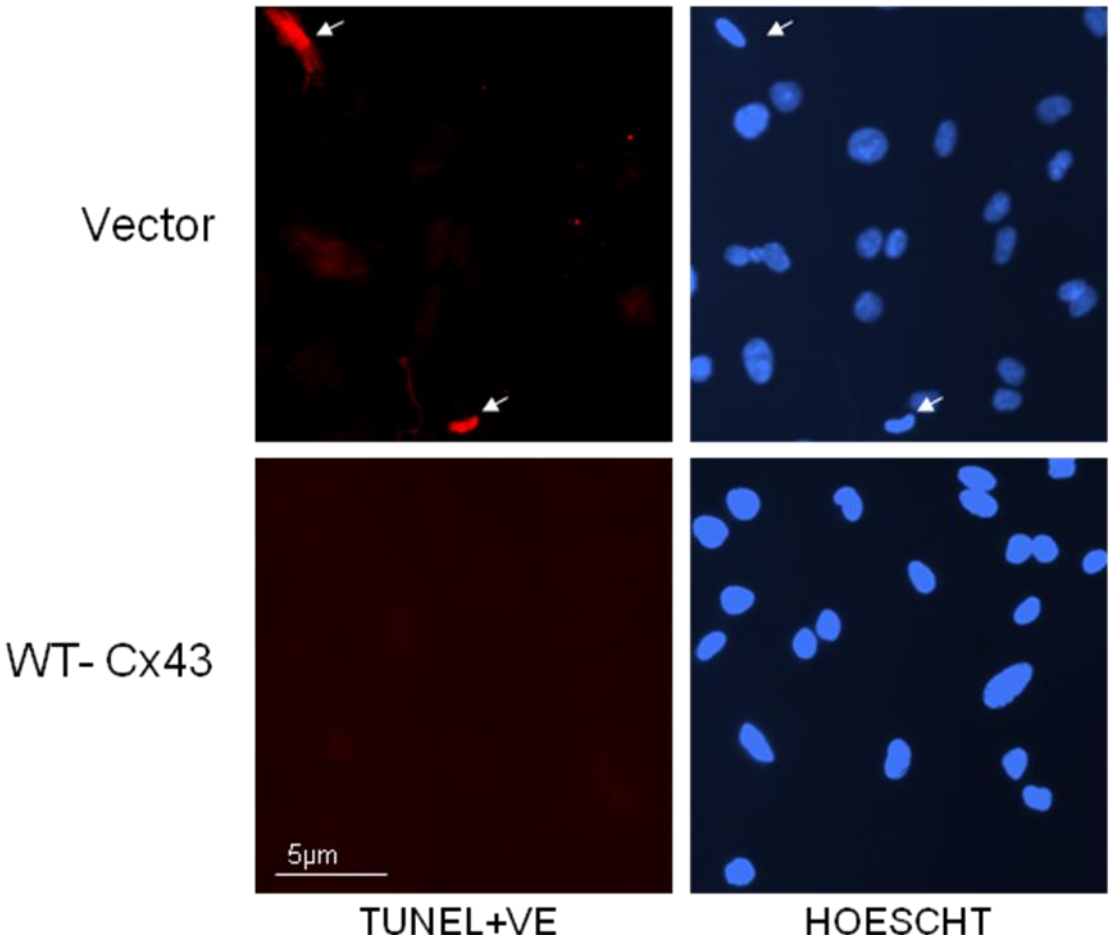
[C] Cx43 expression after gene transfer



[D] Effect of overexpression of Cx43CT on vulnerability of NCM to ischemic injury



[E] Representative images of TUNEL Staining



III.A-3. Effect of phosphorylation mutants of Cx43 on NCM vulnerability to ischemic injury

We expressed S262A-Cx43 (as Ad-S262A-Cx43), simulating constitutive lack of phosphorylation at that site, and S262D-Cx43 (as Ad-S262D-Cx43), simulating the charge effect of constitutive phosphorylation at that site, and then subjected NCM to simulated ischemia.

Before simulated ischemia, expression of S262A/D-Cx43 mutants had no significant effect on cellular injury (LDH release), compared to Ad-Vector treated cultures (Fig.I-6) ($P>0.05$). After simulated ischemia, expression of S262A-Cx43 promoted a significant increase in cell death (TUNEL-staining) and injury (LDH release), compared to Ad-Vector-treated cultures (Fig.I-7B). In contrast, cultures expressing S262D-Cx43 had significantly decreased TUNEL staining index compared to S262A-Cx43 expressing cultures; in fact their TUNEL staining index was not significantly different to that of Ad-Vector expressing cells. Also, cultures expressing S262D-Cx43 had significantly decreased LDH release compared to S262A-Cx43 expressing cultures. Nevertheless, LDH release in S262D-Cx43 cultures showed a statistically significant increase compared to Ad-Vector expressing cells ($P<0.001$).

Expression of Cx43 phosphorylation mutants was assessed by immunofluorescence (Figure.1-7D) and western blotting (Fig.I-7C). Increased anti-Cx43 immunostaining, and appropriate cell-cell contact localization were observed in cultures expressing the phosphorylation mutants compared to controls. Increased Cx43 levels were also shown in western blot data: both

S262A-Cx43 and S262D-Cx43 expressing cultures showed a ~3 fold increase in immunoreactive Cx43 compared to controls (Ad-Vector infected) cultures, before ischemia.

After simulated ischemia, S262A-Cx43 and S262D-Cx43 expressing cultures continued to express increased levels of immunoreactive Cx43 compared to Ad-Vector-treated cultures (Fig.I-7D). Cultures expressing S262A-Cx43 accumulated predominantly dephosphorylated 41 KDa Cx43, while cultures expressing S262D-Cx43 seemed to accumulate more 43-45 KDa Cx43 (Fig.I-7E). Western blot data shown in Fig.I-7E were obtained from pooling 3 samples per lane, and thus each lane represents the cumulative result from 3x plates. The whole experiment was repeated once in its entirety, with similar results.

We also examined the effect of Cx43 phosphorylation mutants on NCM death and injury after incubation only in ‘ischemic medium’, for 24 h. As seen in Fig.I-8, S262A-Cx43 expressing cultures had a significant 3.5-fold and 2.5-fold increase in TUNEL-staining nuclei, and LDH release, respectively, compared to Ad-Vector cultures. Cultures expressing S262D-Cx43 showed significantly less TUNEL-staining, and LDH release compared to S262A-cultures. There was no significant difference in TUNEL staining between S262D-expressing and Ad-Vector cultures. Nevertheless, expression of S262D-Cx43 was accompanied by 1.7-fold (significant) increase in LDH release after 24 h in ischemic medium compared to Ad-Vector cultures (Fig.I-8).

<u>Figure I-6:</u> Overexpression of S262A- or S262D- Cx43 in NCM has no effect on LDH release in the absence of ischemia

The y-axis shows normalized LDH release (fold-effect) of groups indicated in the x-axis. These include NCM infected with Ad-Vector; Ad-S262A-Cx43; Ad-S262D-Cx43 and incubated in DMEM under normoxic conditions. In each set of experiments, values from control (Ad-Vector-only infected) cultures were arbitrarily set to 1 and all other values were normalized accordingly. The graph shows no difference in the LDH release in NCM overexpressing S262A-Cx43 or S262D-Cx43 compared to Ad-Vector infected cardiomyocytes. (P>0.05, ANOVA, n=9). Data are mean+SEM.

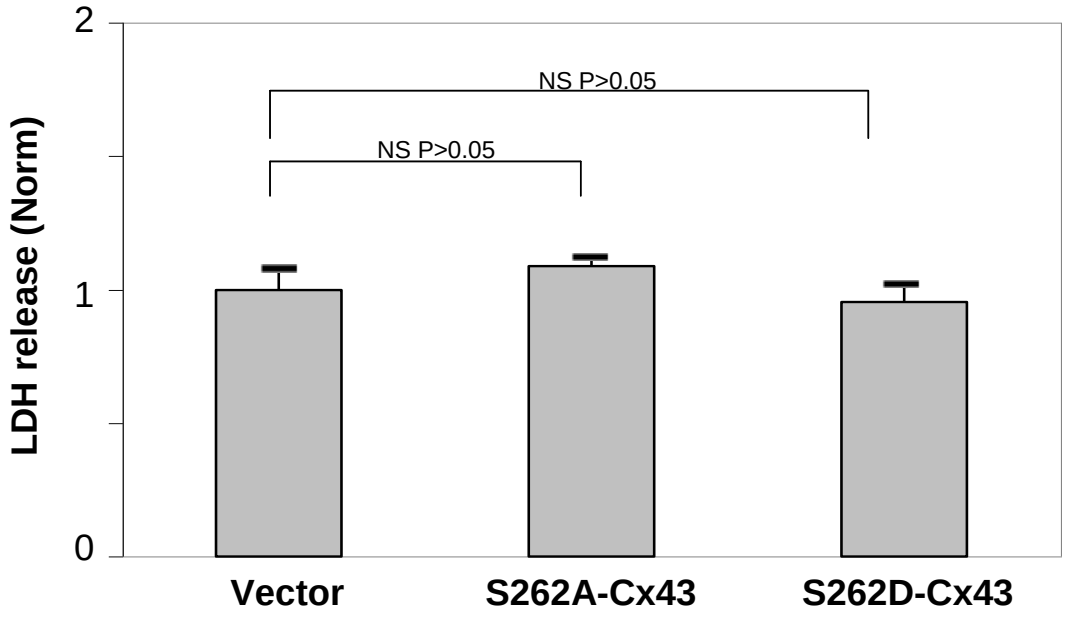


Figure I-7: S262A-Cx43 increases vulnerability of NCM to ischemic injury

[A] Representative dual-fluorescence images of NCM stained for TUNEL (red; to identify nuclei in dying cells) and Hoechst (blue; to identify all nuclei). Cultures were treated with Ad-Vector (Vector; upper panels) or Ad-S262A-Cx43 (middle panels) or Ad-S262D-Cx43 (lower panels) as indicated. Arrows indicate examples of TUNEL positive nuclei.

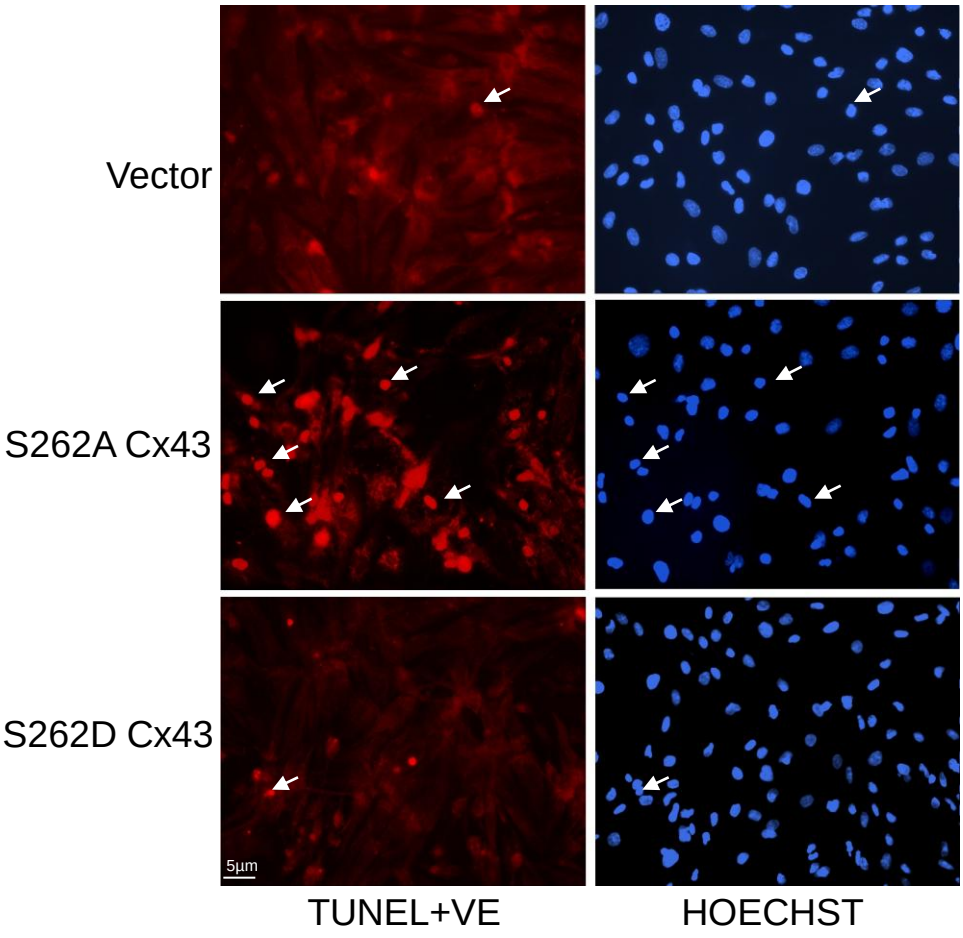
[B] The y-axis shows either the LDH release (upper panel) or TUNEL-staining index (lower panel) of NCM infected with Ad-Vector or Ad-S262A/D-Cx43 and incubated in ischemic medium+hypoxia for 6 h as indicated in the x-axis. In each set of experiments, values from control (Ad-Vector-only infected) cultures were arbitrarily set to 1-fold and all other values were normalized accordingly. The graph shows a significant increase in the LDH release and TUNEL-staining index in NCM overexpressing S262A-Cx43 compared to Ad-Vector infected cardiomyocytes. All comparisons for TUNEL-staining index are as follows. Vector versus S262A (*$P<0.05$, ANOVA, n=6), Vector versus S262D (ns $P>0.05$), S262A versus S262D (*$P<0.05$, ANOVA, n=6). Data are mean+SEM.

[C] The y-axis shows relative levels (fold-effect) of Cx43 expression in the NCM groups indicated in the x-axis. These include NCM infected with either Ad-Vector, Ad-S262A-Cx43, or Ad-S262D-Cx43 as indicated. The graph shows ~3 fold increase in Cx43 expression in NCM infected with Ad-S262A-Cx43, or Ad-S262D-Cx43 compared to Ad-Vector infected cardiomyocytes. (NS, student t test n=3). Data are mean+SEM.

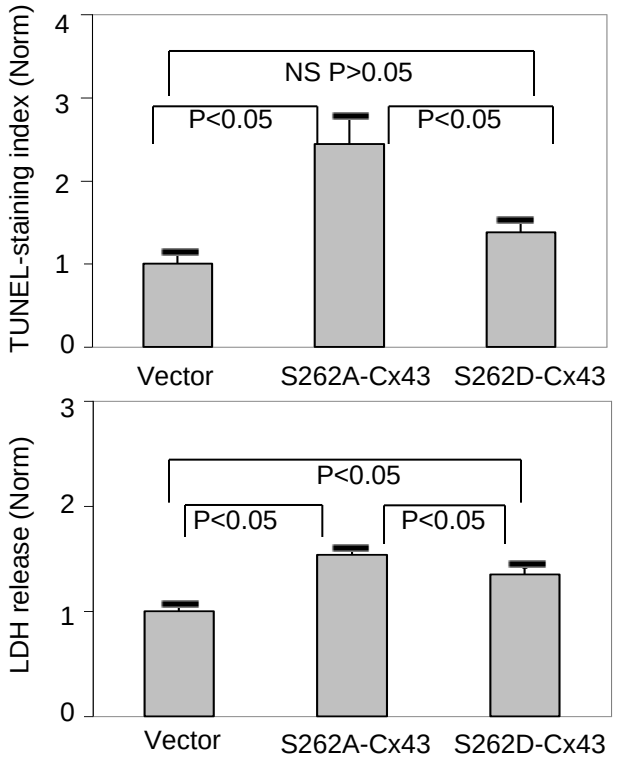
[D] Representative double-immunofluorescence images show NCM before or after hypoxia, infected with Ad-Vector alone, Ad-S262A-Cx43 alone or Ad-S262D-Cx43 alone, and stained (green) with a polyclonal antibody for total Cx43 and counterstained with Hoechst (blue) to detect nuclei.

[E] Representative western blot of NCM before and after hypoxia, probed for total Cx43 in Ad-Vector, Ad-S262A/S262D-expressing cultures, as indicated. Both the panels show migration of extensively phosphorylated (43-45 kDa) and dephosphorylated (or minimally phosphorylated) Cx43. Staining for actin or GAPDH was used to demonstrate even loading.

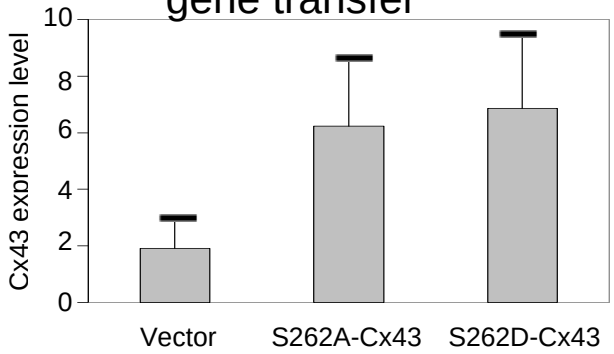
[A] Representative images of TUNEL-staining



[B] Effect of overexpression of S262A/D on vulnerability of NCM to ischemic injury

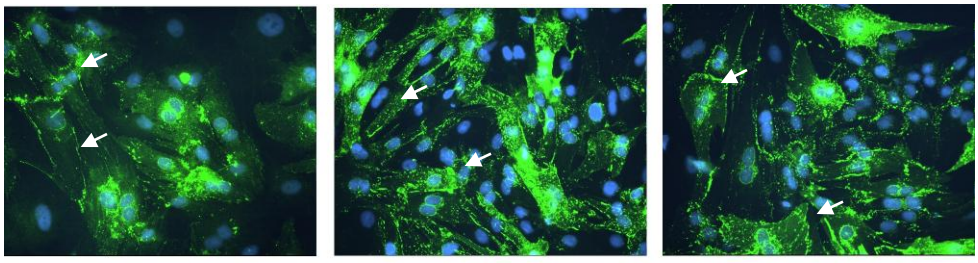


[C] Cx43 expression after gene transfer



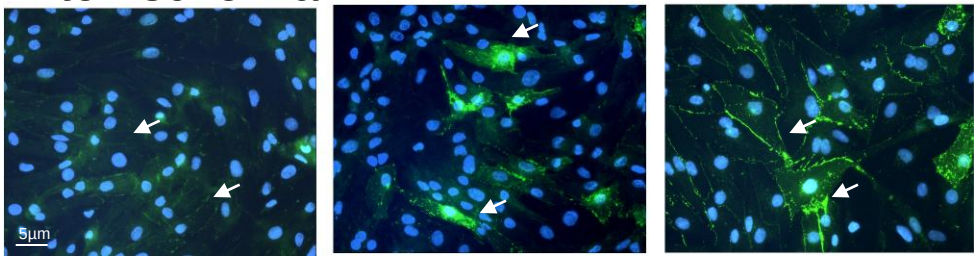
[D] Representative immunofluorescence images

Before Ischemia



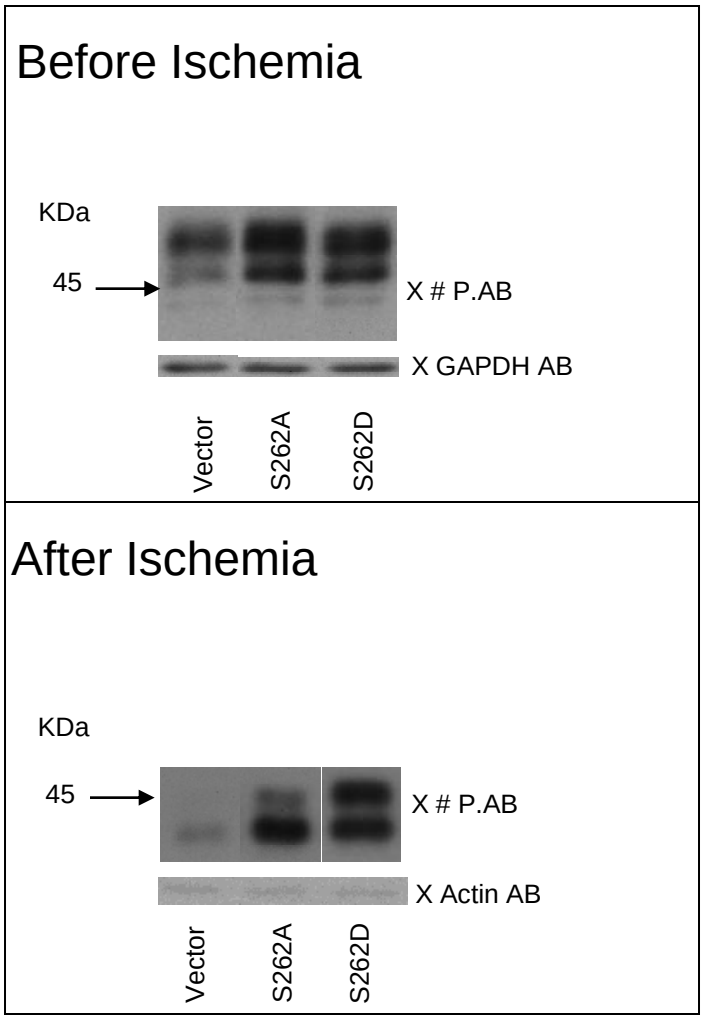
Vector S262A-Cx43 S262D-Cx43

After Ischemia



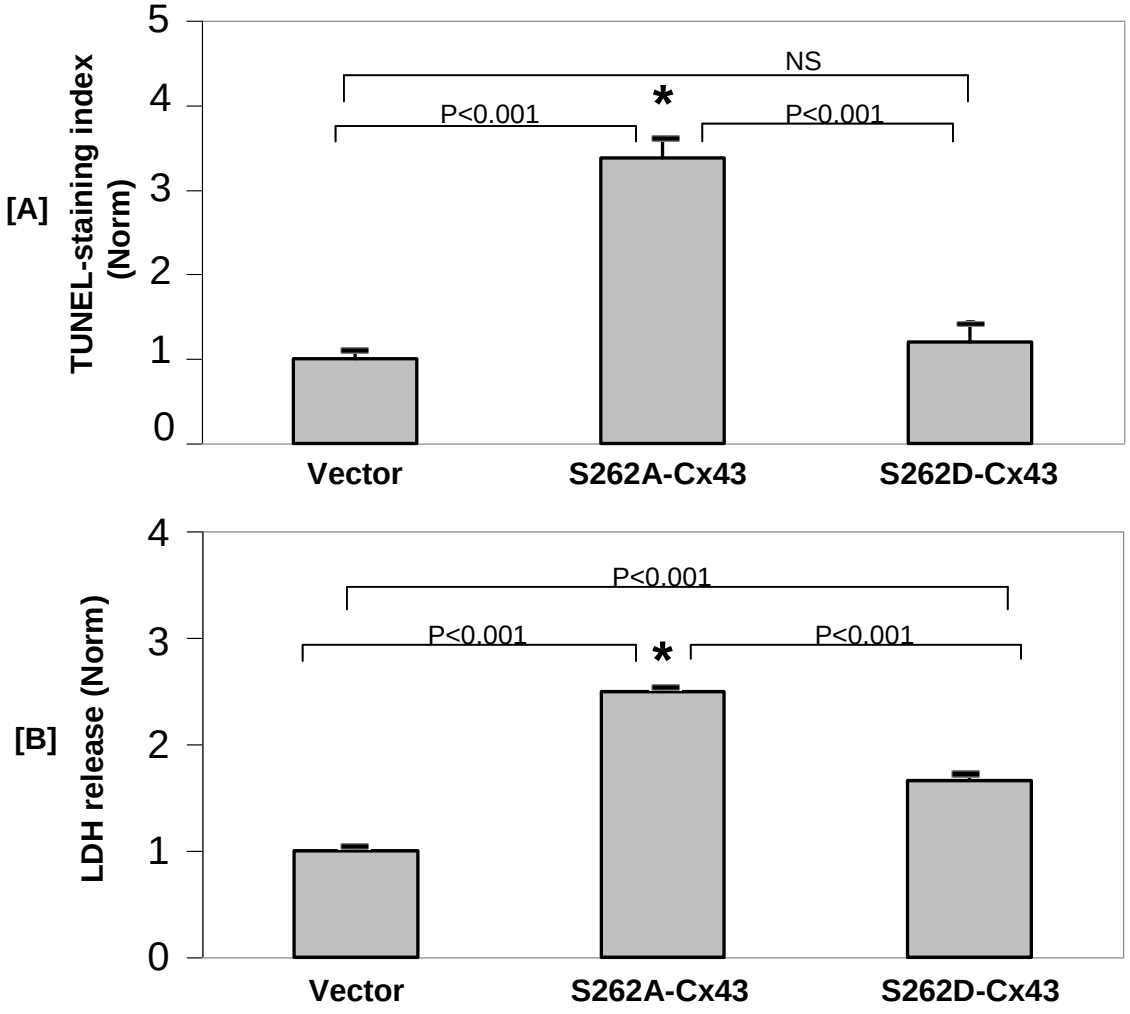
Vector S262A-Cx43 S262D-Cx43

[E] Cx43 levels after gene transfer



<u>Figure I-8</u>: S262A-Cx43 renders NCM vulnerable to ischemic medium exposure

The y-axis shows normalized (fold-effect), [A] TUNEL-staining index or [B] LDH release of the NCM groups shown in the x-axis. These include NCM infected with Ad-Vector; Ad-S262A-Cx43; Ad-S262D-Cx43 and incubated in ischemic medium for 24 h as indicated in the x-axis, under normoxic conditions. In each set of experiments, values from control (Ad-Vector-only infected) cultures were arbitrarily set to 1 and all other values were normalized accordingly. The graph shows a significant increase in the LDH release and TUNEL-staining index in NCM overexpressing S262A-Cx43 compared to Ad-Vector infected cardiomyocytes. Brackets indicate comparisons between groups (*P<0.001, ANOVA, n=6). Data are mean±SEM.



III.A-4. The effect of 'tagged' Cx43 (Cx43-HA) on NCM susceptibility to ischemic injury

Previous studies in our laboratory had resulted in the creation of an adenoviral vector carrying a construct expressing 'tagged' WT-Cx43, that is Cx43 containing an HA peptide (YPYDVPDYA) at its C-terminal end (X. Dang, E. Kardami, unpublished data). We hypothesized that the tagged WT-Cx43 would have similar properties as the non-tagged Cx43. If so, it would enable us to replace the Ad-WT-Cx43 vector with the Ad-Cx43-HA vector, allowing us to determine the localization of the introduced (compared to endogenous) Cx43 by using antibodies specific for the HA tag. Typical results are shown in Fig.I-9.

Treatment of NCM with Ad-Cx43-HA resulted in increased anti-Cx43 immunoreactivity (x P.AB), consistent with about 2.5-fold increase over Ad-Vector cultures. In the absence of ischemia, Cx43-HA was detected at cell-cell contact sites, as well as around the cell nucleus (Fig.I-9B). This was similar to the staining pattern obtained upon Ad-WT-Cx43 overexpression and suggested that the 'tagged' protein had similar localization properties as the non-tagged Cx43. By western blotting, most of anti-Cx43 immunoreactivity in the Cx43-HA cultures was at 43-45 kDa, suggesting that Cx43-HA existed mostly as a phosphorylated protein, just like WT-Cx43 (compare Fig.I-9A with Fig.I-5B). After ischemia, cultures expressing Cx43-HA continued to have more immunoreactive Cx43 compared to Ad-Vector cultures, but now most immunoreactivity was at 41 kDa (Fig.I-9A). This is different to our finding with

WT-Cx43-overexpressing cultures that retained mostly phosphorylated Cx43 even after simulated ischemia (Fig.I-5B). After ischemia, Cx43-HA was localized mainly around cell nuclei, unlike the pattern of Cx43 localization in Ad-Vector cultures (Fig.I-9B) or Ad-WT-Cx43 cultures (Fig.I-5A), where Cx43 was localized to cell-cell contact sites.

We also examined the effect of Ad-Cx43-HA expression on simulated ischemia-induced LDH release and TUNEL staining. As shown in Fig.I-10A&B, Ad-Cx43-HA expressing cultures had significantly increased TUNEL staining and LDH release, compared to Ad-Vector cultures, at 6, 9 and 12 h of simulated ischemia. At 24 h of simulated ischemia, values from Ad-Vector cultures were as high as those from Ad-Cx43-HA cultures, almost at 100%. Overall our data show that expression of Cx43-HA potentiates the deleterious effects of ischemia in cardiomyocytes.

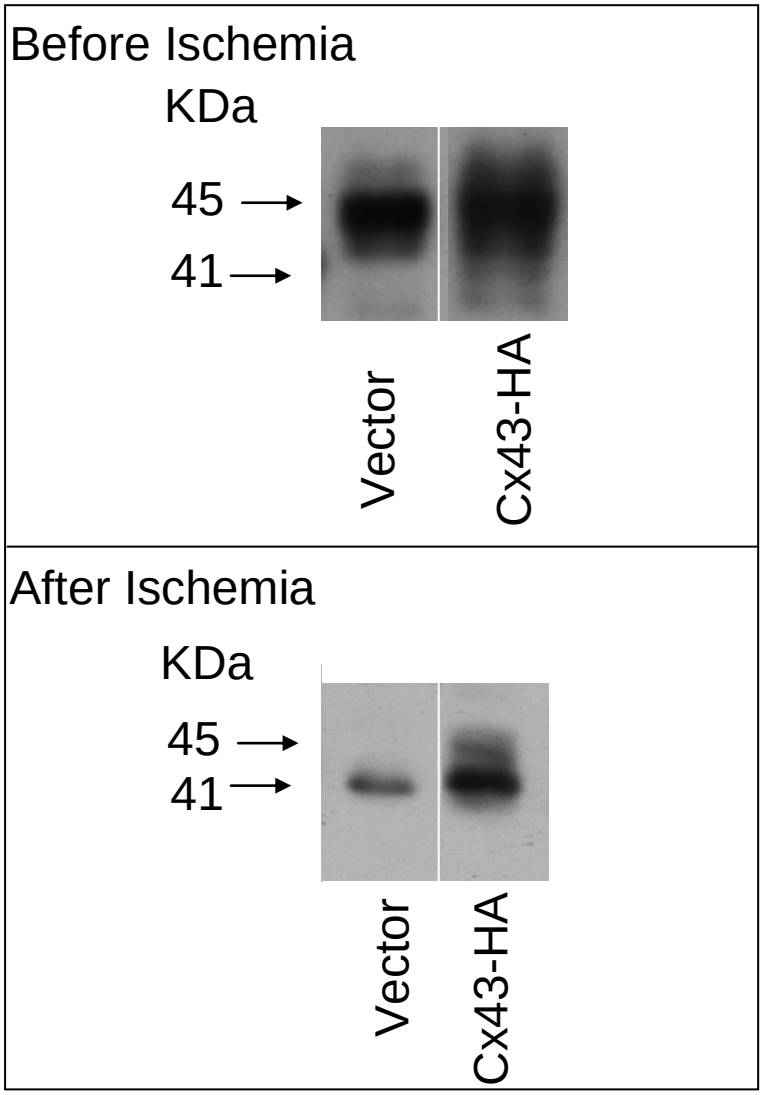
Figure I-9: Detection of Cx43-HA overexpression and TUNEL-staining by fluorescence

[A] Representative western blot of NCM before and after hypoxia, probed for total Cx43 in Ad-Vector and Ad-Cx43-HA expressing cultures, as indicated. The same amount of protein (5µg) was loaded per lane. Both the panels show migration of extensively phosphorylated (43-45 kDa) and dephosphorylated (or minimally phosphorylated) Cx43.

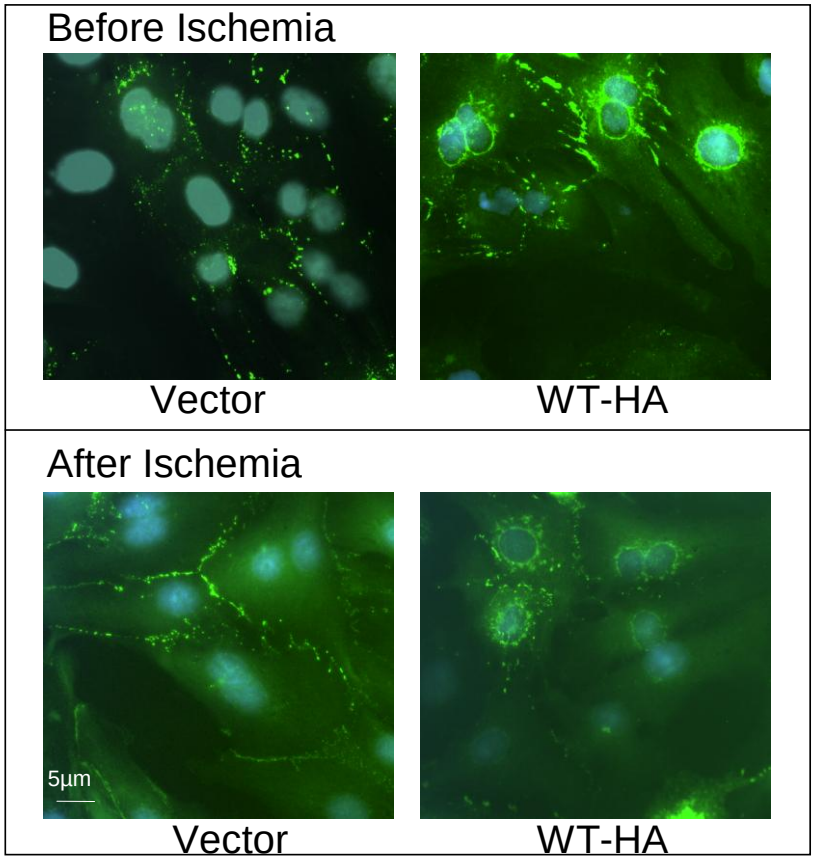
[B] Anti-Cx43 (green) and anti-HA (red) immunostaining of NCMs infected with (A, B) Ad-WT-Cx43, or (C-F) Ad-Cx43-HA and kept under (A,C,D,E) normoxic conditions, or (B, F) under simulated ischemia (ischemic medium plus 6 h hypoxia). Blue indicates nuclear staining. A and B are triple labeled for Cx43, HA (background staining) and nuclei. C, D and E show the same field stained, respectively, for Cx43, HA, or both. A complete overlap of anti-Cx43 and anti-HA staining results in yellow color in (E). Sizing bar in (A) and (C) corresponds to 50 µm.

[C] Representative dual-fluorescence images of NCM stained for TUNEL (red; to identify nuclei in dying cells) and Hoechst (blue; to identify all nuclei). Cultures were treated with Ad-Vector (Vector; upper panels) Ad-Cx43-HA (lower panels) as indicated. Arrows indicate TUNEL positive nuclei.

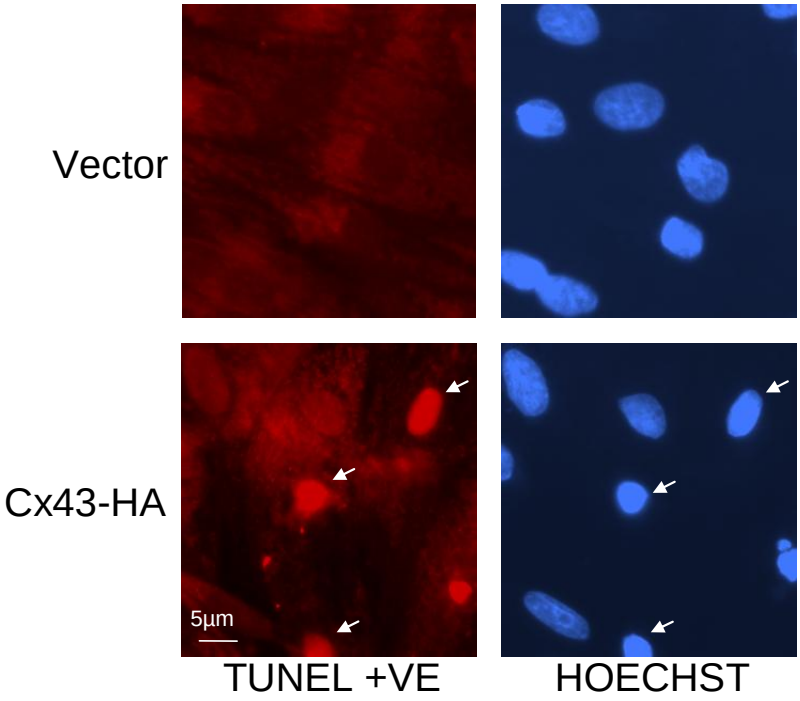
[A] Cx43-HA levels after gene transfer



[B] Representative immunofluorescence images

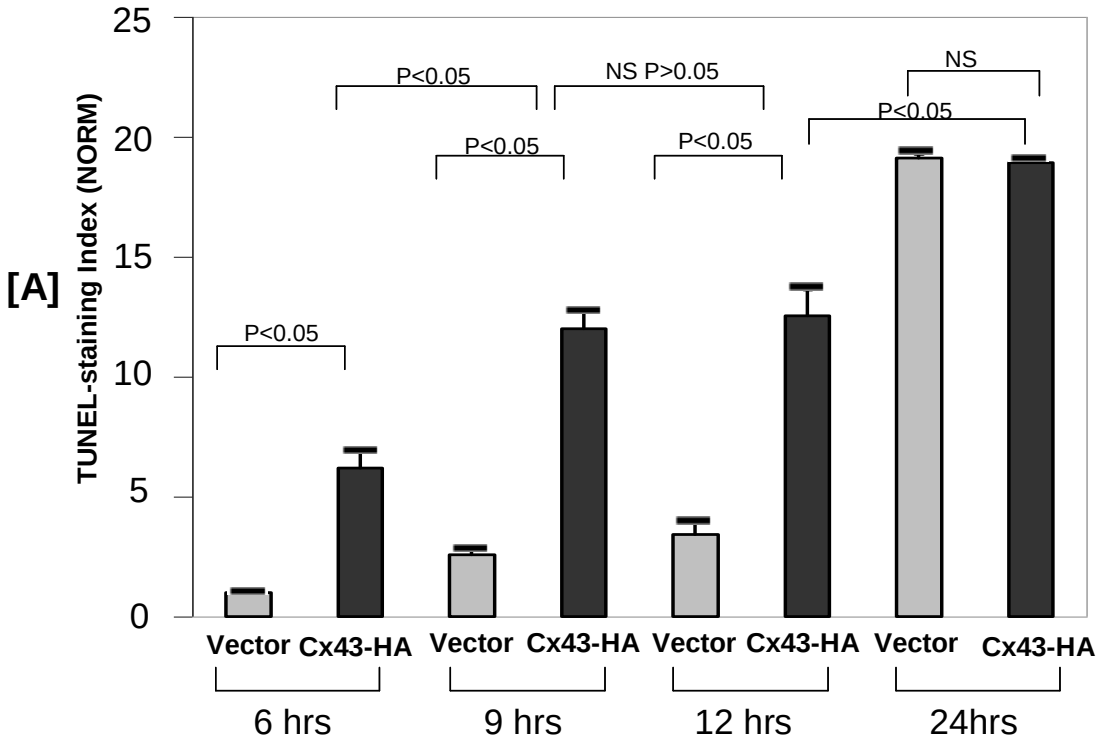


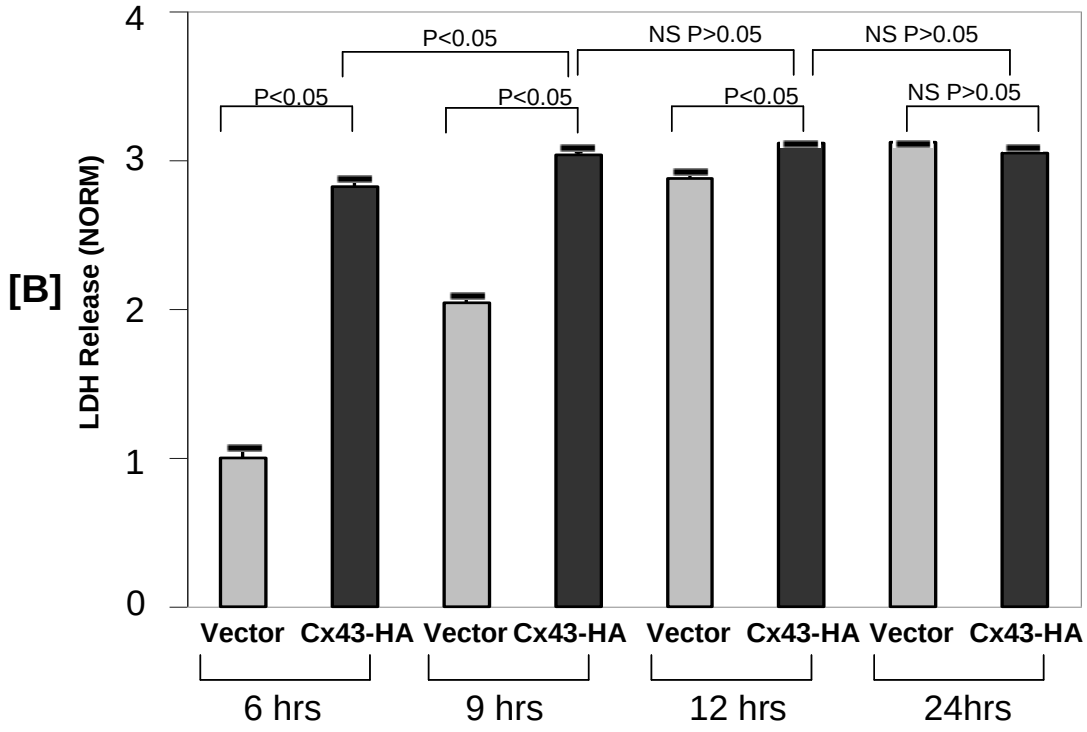
[C] Representative images of TUNEL Staining



<u>Figure I-10A&B:</u> Effect of Cx43-HA on NCM vulnerability to ischemic injury

The y-axis shows either TUNEL-staining index [A] or LDH release [B] of NCM infected with Ad-Vector or Ad-Cx43-HA and simulated ischemia for 6, 9, 12 or 24 h as indicated in the x-axis. In each set of experiments (Fig.I-9A), values from control (Ad-Vector-only infected) cultures were arbitrarily set to 1-fold and all other values were normalized accordingly. The graph shows a significant increase in the LDH release (*P<0.05, ANOVA, n=12) and TUNEL-staining index (*P<0.05, ANOVA, n=18) in NCM overexpressing Cx43-HA compared to Ad-Vector infected cardiomyocytes at all time points. Data are mean+SEM.


[A]
TUNEL-staining Index (NORM)
25
20
15
10
5
0
P<0.05
NS P>0.05
NS
P<0.05
P<0.05
P<0.05
P<0.05
Vector
Cx43-HA
Vector
Cx43-HA
Vector
Cx43-HA
Vector
Cx43-HA
6 hrs
9 hrs
12 hrs
24hrs


[B]
LDH Release (NORM)
4
3
2
1
0
P<0.05
NS P>0.05
NS P>0.05
P<0.05
P<0.05
P<0.05
NS P>0.05
Vector
Cx43-HA
Vector
Cx43-HA
Vector
Cx43-HA
Vector
Cx43-HA
6 hrs
9 hrs
12 hrs
24hrs

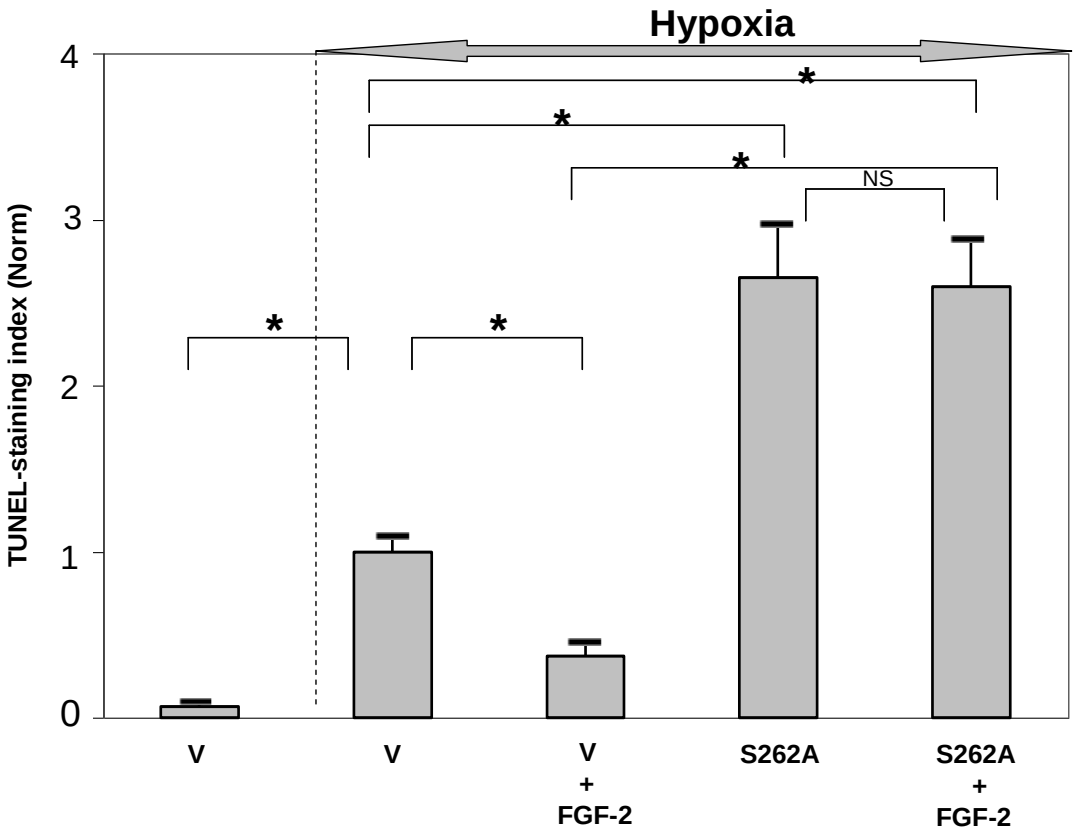
III.A-5. Effect of S262A-Cx43 expression on FGF-2-induced cytoprotection

FGF-2 promotes acute cardioprotection as well as Cx43 phosphorylation at S262 {Doble et al., 1996; Jiang et al., 2002}. As we have shown {Srisakuldee et al., 2009}, cardioprotective treatment such as FGF-2 administration is associated with Cx43 phosphorylation at S262. We used overexpression of S262A-Cx43 to test if Cx43 phosphorylation at S262 is required for the cytoprotective effect of FGF-2. Results are shown in Fig.I-11.

As expected, simulated ischemia caused significant increases in TUNEL staining in control, non-FGF-2 treated cultures. Pre-treatment with FGF-2 (10 ng/ml) significantly decreased ischemia-induced TUNEL-staining, a finding confirming its cytoprotective properties. The protective effect of FGF-2 was no longer evident in the presence of S262A-Cx43 expression. Relative incidence of cell death (after simulated ischemia) was similar between untreated S262A-Cx43-expressing cultures, and FGF-2-pretreated S262A-Cx43 expressing cultures ($P>0.05$). These results indicate that the protective effects of FGF-2 are mediated by Cx43 phosphorylation at S262.

Figure I-11: S262A-Cx43 expression prevents FGF-2 cytoprotection

Fold change in cell death [TUNEL staining (norm)] in cultures infected with Ad-Vector (V) or Ad-S262A-Cx43 (S262A), ±FGF-2 pre-treatment, and subjected to simulated ischemia (double-pointed arrows), as indicated. Values from control (Ad-Vector-only infected) cultures were arbitrarily set to 1-fold and all other values were normalized accordingly. *P<0.05, n=6. Ad-Vector cultures subjected to simulated ischemia displayed 23% TUNEL-positive nuclei.



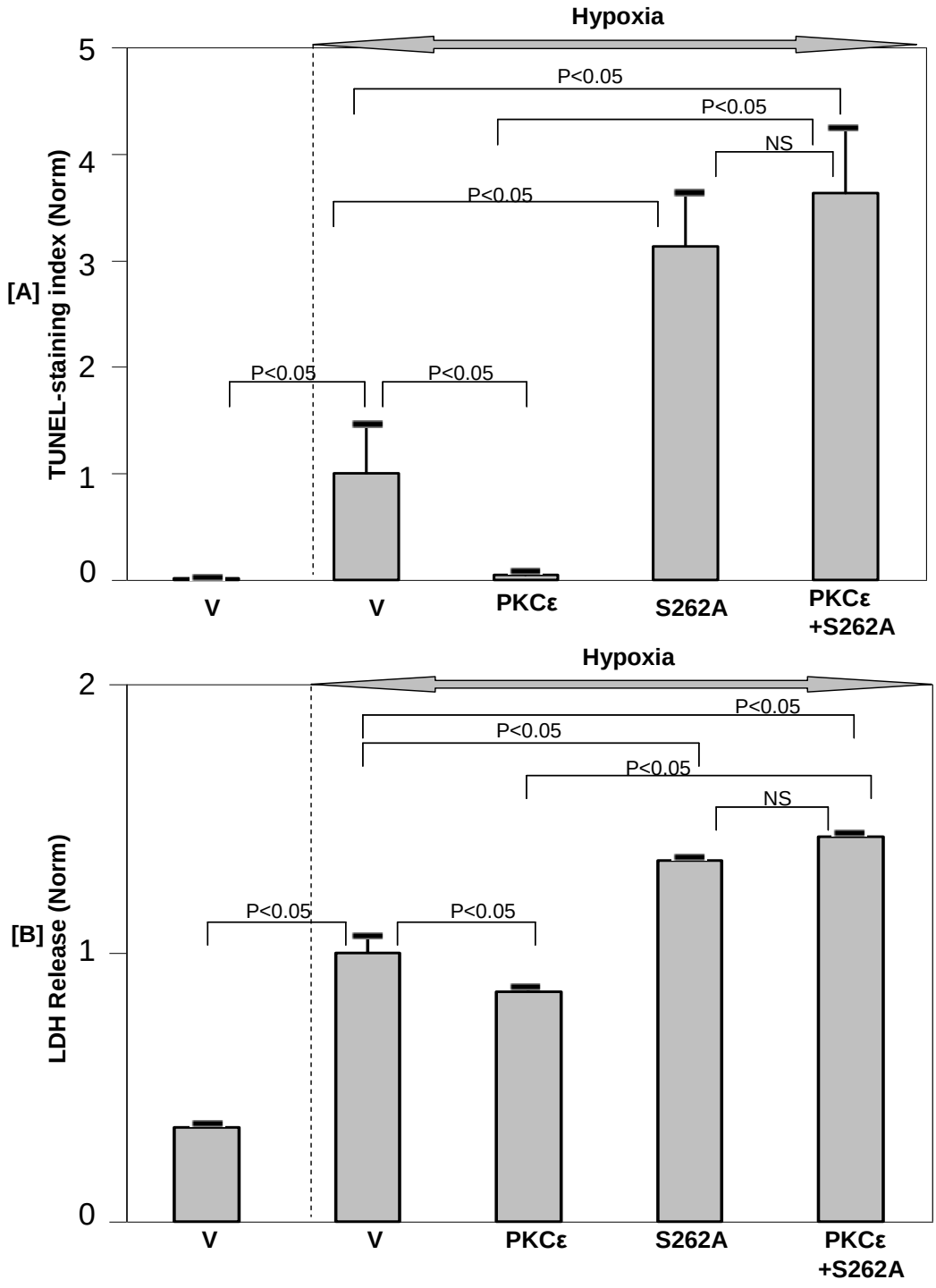
III.A-6. Effect of S262A-Cx43 expression on PKCε-induced cytoprotection

PKCε is a central mediator of cardio-(cyto-) protection by a variety of treatments including FGF-2 {Jiang et al., 2002; Ping et al., 2002}, and is also responsible for Cx43 phosphorylation at S262 {Doble et al., 2000}. Overexpression of PKCε is by itself cardioprotective {Ping et al., 1999}. To determine if the protective effects of PKCε were dependent on downstream Cx43 phosphorylation, we tested the ability of PKCε overexpression to be cytoprotective in the presence of S262A-Cx43. Results (fold-effect on TUNEL staining, or LDH release) are shown in Fig.I-12A and Fig.I-12B respectively.

NCM were infected with Ad-PKCε (50 m.o.i). Overexpression of PKCε alone prevented ischemia-induced cell death significantly compared to Ad-Vector cultures. Overexpression of S262A-Cx43 elicited, as expected from a previous figure (Fig.I-7B), significant increases in TUNEL staining (Fig.I-12A) and LDH release (Fig.I-12B) compared to Ad-vector-only expressing cells, after simulated ischemia. The deleterious effect of S262A-Cx43 expression on both TUNEL staining and LDH release was maintained even in the presence of PKCε overexpression. In other words, in the presence of S262A-Cx43, PKCε overexpressing NCM were unable to develop a protective response against ischemic injury. Overexpression of PKCε was maintained even after simulated ischemia, as seen by immunofluorescence (Fig.I-13). This study confirms that FGF-2 and overexpression of its downstream target PKCε, both elicit protective response in NCM subjected to simulated ischemia and this protective response requires phosphorylation of Cx43 at S262.

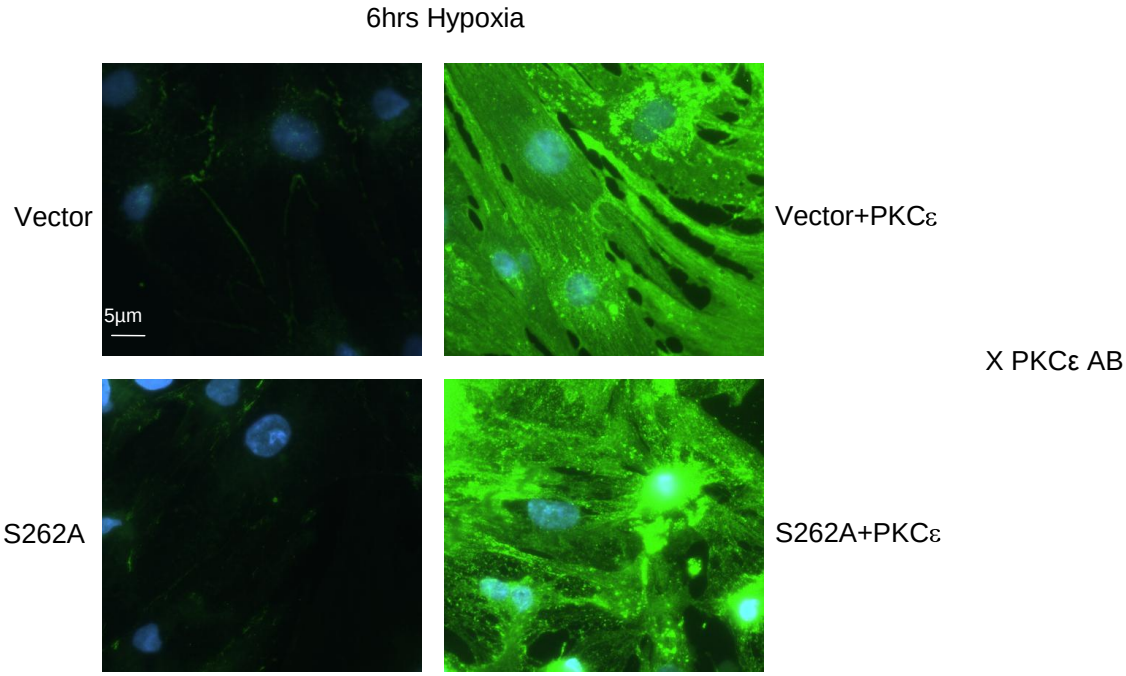
Figure I-12A&B: Effect of S262A-Cx43 on PKCε-induced cytoprotection

The y axis shows normalized (fold-effect): [A] TUNEL staining index and [B] LDH release as a function of simulated ischemia, in the groups shown in the x-axis. These groups include: cultures infected, as indicated, with Ad-Vector (V), Ad-PKCε (PKCε), Ad-S262A-Cx43 (S262A), or Ad-PKCε and Ad-S262A-Cx43 (PKCε+S262A). Double pointed-arrows point to values from groups subjected to simulated ischemia. Brackets indicate comparisons between groups (n=6, 2-way ANOVA, *P<0.05). Data were normalized giving a value of one to measurements from Ad-Vector cultures subjected to simulated ischemia, and are shown as mean ± SEM. The fraction of TUNEL-positive nuclei after simulated ischemia in Ad-Vector cultures was at 20-25% of the total; corresponding arbitrary LDH O.D. units (490nm) varied between 0.9 and 1.2.


[A]
TUNEL-staining index (Norm)
Hypoxia
P<0.05
P<0.05
NS
P<0.05
P<0.05
P<0.05
V
V
PKCε
S262A
PKCε
+S262A
[B]
LDH Release (Norm)
Hypoxia
P<0.05
P<0.05
P<0.05
NS
P<0.05
P<0.05
V
V
PKCε
S262A
PKCε
+S262A

<u>Figure I-13</u>: Immunofluorescence detection of PKCε in NCM

Representative double-immunofluorescence images of NCM infected with Ad-Vector alone, Ad-Vector, Ad-S262A-Cx43, Ad-PKCε or Ad-S262A-Cx43+Ad-PKCε, stained for PKC antibody (green) and Hoechst (Blue-nuclear). The images in the right panel indicate overexpression of PKCε in the NCM compared to vector control.



III.A-7. Effect of S262A-Cx43 on ischemic injury of cardiomyocytes in response to ischemic preconditioning

Ischemic preconditioning (IP) is a potent cardioprotective treatment, which, like FGF-2 treatment, is mediated by PKCε {Ping et al., 2002}. As we have shown {Srisakuldee et al., 2009}, cardioprotective treatments such as IP are associated with Cx43 phosphorylation at S262. We used overexpression of S262A-Cx43 to test if Cx43 phosphorylation at S262 is required for the cytoprotective effect of IP.

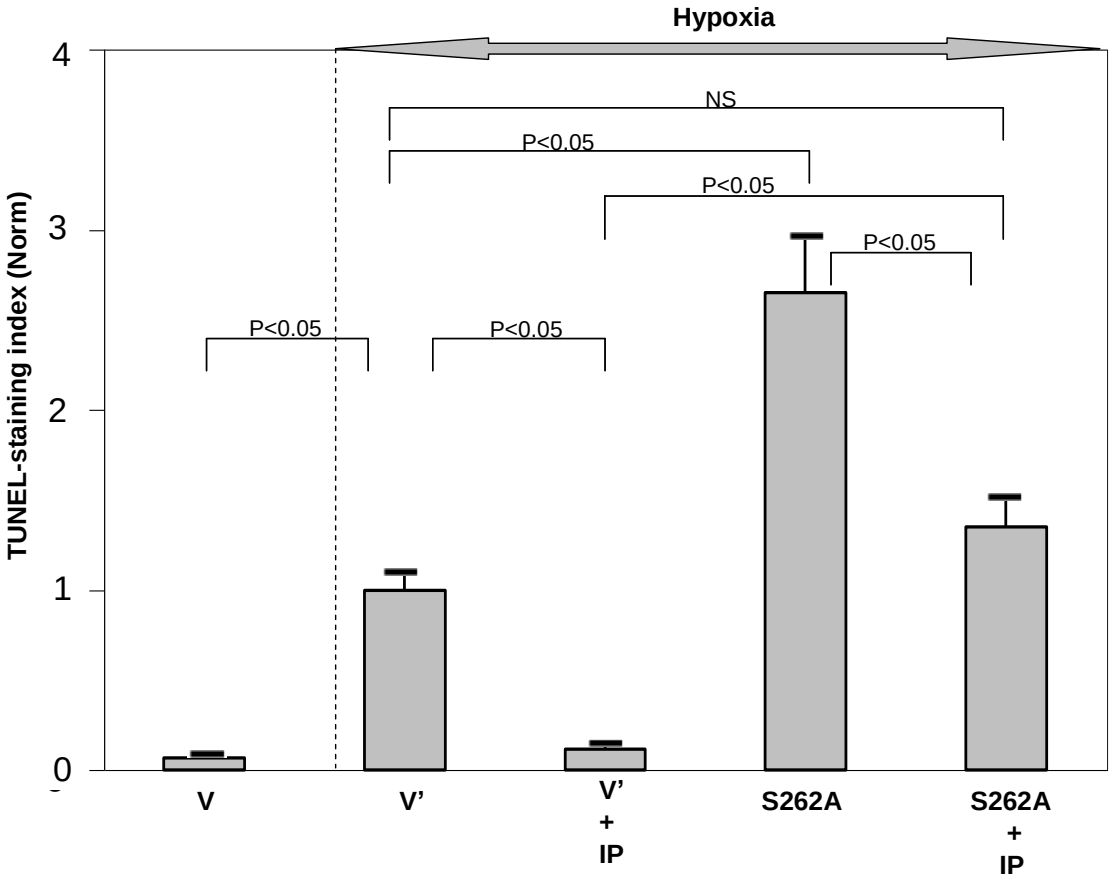
In vitro IP was induced following procedures described in {Sundset et al., 2007}. NCM, infected either with Ad-Vector or with Ad-S262-Cx43, were subjected to 30 min simulated ischemia, followed by 30 min of reperfusion, i.e. re-incubation in non-ischemic, oxygenated buffer; these will be referred to as 'IP-treated' NCM. Control 'untreated' NCM were maintained in oxygenated medium. The 'IP-treated' and 'untreated' NCM groups were subjected to simulated ischemia, as in the preceding studies. Results are shown in Fig.I-14.

As expected, simulated ischemia caused significant increases in TUNEL staining in 'untreated' cultures, compared to their pre-ischemic counterparts. In contrast, simulated ischemia did not increase TUNEL staining of 'IP-treated' cultures compared to non-ischemic cultures ($P>0.05$, ns). Overall, incidence of TUNEL staining decreased by 78% in 'IP-treated' compared to untreated cultures ($^*P<0.05$). Thus *in vitro* IP treatment was indeed cytoprotective. As expected, expression of S262A-Cx43 potentiated the ischemia-induced cell

death: TUNEL staining in S262A-Cx43 expressing NCM was 2.6-fold higher compared to Ad-Vector-only expressing NCM ($P<0.05$); and 44-fold higher than non-ischemic cells. IP treatment of S262A-Cx43 expressing cultures reduced the incidence of TUNEL staining significantly (by about 50%), compared to 'untreated', S262A-Cx43 expressing cultures, indicating that a degree of IP-cytoprotection could be exerted independently of S262A-Cx43 expression. At the same time, compared to 'IP-treated'-Vector-infected cells, incidence of cell death was still significantly higher (by 11-fold, $P<0.05$) in the 'IP-treated'-S262A-Cx43 expressing cells. Taken together our data indicate that the protective effects of IP are partially dependent on Cx43 phosphorylation at S262.

<u>Figure I-14:</u> Effect of S262A-Cx43 on IPC-induced cytoprotection.

The y-axis shows NCM normalized TUNEL-staining (fold-effect) as a function of simulated ischemia. The various NCM groups are indicated in the x-axis. These groups include NCM infected with Ad-Vector (±IPC) or Ad-S262A-Cx43 (±IPC). In each set of experiments, values from control (Ad-Vector-only infected) cultures after simulated ischemia were arbitrarily set to 1.0 and all other values were normalized accordingly. V and V' denote groups infected with Ad-Vector, before and after simulated ischemia, respectively. IPC or S262A denote groups subjected to ischemic preconditioning, or expressing S262A-Cx43, respectively. Brackets indicate comparisons between groups; two-way ANOVA was used, n=16; differences were considered significant (*) if P<0.05. Data are shown as mean+SEM.



III.A.8: Summary of findings

In this chapter it was shown that:

1. Increased expression of WT-Cx43 increases the resistance of NCM against ischemic injury
2. Unlike WT-Cx43, HA-tagged Cx43 has toxic effects on NCM and cannot be used as a substitute
3. Preventing Cx43 phosphorylation at S262 renders NCM more vulnerable to ischemic injury
4. Preventing phosphorylation of Cx43 at S262 also prevents the FGF-2 and/or PKCε induced cytoprotection
5. Cytoprotection by ischemic preconditioning is partially dependent on phosphorylation of Cx43 at S262

III.B. CX43 AND REGULATION OF DNA SYNTHESIS

Studies presented in this chapter address aspects of the mechanism by which Cx43 inhibits DNA synthesis. Results shown in this section have been published in {Dang et al., 2006}, and are reproduced here with permission (see Appendix).

In the first section, previous work {Dang et al., 2003; 2006; Doble et al., 2004} has been extended to address specific structure-function questions. The S262 site on the C-terminal of Cx43 (Cx43CT) has been examined to determine whether it retains the ability to become phosphorylated in the absence of the channel-forming domain of the molecule, and if so, whether it would regulate the ability of Cx43CT to inhibit DNA synthesis. These experiments were done using HEK293 cells which are deficient in endogenous Cx43 expression {Dang et al., 2006}.

In the second section, the relationship between TGFβ1 signal transduction and Cx43-induced inhibition of DNA synthesis was investigated. Recently it was shown that Cx43 potentiates TGFβ1 signaling by binding to microtubules and releasing Smad2 (a downstream signaling molecule that gets activated and phosphorylated upon activation of the TGFβ1 receptor) {Dai et al., 2007}. We investigated if Cx43 mediated inhibition of DNA synthesis requires downstream activation of TGFβ1 signaling pathway, such as its plasma membrane receptors or its downstream signals (Smad2); and the effect of TGFβ1 on the mitogen-induced Cx43 phosphorylation at S262.

III.B-1. Structure-Function Studies: Role of phosphorylation at S262, and channel-forming ability, on Cx43-induced DNA synthesis

Cx43 deficient HEK293 cells were used for this study. It was first assessed whether Cx43 introduced into these cells by transient gene transfer would become phosphorylated at S262 in response to PKC activation by PMA. Lysates from HEK293 cells ectopically expressing Cx43 and stimulated (or not) with PMA, were analyzed by western blotting for total Cx43, as well as P-S262-Cx43. As shown in Fig.II-1A, staining for total Cx43 detects 41–47 KDa bands in unstimulated cells; PMA treatment eliminated bands corresponding to the unphosphorylated or minimally phosphorylated Cx43 (41–42 KDa), promoting an increase in bands corresponding to more extensively phosphorylated Cx43, at 44–47 KDa. The anti-P-S262 antibodies detect faint bands in unstimulated cells, corresponding in electrophoretic mobility to the more extensively phosphorylated Cx43. These bands increased in intensity upon PMA stimulation. Cx43 does therefore become phosphorylated at the PKC target site S262 within the HEK293 cell environment in response to PKC (PMA) stimulation.

To examine if S262 phosphorylation can occur in the absence of the channel forming portion of the molecule, Cx43CT (expected size at 15–17 KDa) was transiently expressed and probed with anti-Cx43 and anti-P-S262-Cx43 antibodies. As seen in Fig.II-1B, antibodies to Cx43 detected immunoreactive bands at around 15–19 KDa in unstimulated cells, and at around 18–19 KDa in stimulated cells, as would be expected by a

phosphorylation-induced shift in apparent electrophoretic motility. In agreement, antibodies to P-S262-Cx43 detected bands at around 18–19 KDa; these bands are faint in unstimulated cells, becoming strong in PMA-stimulated cells. It was concluded that S262 becomes phosphorylated on the ‘free’ Cx43CT, and that, as in the intact molecule, S262 phosphorylation is encountered in bands corresponding to the more extensively phosphorylated Cx43CT.

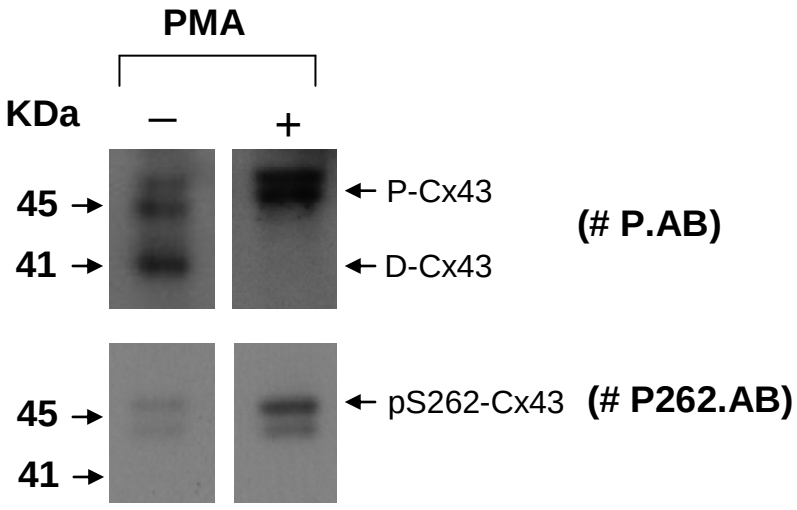
Lysates from cells expressing Cx43CT (S262A) were also examined by western blotting as above. As shown in Fig.II-1B, probing with anti-Cx43 antibodies detected immunoreactive bands with a similar migration pattern as those from cell expressing Cx43CT; a shift in motility towards slower migrating species in response to PMA stimulation was also observed, indicating that although Cx43CT cannot be phosphorylated at S262, phosphorylations at sites other than S262 do occur in the ‘free’ C-tail and can produce slower migrating bands. As expected, probing an identical blot with the anti-P-S262 antibodies did not detect any bands, irrespectively of PMA stimulation, confirming the specificity of the anti-PS262-Cx43 antibodies (Fig.II-1B).

Figure II-1A&B: Cx43 and Cx43CT become phosphorylated on S262

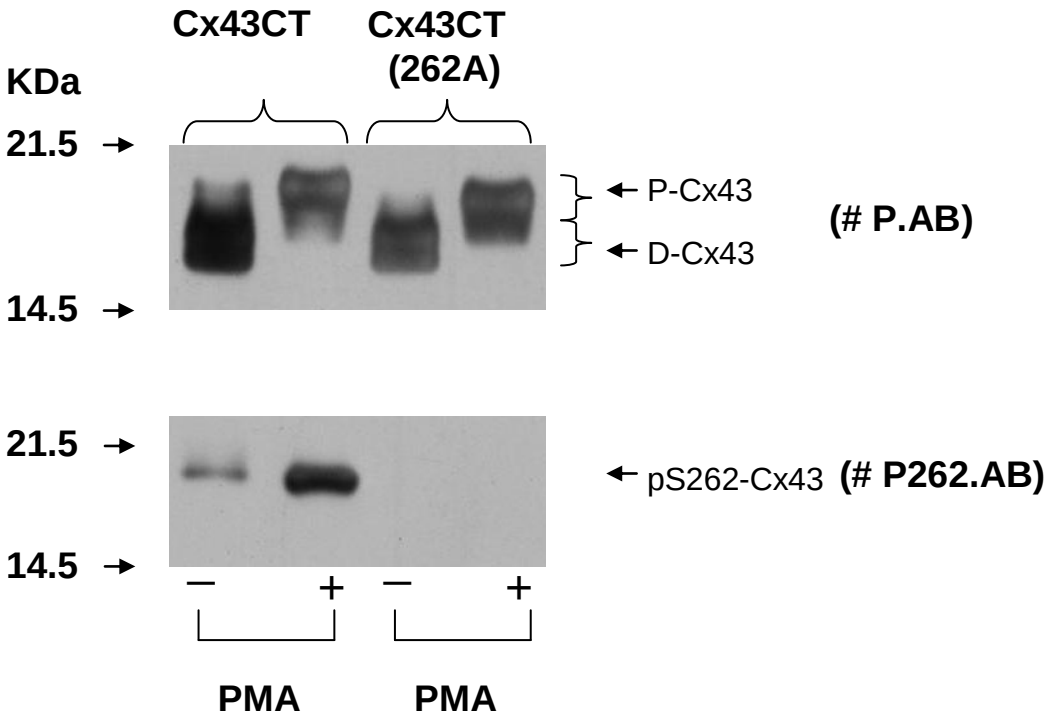
(A)Western blots of lysates (10µg/lane) from HEK293 cells ectopically expressing Cx43, before (−) and after (+) PMA stimulation, and probed with antibodies recognizing all Cx43 (phosphorylated and unphosphorylated), or only Cx43 phosphorylated on S262, as indicated.

(B) Western blot of lysates (10µg/lane) from HEK293 cells ectopically expressing Cx43CT, or Cx43CT (S262A), as indicated, before (−) and after (+) PMA stimulation and probed with antibodies recognizing all Cx43 (phosphorylated and unphosphorylated), or only Cx43 phosphorylated on S262.

[A]



[B]


Cx43CT
Cx43CT (262A)
KDa
21.5
14.5
P-Cx43
D-Cx43
(# P.AB)
21.5
14.5
pS262-Cx43
(# P262.AB)
–
+
–
+
PMA
PMA

As a next step, the question of whether Cx43, and its S262 mutants, were affecting DNA synthesis in HEK293 cells was examined. Cx43, S262A-Cx43 and S262D-Cx43 were expressed transiently and BrdU labeling index at 2 days after transfection was determined. Quantitative data are shown in Fig.II-2A&B. Expression of Cx43 decreased the fraction of cells synthesizing DNA by about 30% compared to non-expressing cells. Expression of S262A-Cx43 resulted in an even more pronounced 65% decrease in BrdU labeling index (Fig.II-2A). On the other hand expression of S262D-Cx43 elicited no inhibition. These data therefore show that Cx43 inhibits DNA synthesis in HEK293 cells in a manner dependent on S262, and its state of phosphorylation.

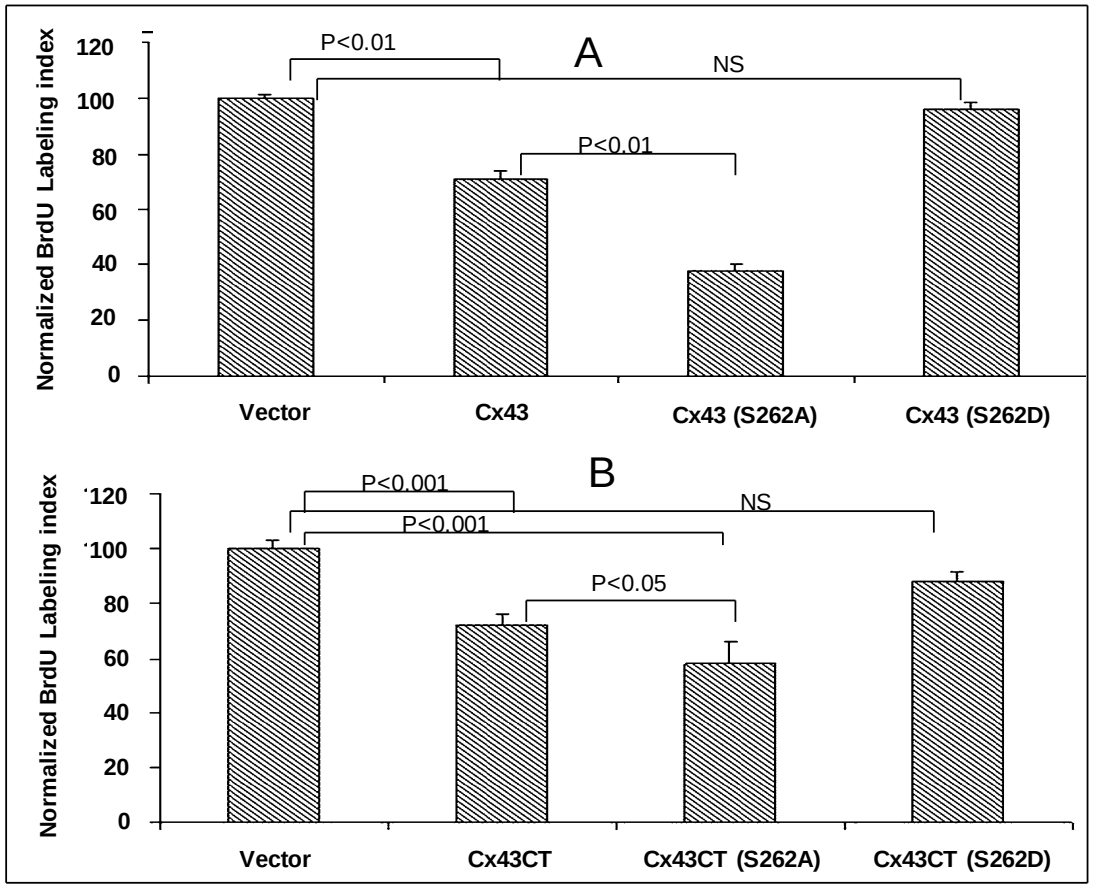
Both PKC as well as the S262 site have been shown previously to affect gap junction mediated metabolic coupling {Dang et al., 2003; Doble et al., 2004; 2000}, thus one might surmise that changes in coupling are responsible for growth inhibition and its regulation by S262. To investigate this notion the effects of Cx43CT, and corresponding S262 mutations, were tested on BrdU incorporation. These Cx43 truncation mutants lack the gap junction domain, thus any effect they may exert cannot be attributed to channel-formation and intercellular communication. Lack of endogenous Cx43 expression in HEK293 cells renders them a good model system for testing the effects of Cx43CT in isolation: this system eliminates any interference from interactions between endogenous intact Cx43 and the introduced truncations. As shown in Fig.II-2B, ectopically expressed Cx43CT inhibited DNA synthesis by 28% compared to controls. Expression of Cx43CT (S262A) inhibited DNA synthesis by 42%,

significantly more pronounced than that by Cx43CT ($P<0.05$). On the other hand, Cx43CT (S262D) had no significant effect on DNA synthesis compared to controls.

As was the case for Cx43, Cx43CT inhibited DNA synthesis of HEK293 cells in a manner that was regulated by S262. Thus it was concluded that Cx43-mediated growth inhibition and its regulation by S262 do not require the channel-forming domain of the molecule, and are independent of gap junction channel-mediated intercellular communication.

<u>Figure II-2:</u> Effect of S262 mutations on Cx43 and Cx43CT mediated inhibition of DNA synthesis in HEK293 cells

Normalized BrdU Labeling Index of the HEK293 ectopically expressing: (A) Cx43, Cx43 (S262A) and Cx43 (S262D) as indicated; (B) Cx43CT, Cx43CT (S262A), Cx43CT (S262D), as indicated. C = control mock-transfected cultures. Bars = SEM, n = 4. Labeling Index represents the fraction of cells incorporating BrdU in their DNA, and, while it's actual value was at 0.35 in the control group, it was arbitrarily set (normalized) to 100. P values in (A) are: Control versus WT-Cx43 and Cx43 versus Cx43 (S262A) **P<0.01, control versus (S262D) ns P<0.05, Cx43 versus Cx43 (S262D) **P<0.01. P values in (B) are: Control versus Cx43CT or Cx43CT (S262A) P<0.001, control versus Cx43CT (S262D) ns >0.05, Cx43CT versus Cx43CT (S262A) P<0.05.



III.B-2. Potential interactions between Cx43- and TGFβ1-mediated inhibition of DNA synthesis

III.B-2a: Cx43-mediated inhibition of DNA synthesis does not require downstream activation of TGFβ1 triggered signal transduction

TGFβ1 is a known inhibitor of cardiomyocyte DNA synthesis {Kardami, 1990; Sheikh et al., 2004}. This was also confirmed in our experimental system, as shown in Fig.II-3. The BrdU labeling index in the presence of 5 ng/ml of added TGFβ1 was at 71% of control levels. Fig.II-3 and subsequent figures show normalized data (fold-effect), arbitrarily assigning a value of 1.0 in the control group. Absolute values of NCM labeling index (percent myocytes staining positive for BrdU) ranged between 20-35% of the total in different experiments. It was hypothesized that the Cx43-associated inhibition of NCM is linked to the TGFβ1 signal transduction pathway. As a first step towards addressing this, we hypothesized that Cx43 mediated inhibition of DNA synthesis is dependent on downstream activation of signals associated with TGFβ1, such as activation of TGFβ1 receptors (TGFβRI, TGFβRII), and/or Smad2.

SB431542 is a pharmacological antagonist of TGFβRI {Waghabi et al., 2007}, while over-expression of DNTGFβRII by adenoviral gene transfer inhibits TGFβRII {Sheikh et al., 2004}. These reagents were used on NCM, to examine whether they would prevent Cx43-induced inhibition of DNA synthesis. Results using SB431542 are shown in Fig.II-4. Treatment of NCM

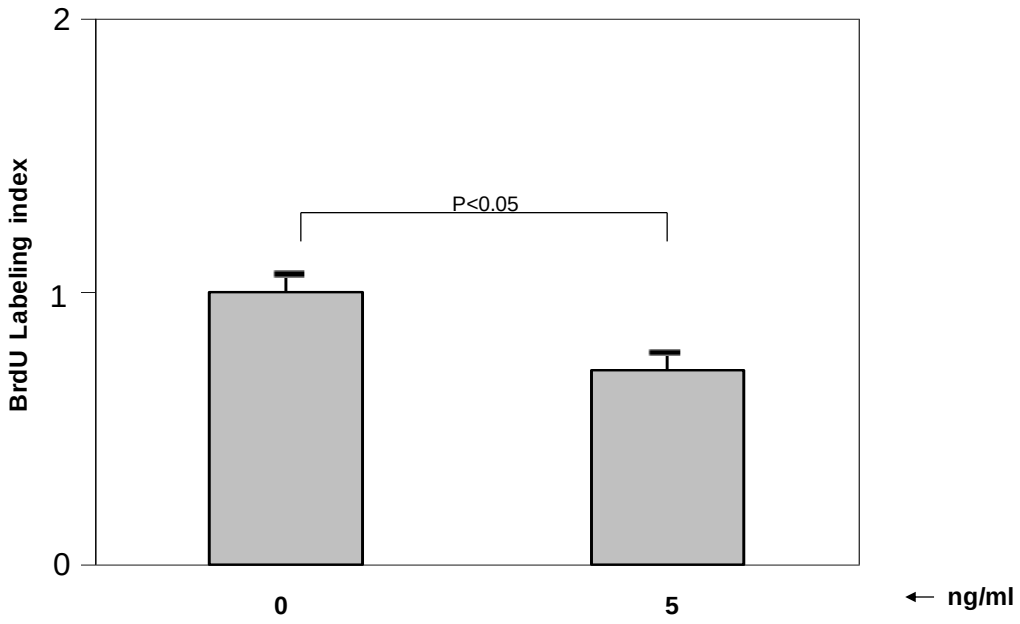
with SB431542 significantly increased DNA synthesis, compared to vehicle-only (DMSO) treated cells, as expected from successful inhibition of serum-derived or cell-derived TGFβ1-signal transduction. Again as expected, over-expression of Cx43 significantly decreased DNA synthesis compared to vector-infected controls. The Cx43-inhibition remained unchanged in the presence of SB431542, indicating that it is not dependent on the activation of TGFβRI.

Fig.II-4 also shows the effect of expression of truncated (non-channel forming) Cx43 (Cx43CT) on NCM DNA synthesis: as expected from the previous section, Cx43CT induced a significant decrease in DNA synthesis compared to Vector-treated cells. Presence of SB431542 did not change Cx43CT induced inhibition of DNA synthesis, indicating that it is not dependent on the activation of TGFβRI.

To verify that SB431542 was effective as an inhibitor of TGFβ1-TGFβRI signal transduction, its effects on Smad2 phosphorylation were examined by western blotting with phospho-Smad2 antibodies (P-Smad2 AB). As shown in Fig.II-5, control (Vector-infected) cells present a clear signal for P-Smad2, and this signal was abolished by SB431542. Expression of WT-Cx43 had no apparent effect on P-Smad2; SB431542 remained capable of preventing Smad2 phosphorylation in the presence of WT-Cx43 expression. Similar results were obtained when S262A-Cx43 was used instead of WT-Cx43. As seen in Fig.II-5, none of the treatments affected total levels of Smad2; probing for actin confirmed even loading.

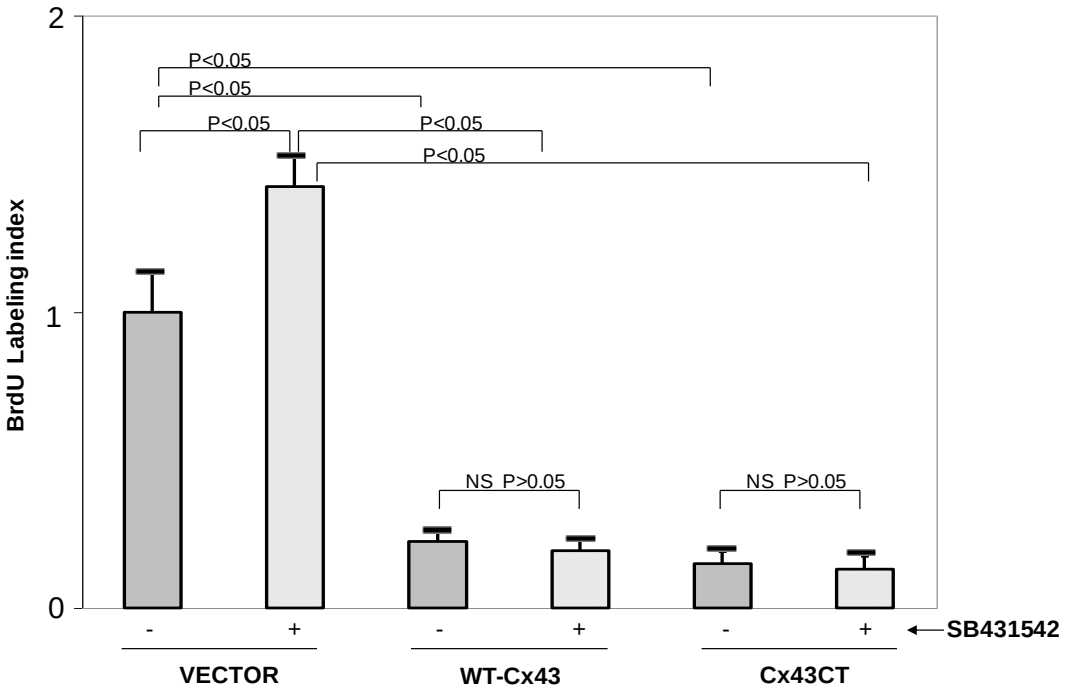
Figure II-3: TGFβ$_1$ inhibits DNA synthesis in NCM

The y-axis shows normalized BrdU labeling index (fold-effect) of NCM treated or not with TGFβ1 (0-5 ng/ml) as indicated in the x-axis. TGFβ1 significantly inhibited DNA synthesis at a concentration of 5 ng/ml (*P <0.01, Student 't' test, n=24). Data are mean+SEM.



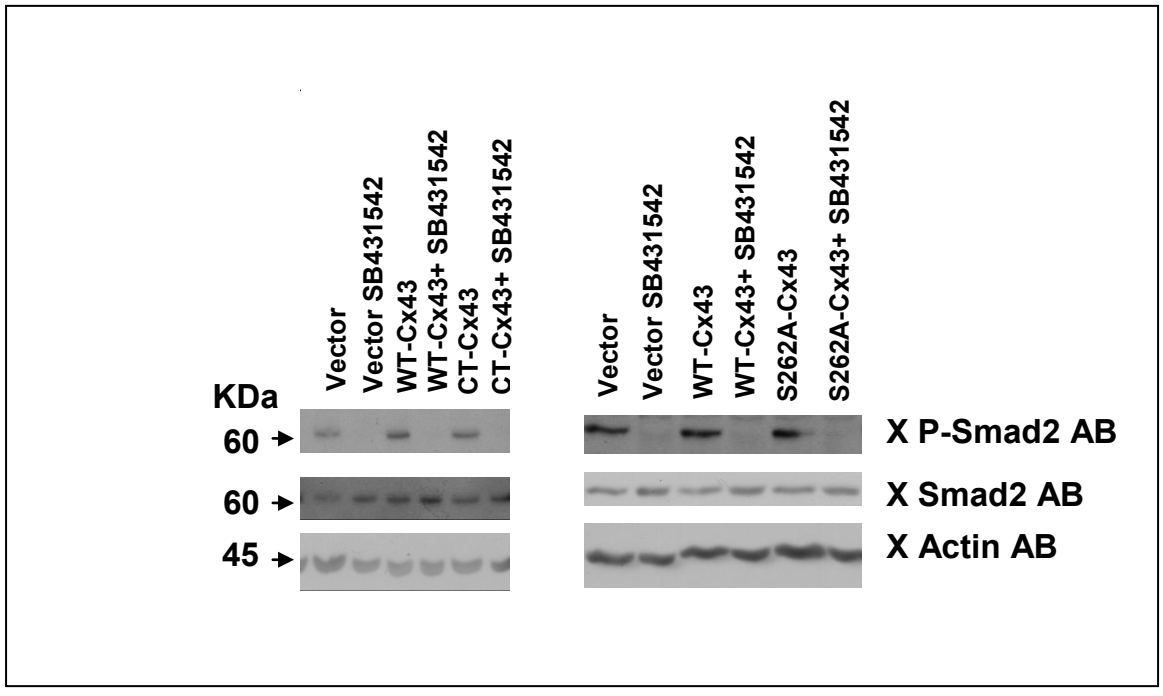
<u>Figure II-4:</u> Pharmacological inhibition of TGFβRI does not prevent WT or Cx43CT induced inhibition of DNA synthesis

The y-axis shows the normalized BrDU labeling index of NCM infected (as indicated in the x-axis) with Ad-Vector, Ad-WT-Cx43 or Ad-CT-Cx43 in the presence or absence of SB431542 (20µM). Cardiomyocytes infected with Ad-WT-Cx43 or Ad-Cx43CT exhibit significant decrease in BrDU labeling compared to vector-infected controls irrespectively of the presence (+) or absence (-) of SB431542. Comparisons between groups are indicated by brackets (*P<0.05, Two way ANOVA, n=24). Data are mean+SEM).



<u>Figure II-5:</u> Pharmacological inhibition of TGFβRI prevents phosphorylation of SMAD2 while Cx43 overexpression has no effect

Representative western blot shows the effect of SB431542, on the phosphorylation of Smad2, in the presence or absence of overexpression of Cx43 in neonatal cardiomyocytes as indicated. The upper, middle and lower panels were probed with P-Smad2, total Smad2 and Actin (loading control) antibodies respectively. Irrespectively of the presence or absence of overexpression of Cx43, SB431542 inhibited the phosphorylation of Smad-2. Cx43 expression had no effect on levels of total or phosphorylated Smad2.



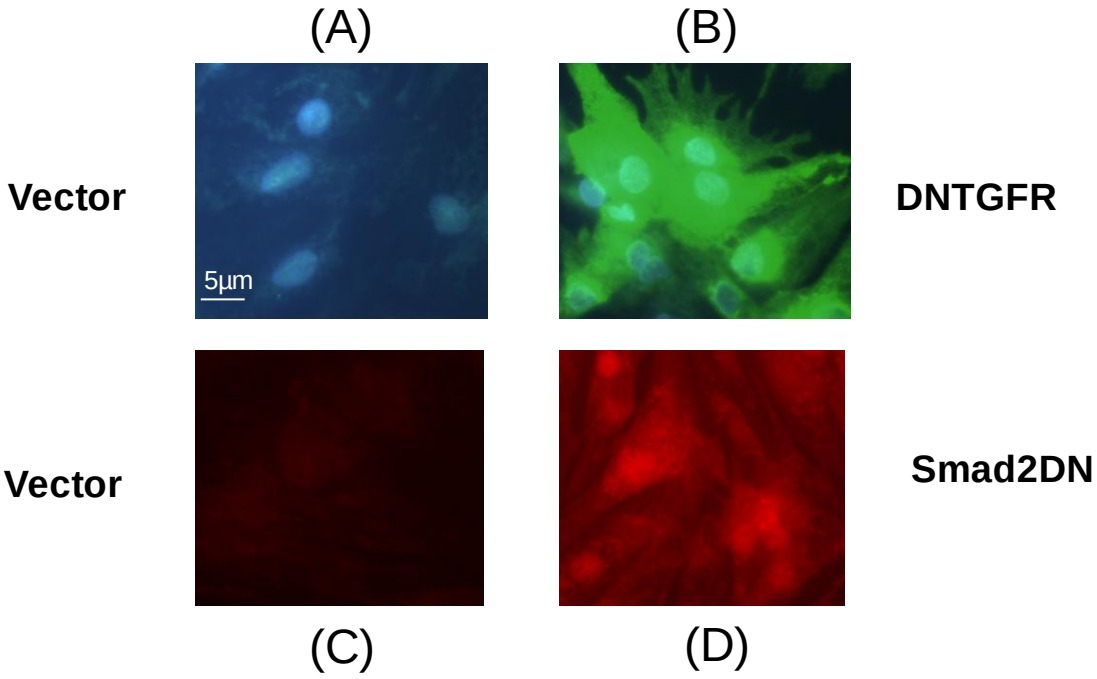
To address potential involvement of additional signals associated with TGFβ1 overexpression of dominant negative versions of TGFRII and Smad2 (Ad-DNTGFRII and Ad-DNSmad2) were used. Successful expression of these proteins was confirmed by immunofluorescence with corresponding antibodies, as illustrated in Fig.II-6.

Fig.II-7 shows the effect of WT-Cx43 on BrdU labeling index, in the presence or absence of Ad-DNTGFRII overexpression. As expected, expression of WT-Cx43 decreased DNA synthesis compared to controls. Expression of DNTGFRII alone increased DNA synthesis significantly compared to Vector-infected controls. The inhibitory effect of WT-Cx43 was unchanged in the presence of DNTGFRII expression.

Fig.II-8A shows the effect of S262A-Cx43 on BrdU labeling index, in the presence or absence of Ad-DNSmad2 overexpression. Expression of Ad-DNSmad2 alone increased DNA synthesis significantly compared to Vector-infected controls. As expected, expression of S262A-Cx43 decreased DNA synthesis compared to controls. This effect was unchanged in the presence of DNSmad2 expression. Representative immunofluorescence images in support of these data are shown in Fig.II-8B.

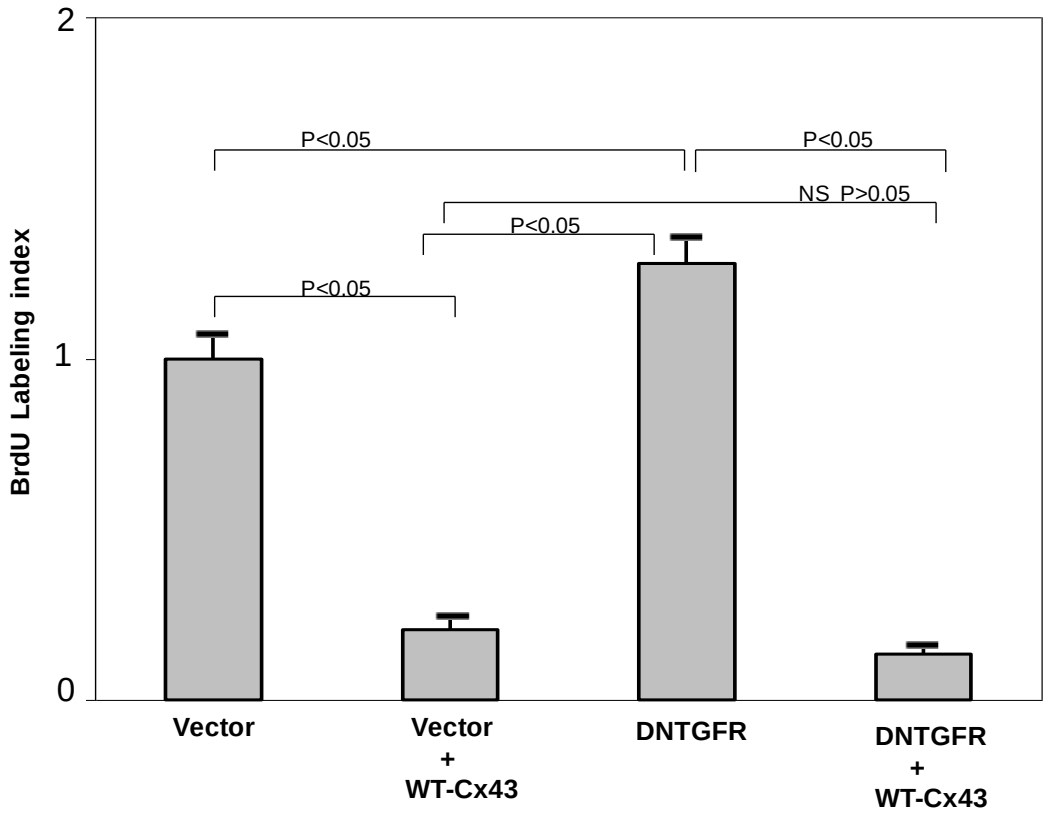
Figure II-6: Immunofluorescence detection of the protein products of the introduced genes (DNTGFRII, DNSmad2)

Representative immunofluorescence images of cardiomyocyte cultures. Cardiomyocyte cultures infected with (A) Ad-Vector alone and probed with antibody for TGFRII (green) and nuclear staining with Hoechst (blue) (B) Ad-DNTGFRII and probed with antibody for TGFRII (green) and nuclear staining with Hoechst (blue) (C) Ad-Vector alone and probed with antibody for Smad2 (red) (D) Ad-DNSmad2 and probed with antibody for Smad2 (red).



<u>Figure II-7:</u> Inhibition of TGFβRII does not prevent Cx43-induced inhibition of DNA synthesis

The y-axis shows the normalized BrdU labeling index of NCM infected with Ad-Vector alone, Ad-WT-Cx43, Ad-DNTGFRII or Ad-WT-Cx43 and Ad-DNTGFRII simultaneously as indicated in the x-axis. WT-Cx43 expression significantly decreased DNA synthesis compared to non-expressing cardiomyocytes irrespectively of the presence or absence of DNTGFRII. Brackets indicate comparisons between groups (*P<0.05, two-way ANOVA, n=12). Data are mean+SEM.



<u>Figure II-8A:</u> Inhibition of Smad2 does not prevent S262A-Cx43 mediated inhibition of DNA synthesis

The y-axis shows the normalized BrdU labeling index of NCM infected with Ad-Vector alone, Ad-S262A-Cx43, Ad-DNSmad2 or Ad-S262A-Cx43 and Ad-DNSmad2 simultaneously as shown in the x-axis. S262A-Cx43 expression significantly decreased DNA synthesis compared to non-expressing cardiomyocytes irrespectively of the presence or absence of DNSmad2. All comparisons between groups (*P<0.05, two way ANOVA, n=24). Data are mean+SEM.

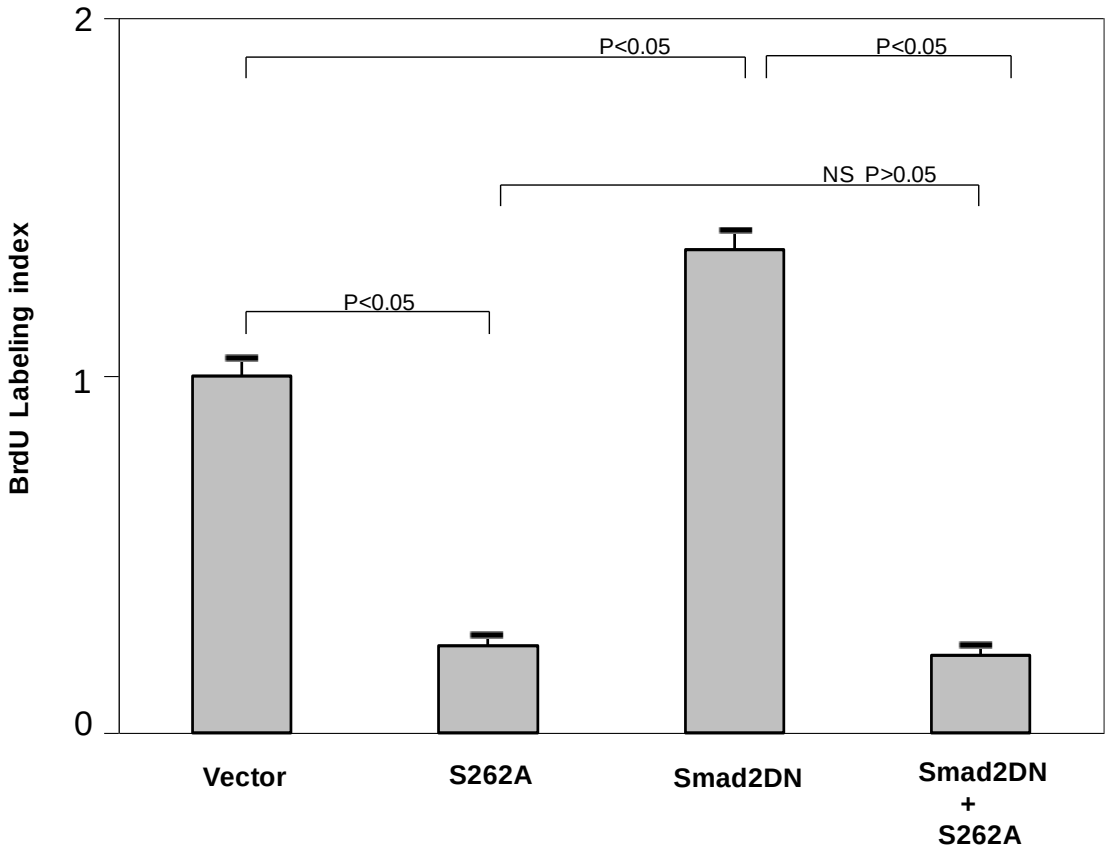
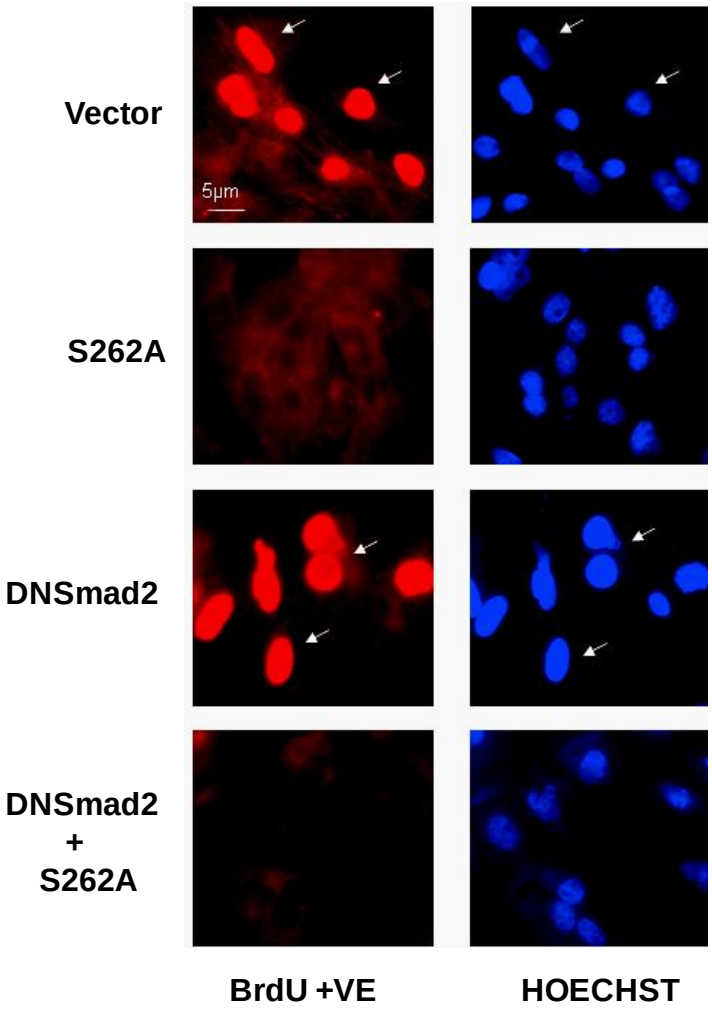


Figure II-8B: Representative Immunofluorescence pictures (corresponding to Fig.II-8A)

Left panel: Representative immunofluorescence images of neonatal cardiomyocytes infected with Ad-Vector, Ad-S262A-Cx43, Ad-DNSmad2 or Ad-S262A-Cx43+ DNSmad2 and probed for BrdU (red-nuclear) and α-actinin (red-myocyte). Myocyte cultures infected with Ad-S262A-Cx43 and S262A-Cx43+ DNSmad2 show decreased BrdU staining compared to control.
Right panel: These are the same immunofluorescence images from the left panel but showing nuclear staining with Hoechst dye.



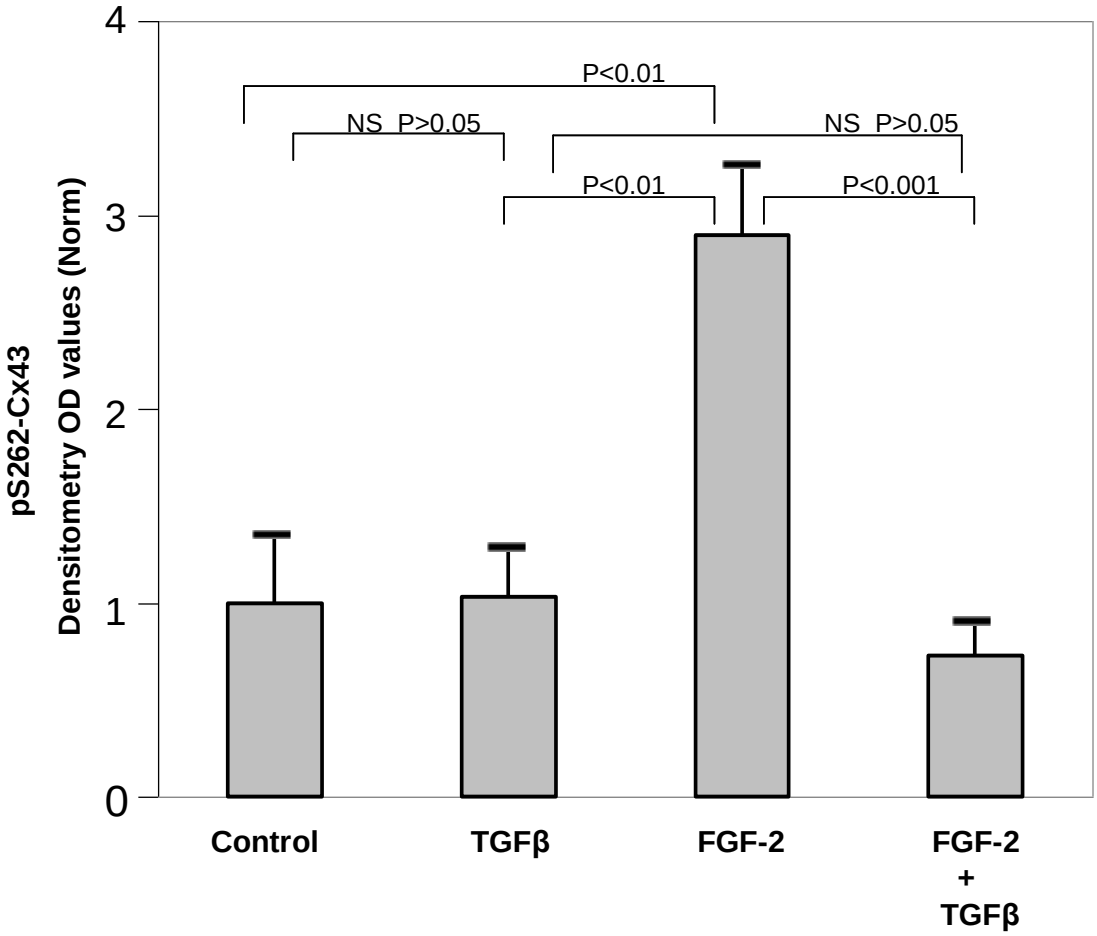
III.B-2b: TGF β1 prevents mitogen-induced Cx43 phosphorylation at S262

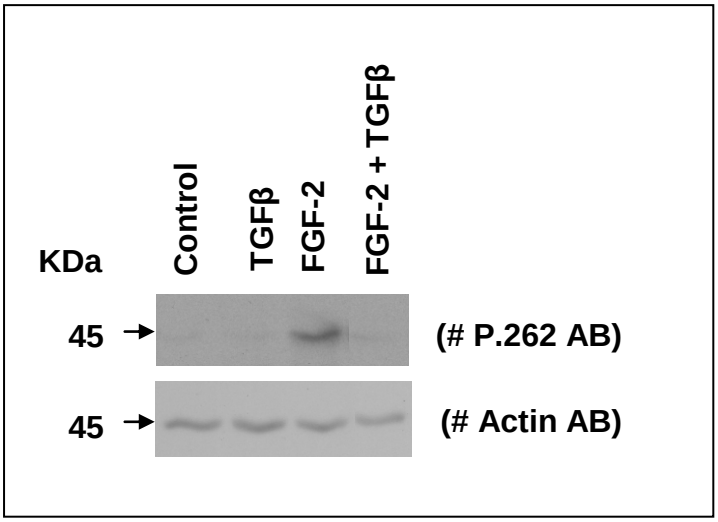
TGFβ1 has been shown to prevent the FGF-2-induced stimulation of cardiomyocyte DNA synthesis and proliferation {Kardami, 1990}. The mechanism by which this occurs is not well understood. As shown previously {Doble et al., 1996}, mitogenic stimulation of cardiomyocytes promotes Cx43 phosphorylation at S262 {Dang et al., 2006}. This mitogen-induced phosphorylation cancels the inhibitory effect of Cx43 on DNA synthesis {Doble et al., 2004} (Fig.II-2). Taking all this into account it was hypothesized that TGFβ1 promotes the 'growth inhibitory' state of Cx43, preventing its phosphorylation at S262. To study this NCM were incubated with TGFβ1 (5 ng/ml); or FGF-2 (10 ng/ml); or FGF-2 + TGFβ1. Results are shown in Fig.II-9: As expected, FGF-2 significantly increased the phosphorylation of Cx43 at S262 compared to untreated controls ($P<0.01$). TGFβ1 alone did not have any effect on the phosphorylation of Cx43 at S262. On the other hand TGFβ1 significantly decreased the ability of FGF-2 to stimulate phosphorylation of Cx43 at S262 ($P<0.001$). The above results indicate that TGFβ1 blocks mitogen (FGF-2) induced phosphorylation of Cx43 at S262.

Figure II-9: TGFβ1 prevents the FGF-2-induced phosphorylation of Cx43 at S262

(A) The y-axis represents normalized densitometric O.D. values from immunoreactive bands resulting from cardiomyocyte treatments indicated in the x-axis. These include treatments with TGFβ1 (5ng/ml) alone, FGF-2 (10µg/ml) alone, FGF-2+ TGFβ1 or control untreated cells. The data indicates that TGFβ1 significantly decreases FGF-2 induced increase in the phosphorylation of Cx43 at S262. FGF-2 VS FGF-2+ TGFβ1 (# $P<0.001$, ANOVA, n=3). TGFβ1 VS FGF-2 and Control VS FGF-2 (*$P<0.01$, ANOVA, n=3). Data are mean+SEM.

(B) Representative western blot showing lysates (40µg/lane) from either control neonatal cardiomyocytes or neonatal cardiomyocytes incubated in TGFβ1, FGF-2 or FGF-2+ TGFβ1. The blots were analyzed for the phosphorylation of Cx43 at S262 using P-262 Cx43 antibody. Actin antibody was used to demonstrate equal loading.


4
3
2
1
0
pS262-Cx43
Densitometry OD values (Norm)
P<0.01
NS P>0.05
NS P>0.05
P<0.01
P<0.001
Control
TGFβ
FGF-2
FGF-2
+
TGFβ


KDa
Control
TGFβ
FGF-2
FGF-2 + TGFβ
45
(# P.262 AB)
45
(# Actin AB)

III.B-3: Summary of main findings

In this chapter we have shown that:

1. In HEK293 cells both Cx43 and Cx43CT can become phosphorylated at S262.

2. In HEK293 cells both Cx43 and Cx43CT inhibited DNA synthesis and this is regulated by phosphorylation at S262, indicating that growth inhibition by Cx43 do not require channel forming domain and is independent of gap junctional intercellular communication.

3. In NCM, Cx43 mediated inhibition of DNA synthesis does not require activation of components of TGFβ_1 signaling pathway such as TGFβ_1 receptors or Smad2.

4. In NCM TGFβ_1 inhibits FGF-2–induced phosphorylation of Cx43 at S262 indicating a novel mechanism by which TGFβ_1 could inhibit DNA synthesis.

CHAPTER IV: DISCUSSION

The overall purpose of my studies was to investigate the relationship between Cx43, its phosphorylation at PKCε-target sites such as S262, and PKCε-mediated end-points such as cytoprotection/cardioprotection, and stimulation of DNA synthesis. The role of TGFβ1 signaling in Cx43 mediated inhibition of DNA synthesis was also investigated.

IV.A. CX43 AND CARDIOMYOCYTE RESISTANCE TO INJURY

Work in my supervisor's laboratory has shown that several experimental treatments promoting PKC-dependent cardioprotection also cause above-normal Cx43 phosphorylation at PKC target sites. Ischemic preconditioning, FGF-2 treatment before ischemia {Srisakuldee et al., 2006} as well as FGF-2 treatment post-ischemia were characterized by robust increases in P-S262-Cx43 and P-S368-Cx43, compared to normal hearts. The same was true when hearts were perfused with diazoxide, a compound, reported to promote ischemic preconditioning-like cardioprotection {Costa and Garlid, 2008}. Using an *in vitro* model of cardiomyocyte ischemic injury a cause-and-effect relationship between Cx43 phosphorylation at S262 and cardiomyocyte cytoprotection was demonstrated. In this section the validity of the experimental model used will be discussed in detail in addition to the findings on the effects of overexpressed Cx43, and Cx43 phosphorylation mutants on

the ability of cardiomyocytes to raise their threshold to ischemic injury in response to several manipulations.

IV.A-1. *In vitro* model of ischemic injury

To simulate aspects of cardiomyocyte ischemic injury confluent primary NCM cultures were placed in 'ischemic medium' containing sodium lactate, no glucose, maintained at a low pH of 6.2, and then incubated these cultures in a hypoxia chamber for various time points. These conditions mimic *in vivo* ischemia {Wu et al., 2004}. Indeed, these findings showed that NCM placed under these conditions manifested Cx43 changes that were very similar to those encountered *in vivo* and described by {Beardslee et al., 2000; Clarke et al., 2009}. Specifically it was found that in NCM Cx43 became dephosphorylated at the earlier time points, followed by Cx43 degradation at later time points (Fig.I-2). The progressive Cx43 loss was accompanied by evidence of increased incidence of apoptotic cell death (TUNEL staining) and overall cell death (LDH release), as would be expected *in vivo*, and as reported previously {Regula et al., 2002}. Loss of Cx43 at later stages of ischemia is in agreement with several previous studies {Luke and Saffitz, 1991; Peters et al., 1993; Ruiz-Meana et al., 2008}. This model was used therefore for later experiments. Based on the findings, subsequent experiments exposed NCM to moderate length of simulated ischemia (ischemic medium plus 6 h in hypoxia chamber) as it was felt that these conditions (causing intermediate levels of cell injury and cell death, and Cx43

dephosphorylation, but not substantial Cx43 loss) allowed effect of manipulations over a broader range of possible outcomes to be tested.

IV.A-2. Increased Cx43 expression is cardioprotective

A modest 2.5-fold overexpression of wild type Cx43, achieved by adenoviral infection, was found to promote a small but significant increase in resistance to simulated ischemic injury (decreased TUNEL staining and LDH release) of NCMs. These findings are in agreement with several other studies showing a positive relationship between Cx43 levels and development of resistance to injury. For example, incidence of apoptosis in cultured NCM was shown to be aggravated in association with the downregulation of Cx43 by antisense oligonucleotides {Yasui et al., 2000}. Hearts from Cx43 (+/-) mice, expressing half the normal levels of Cx43, failed to develop a protected phenotype in response ischemic preconditioning-type of treatment {Heinzel et al., 2005; Schulz and Heusch, 2004}. Ischemic preconditioning failed to elicit protective effects in aged hearts that have a marked reduction in a Cx43 levels {Boengler et al., 2006a}.

Overexpressed wild type Cx43 localized mainly to cell-cell contact and plasma membrane sites, indicating that its localization and trafficking was similar to that of endogenous Cx43. Also, these findings suggested that targeting of Cx43 to membrane sites, and the attendant channel and hemi-channel-forming ability were important for the exertion of cytoprotection. In agreement, absence of the channel forming domain eliminated the protective

function of Cx43: as seen in Fig.I-5 (D) expression of Cx43CT (lacking the channel forming and membrane targeting domain) had no protective effect and in fact rendered NCMs vulnerable to mild ischemic injury. Work in my supervisor's laboratory has shown that this was associated with stimulation of p53 expression {Kardami et al., 2007a}, an anti-oncogenic protein which is also known to mediate ischemia-induced cell death in cardiomyocytes {Regula and Kirshenbaum, 2001}. There is some evidence that subjecting isolated hearts to global ischemia causes accumulation of endogenously produced truncated Cx43 which may directly contribute to myocardial ischemic injury {Kardami et al., 2007a}. Thus it would appear that the intact Cx43 structure, including both the membrane-targeting channel forming domain, and the regulatory C-terminal domain, are required for Cx43 cytoprotection.

Following ischemia, most of Cx43 in wild type Cx43 overexpressing cultures remained phosphorylated, as indicated by its migration at 43-45 kDa. Retention of phosphorylated Cx43 at membrane sites may be important for the protective response: cardioprotective treatments like ischemic preconditioning {Schulz et al., 2007} or FGF-2 administration {Srisakuldee et al., 2006} have been shown to prevent the ischemia-induced Cx43 dephosphorylation and to preserve relative levels of phosphorylated Cx43. These studies indicated that phosphorylated Cx43 (and phosphorylation-induced changes in metabolic coupling) may play a key role in protection, perhaps by preventing the spread of injurious metabolites caused by ischemic injury. Preservation of Cx43 phosphorylation, furthermore would be expected to maintain the integrity of

gap junction plaques and overall intercalated disk architecture, and thus contribute to proper cardiomyocyte function and viability. It is also possible that the preservation of phosphorylated Cx43 observed in our overexpressing cultures after ischemia may be a secondary effect of a primary protective mechanism that prevented ATP loss and subsequent activation of phosphatases.

IV.A-3. Expression of Cx43-HA renders cardiomyocytes more vulnerable to ischemic injury

While investigating the question of Cx43 expression and phosphorylation in ischemic injury a series of pilot studies were conducted and expected to demonstrate that Cx43-HA (tagged at its C-terminal with the short tag HA which would allow detection with anti-HA-antibodies) would behave in a similar manner as endogenous, or wild type Cx43. Previous use of tagged Cx43 had indicated that attaching a small label to the C-tail allows proper subcellular localization and does not interfere with the gap junction forming ability {Laird et al., 2001}. Surprisingly, it was found that Cx43-HA localized not only at cell-cell contact sites but also around the nucleus and this was unlike the distribution seen with overexpressed wild type Cx43, which localized mainly at plasma membrane sites. This peri-nuclear localization of Cx43-HA became more prominent during ischemia and was associated with Cx43-HA dephosphorylation as well as significant toxicity to the cells. Myocytes

expressing Cx43-HA were significantly more vulnerable to simulated ischemia compared to non-overexpressing controls. Overall these findings showed that Cx43-HA could not be used as a substitute for wild type Cx43, as the addition of the 9 amino-acid HA tag at its C-terminal appeared to interfere with its trafficking and its functionality during ischemic conditions. The Cx43-HA toxicity offers further support to the argument made in the previous section, that appropriate subcellular/ plasma membrane localization (and/or channel formation) and phosphorylation status are important elements in the protective effect of wild type Cx43.

IV.A-4. Preventing phosphorylation of Cx43 at S262 renders cardiomyocytes more vulnerable to ischemic injury

Cx43 phosphorylation mutants, S262A-Cx43, and S262D-Cx43 were used to examine the role of phosphorylation at S262 in NCM resistance to ischemic injury. In the absence of any protective manipulation, over-expression of S262A-Cx43 significantly increased the baseline vulnerability of cardiomyocytes to ischemic injury. It is likely that the injury promoting effect of S262A-Cx43, introduced into NCM by adenoviral infection, is a non-specific consequence of overexpression, since, as discussed earlier in this section, similar levels of expression of wild type Cx43 had the opposite effect, being protective. Furthermore, ectopically expressed S262A-Cx43 localizes in a manner similar to its wild-type counterpart, shown for both Cx43-deficient

{Dang et al., 2006}, and Cx43 expressing cells such as myocytes {Doble et al., 2004}, thus its effects are not likely to result from aberrant localization.

The data obtained imply that a relatively modest (2.5-fold) overexpression of the S262A-Cx43 has a ‘dominant-negative’ effect over endogenous Cx43. S262A-Cx43, having an intact channel-forming domain, can interact with endogenous Cx43 to form mixed, and thus ‘altered’, connexons or aggregates. Studies have shown that all six components of a connexon need to be capable of phosphorylation to achieve full effect on connexon properties {Bao et al., 2007}. It is possible that all Cx43 monomers in an aggregate may need to become phosphorylated at S262 for developing PKCε-mediated cytoprotection.

The S262D-Cx43 mutation simulates the charge effects of constitutive phosphorylation at S262, and was used on the assumption that it exerts effects similar to those of P262-Cx43. If that were the case, S262D-Cx43 overexpression should be protective against simulated ischemia. In contrast, S262D-Cx43 expression was found to have no protective effect, resulting in similar levels of TUNEL staining (Fig.I-7B, Fig.I-8A) as those of vector-infected cells, and in some cases somewhat increased levels of LDH release compared to vector-infected cells. One possible explanation for these results is that, in contrast to a basic assumption, the S262D substitution does not adequately represent the consequences of phosphorylation at S262, which may not only rely on the addition of a negative charge but also to an overall

conformational change. S262 phosphate is indispensable for antibody specificity.

Nevertheless, the S262D mutation was not as toxic, and in fact elicited significantly less TUNEL staining than the S262A mutation, suggesting that the added negative charge counteracted, in part, the toxic consequences of the S262A mutation.

IV.A-5. The FGF-2-induced and PKCε-mediated cardiomyocyte protection from ischemic injury are mediated by Cx43 phosphorylation at S262

FGF-2 is a well established cytoprotective/cardioprotective agent, exerting preconditioning- and post-conditioning like effects {Detillieux et al., 2003; Kardami et al., 2007b}, as well as stimulating the PKCε-mediated Cx43 phosphorylation at S262 {Doble et al., 2001; 2000}. In agreement with these previous reports, it was also found that FGF-2 pre-treatment protected NCMs from simulated ischemia, resulting in decreased incidence of cell death. Nevertheless, since FGF-2 was unable to exert any protective effect in a background of S262A-Cx43 overexpression (when the majority of Cx43 cannot become phosphorylated at S262) it was concluded that its cytoprotection is mediated by Cx43 via its phosphorylation at S262.

In a similar manner, overexpression of a signaling molecule activated downstream of FGF-2, PKCε, was also found to be protective from cell death in the NCM model, in agreement with several previous reports {Bright and Mochly-Rosen, 2005; Ping et al., 2001}. The protective effects of PKCε were

eliminated in a background of S262A-Cx43 expression, thus it was concluded that they are largely mediated by downstream phosphorylation of Cx43 at S262.

Although PKCε overexpression virtually eliminated incidence of cell death (TUNEL staining, suggestive of apoptosis), it only caused a small (albeit significant) decrease in ischemia-induced LDH release. LDH release is an indicator of plasma membrane permeability, which implies presence of cellular injury and potentially necrotic cell death. One possible interpretation of the data is that the pathways leading to LDH release (cell necrosis) and TUNEL staining (cell apoptosis) are not identical, and that PKCε is more effective in the latter.

The studies presented here cannot exclude the possibility that Cx43 phosphorylation at additional PKC (or other kinase) target sites may also participate in protective effects. Indeed, FGF-2 stimulates robust phosphorylation of Cx43 at the PKC target site S368 {Srisakuldee et al., 2009; Srisakuldee et al., 2006} in the perfused heart. Furthermore, FGF-2 activates other cell survival associated kinases like ERK {Bogoyevitch et al., 1994; Disatnik et al., 1995; Padua et al., 1998} and Akt {Jiang et al., 2009} and these may also affect Cx43 phosphorylation. ERK stimulates Cx43 phosphorylation at S255/279/282 {Warn-Cramer et al., 1998; Warn-Cramer et al., 1996} while Akt stimulates Cx43 phosphorylation at S373 {Park et al., 2007}. The role of these Cx43 phosphorylation events, and indeed of other downstream targets of FGF-2 and PKCε signal transduction remains to be investigated.

Nevertheless, the data indicate that Cx43 is an important end-effector of FGF-2, and PKCε cytoprotection.

IV.A-6. Cx43 phosphorylation at S262 contributes to ischemic preconditioning-induced cardiomyocyte protection from simulated ischemia

Ischemic preconditioning, which is also mediated by PKCε activity, was recently shown to promote robust phosphorylation of Cx43 at S262, in the isolated perfused heart {Srisakuldee et al., 2009}. These findings suggested that as was the case for FGF-2, Cx43 phosphorylation at S262 may be required for ischemic preconditioning protection. The preconditioning protocol used was adapted and modified from Yamada and colleagues {Sundset et al., 2007}. Our *in vitro* model system confirmed that ischemic preconditioning did indeed promote nearly complete resistance to simulated ischemia-induced cell death, by reducing incidence of TUNEL staining to about 6% of that induced by ischemia. When however ischemia was exerted in a background of S262A-overexpression (which elicited a 2.6-fold higher incidence of cell death compared to ischemia alone), ischemic preconditioning reduced the incidence of TUNEL staining to a lesser degree, by about 50%. The findings in this thesis indicated that the protective effects of ischemic preconditioning may partially depend on Cx43 phosphorylation at S262, and they likely represent the combined effect of both PKCε-Cx43-S262 dependent and independent

pathways. It would also appear that the signal transduction pathway of FGF-2- or PKCε- induced cardiomyocyte protection is not identical but has common elements (those pertaining to PKCε-Cx43-S262 phosphorylation) with pathways activated by ischemic preconditioning.

Ischemic preconditioning cardioprotection requires the production of short bursts of reactive oxygen species (ROS) to activate downstream signals required for the net protective effect. There is a recognition that mitochondria-derived ROS (which is stimulated by diazoxide) make a major contribution to ischemic preconditioning protection {Forbes et al., 2001; Pain et al., 2000}, and that diazoxide-dependent preconditioning is dependent on appropriate Cx43 expression, becoming inoperable in hearts with reduced Cx43 content {Heinzel et al., 2005}. On the other hand, hearts with reduced Cx43 are still capable of a preconditioning response when ROS production was elicited by a different, non-mitochondrial- exclusive route {Heinzel et al., 2005}. This information, and the data presented here suggest the following hypothetical scenario. Ischemic preconditioning triggers pathways and ROS bursts from mitochondrial and non-mitochondrial sources. Mitochondrial ROS production stimulates PKCε activity and subsequent Cx43 phosphorylation at S262 and this component of the pathway is shared by FGF-2 stimulation, or PKCε overexpression. The non-mitochondrial produced ROS can exert protective effects that are independent of Cx43, or at least mitochondrial-Cx43 {Heinzel et al., 2005}, and this component of the pathway may not be addressed by

FGF-2 stimulation or PKCε overexpression, at least under the conditions tested.

Ischemic preconditioning was shown to stimulate phosphorylation of Cx43 at S368 as well {Srisakuldee et al., 2009; Srisakuldee et al., 2006}. The phosphorylation of Cx43 at S368 has been shown to promote both metabolic and electrical uncoupling of gap junctions {Lampe and Lau, 2000} and to close hemichannels {Bao et al., 2004}. It would seem unlikely that this phosphorylation event is responsible for the ability of ischemic preconditioning to partially reverse the detrimental effects of S262A-Cx43 expression during ischemia, because it is also stimulated by FGF-2 (and PKCε) {Srisakuldee et al., 2009; Srisakuldee et al., 2006}.

IV.A-7. Potential mechanism(s) by which Cx43, and its phosphorylation at S262, may promote cardiomyocyte protection from ischemic injury

Cx43 in the normal heart is constitutively phosphorylated at multiple sites (at least 13) {Solan and Lampe, 2009}, migrating at ~43-45 kDa, but is minimally phosphorylated at S262. Global ischemia of the perfused heart (or simulated ischemia in our *in vitro* system) results in a shift to ~41 kDa Cx43, indicative of dephosphorylation at all sites. If however hearts are treated with FGF-2, or subjected to ischemic preconditioning, Cx43 becomes hyper-phosphorylated at S262, and this phosphorylation (i) remains elevated even after 30 min of global ischemia in the perfused heart, and (ii) preserves the

overall phosphorylation status of Cx43, as this protein continues to migrate as an extensively phosphorylated Cx43 at ~44-45 kDa {Srisakuldee et al., 2009}. Similarly, (iii), as shown here (Fig1-7) overexpression of S262D-Cx43, simulating constitutive phosphorylation at that site, prevented the ischemia-induced overall dephosphorylation of Cx43, as there was no major shift to a ~41 kDa Cx43 after ischemia. In contrast, overexpression of S262A-Cx43 resulted in accumulation of mostly ~41 kDa fully dephosphorylated Cx43 after ischemia. Taken together, these data suggest that Cx43 phosphorylation at S262 protects from ischemia-induced dephosphorylation, and eventual degradation, and this may be a principal mechanism of its protective effect.

Reducing relative levels of dephosphorylated Cx43 during ischemia would be expected to preserve the structural integrity of intercalated disks {Beardslee et al., 2000}. It would also prevent undesirable increases in permeability not only of GJ but also hemi-channels. While hemi-channels are normally closed, Cx43 dephosphorylation causes them to open, resulting in injury and death during ischemia-reperfusion {Shintani-Ishida et al., 2007}.

Ischemia leads to ATP loss followed by activation of phosphatases and Cx43 dephosphorylation {Jeyaraman et al., 2003}. The preservation of phosphorylation at S262-Cx43 even after 30 min ischemia {Srisakuldee et al., 2009}, implies either that phosphatase activation did not occur or that phosphorylation of S262-Cx43 became resistant/inaccessible to phosphatases. Both possibilities merit consideration. Global cytoprotective pathways activated by FGF-2 and ischemic preconditioning would preserve

mitochondrial integrity and energy stores and prevent activation of phosphatases. It is also known that FGF-2 renders Cx43 inaccessible to antibodies recognizing epitopes within residues 260–270, a region containing the S262 site {Doble et al., 1996}. Phosphorylation of Cx43 at S368, another event caused by FGF-2 and ischemic preconditioning causes conformational changes decreasing accessibility to trypsin {Bao et al., 2004}. It is logical to expect that molecular changes causing 'masking' of Cx43 domains from antibodies or proteolytic enzymes may also protect those domains from phosphatases. Phosphatase inhibition exerts cardioprotection in the absence of a preconditioning stimulus {Fenton et al., 2005}. It would be interesting to examine whether phosphatase inhibitors increase baseline levels of P-S262-Cx43, an event which, based on the results of this thesis, would promote cytoprotection.

Relative levels of PS262-Cx43 are comparatively low in the non-'protected' cardiomyocyte {Miura et al., 2007; Srisakuldee et al., 2006} indicating that the normal, in contrast to the 'injury-resistant' state, does not support extensive constitutive Cx43 phosphorylation at S262. This may be a reflection of relatively low levels of PKCε activation in the normal heart. It is suspected that chronic, sustained activation of PKCε, and phosphorylation of S262-Cx43, may cause them to lose their effectiveness as protective responses, by perhaps causing some sort of desensitization of the cell.

Phosphorylation of Cx43 at S262 has been shown to decrease (but not eliminate) metabolic GJ coupling in NCM {Doble et al., 2000} and this could

contribute to protection by limiting the spread of injurious metabolites between cells, as is the case for anesthetics {Zaugg et al., 2003}.

Cx43 has also been shown to be localized in the inner membrane of sub-sarcolemmal mitochondria with the C-terminal part of Cx43 that contains all the phosphorylation sites facing the intermembrane space {Boengler et al., 2009}. PKCε has been shown to be located at the outer or intermembrane space of mitochondria {Baines et al., 2002; Costa and Garlid, 2008} and mitochondrial Cx43 has been shown to play a key role in the protection by ischemic preconditioning and diazoxide {Rodriguez-Sinovas et al., 2006a}. Hence the interaction between mitochondrial protein kinases and Cx43 could play a key role in conferring a protective phenotype. Since S262 site of Cx43 is a target of PKCε and PKCε plays a central role in cardioprotection, it is possible that S262A-Cx43 could prevent the baseline phosphorylation of mitochondrial Cx43 and thus make cells more vulnerable to injury.

In conclusion: The precise mechanism by which Cx43 phosphorylation at S262 contributes to cardioprotection needs to be determined. The effect may be a consequence of subtle changes in GJ coupling, and/or Cx43 protein–protein interactions. Alternatively, or concurrently, it is possible that Cx43 hemi-channels and/or mitochondrial Cx43 are involved. Certainly, PKCε is present at all subcellular sites where Cx43 is found including intercalated disks, plasma membrane, and mitochondria, and thus it is probable that it can stimulate above- normal Cx43 phosphorylation at S262 at all these locations.

IV.B. CX43 AND REGULATION OF DNA SYNTHESIS

IV.B-1. Phosphorylation at S262 prevents inhibition of DNA synthesis by Cx43 in a manner independent of channel-forming ability

Using overexpression of Cx43 and Cx43 phosphorylation mutants it was previously reported that the PKC target site S262 of Cx43 regulates Cx43-mediated growth inhibition in rat primary neonatal cardiomyocytes {Doble et al., 2004}. Cardiac myocytes display very robust endogenous Cx43 expression, which may interfere/combine with the effects of the modified, ectopically expressed connexins. To eliminate the influence of endogenous Cx43 expression, a series of studies were conducted using HEK293 cells which are Cx43 deficient. The effect of Cx43, Cx43 phosphorylation mutants, truncated Cx43 (Cx43CT), and truncated Cx43 phosphorylation mutants on DNA synthesis were tested following transient gene transfer.

The finding that ectopically expressed Cx43 in HEK293 cells became phosphorylated at S262 following PMA stimulation is in agreement with previous observations on rat cardiomyocytes {Doble et al., 2004}, and HeLa cells {Kardami et al., 2003}. Taken together these data show that phosphorylation at the S262 site is neither species-, nor cell type-specific. Furthermore, as the truncated Cx43CT was also capable of becoming phosphorylated at S262 it is concluded that presence of the channel-forming domain, and/or plasma membrane localization are not required for S262 to become phosphorylated.

The anti-PS262-Cx43 immunoreactive bands in western blots corresponded to the slower migrating, more extensively phosphorylated Cx43, as well as Cx43CT. These data suggest that several previous phosphorylations at different sites may be required before Cx43 can become phosphorylated at S262. Also, preventing phosphorylation at S262 does not prevent phosphorylation at additional sites on either Cx43 or Cx43CT, as PMA stimulation shifted S262A-Cx43, as well as Cx43CT (S262A) to slower electrophoretic motilities.

Preventing phosphorylation at S262 in Cx43 and/or Cx43CT maximized inhibition of DNA synthesis, while simulating phosphorylation (by using the S262D mutants) eliminated the inhibitory effect of Cx43, as well as Cx43CT. It is concluded that the S262 site acts as a switch in regards to Cx43-mediated inhibition of DNA synthesis. Lack of phosphorylation at S262 allows Cx43 to exert inhibition of DNA synthesis, while phosphorylation at S262 blocks the inhibitory effect. Thus these data with HEK293 cells are in agreement with previously published studies on cardiomyocytes {Doble et al., 2004}. As the S262 site was also able to regulate the inhibitory effect of Cx43CT, it is concluded that its effects on DNA synthesis are not dependent on channel-forming ability, and/or intercellular communication.

Regulation of Cx43, and Cx43CT growth inhibition by a PKC target site suggest that growth factors and oncogenes may overcome Cx43 growth inhibition by stimulating its phosphorylation by PKC. Thus the ability for enhanced inhibition of DNA synthesis when this phosphorylation step is

prevented makes the S262A Cx43 (or Cx43CT) mutants more effective candidates (compared to the wild type molecules) for consideration in situations where inhibition of DNA synthesis is desirable, as, for example, in tumor growth suppression strategies. Reversal of Cx43-mediated growth inhibition by phosphorylation at S262 furthermore points to a likely explanation for the sometimes conflicting data concerning the extent of growth suppression by Cx43 {Kardami et al., 2007a} since growth suppression may depend not as much on the absolute levels of Cx43 expression but rather on the ability of the cell to sustain phosphorylation (or lack of phosphorylation) at S262.

It would be important to dissect the molecular mechanism by which the S262 site at the C-tail of Cx43 regulates inhibition of DNA synthesis. The C-portion of Cx43 interacts with a number of proteins, including PKCε, PKCα, src, and other kinases, structural proteins (β-tubulin), and proteins with transcription-factor properties such as beta-catenin and NOV (Nephroblastoma overexpressed), several of which are implicated in growth regulation {Dang et al., 2006; Fu et al., 2004; Giepmans, 2004}. Interaction with the C-terminal of Cx43 may effectively decrease 'free' intracellular levels of any one of these proteins, and/or sequester them from their appropriate site(s) of action. In this scenario, the S262 site and its phosphorylation could alter protein or protein-complex binding properties of Cx43, allowing or preventing growth inhibition.

The channel forming ability of Cx43 resides at the N-terminal portion of the molecule, while the cytosolic C-terminal tail (Cx43CT) plays a regulatory role

on channel permeability but cannot form channels. A number of studies have investigated whether the growth inhibitory activity of Cx43 is linked with the channel-forming or the regulatory domains. While there are some reports indicating that the channel-forming domain of Cx43 can exhibit growth inhibitory activity {Moorby, 2000; Moorby and Gherardi, 1999; Omori and Yamasaki, 1999}, several laboratories have demonstrated that Cx43CT is capable of inhibiting cell proliferation {Gellhaus et al., 2004; Moorby and Patel, 2001; Zhang et al., 2003}. Data presented here are in agreement with the latter group, but do not exclude the possibility that both channel-forming and regulatory domains can independently exert inhibitory effects on DNA synthesis.

IV.B-2. Cx43-mediated inhibition of cardiomyocyte DNA synthesis is not dependent on downstream activation of TGFβ1 signal transduction

Both Cx43 and TGFβ1 exert inhibitory effects on cardiomyocyte DNA synthesis. Thus, it is reasonable to suspect that they may activate similar pathways. In agreement, a report by Dai and colleagues {Dai et al., 2007} indicated that Cx43 potentiates TGFβ1 signal transduction in the atrial cardiomyocyte cell line HL-1. They provided evidence that Cx43 could bind to the same site in microtubules as Smad2, and that it could in fact displace Smad2 from microtubules. As a consequence, increases in Cx43 expression would result in Smad2 being released from its binding site and becoming

available for phosphorylation, nuclear translocation, and activation of TGFβ1-responsive gene expression {Dai et al., 2007}. TGFβ1-responsive gene expression leads to suppression of proliferation {Medrano, 2003}.

Therefore, Cx43 was examined to see if it could exert its inhibitory effect under conditions where several components of TGFβ1-linked signal transduction (TGFβR1, TGFβRII, Smad2) were blocked. Inhibition at the level of TGFβR1 was achieved through the use of a potent pharmacological inhibitor, SB431542, which inhibits TGFRI (ALK5) by acting as a competitive ATP binding site kinase inhibitor with an IC50 value of 0.75μM {Inman et al., 2002}. In cardiomyocytes, (which express ALK5), SB431542 is effective in preventing downstream activation (phosphorylation) of Smad2 as shown by {Waghabi et al., 2002}, and confirmed here (Fig.II-4). This reagent, furthermore, increased cardiomyocyte DNA synthesis, validating its effectiveness in inhibiting endogenous and/or serum-derived TGFβ1 signaling via TGFβRI-Smad2. The data showed that the inhibitory effects of Cx43, as well as Cx43CT, on cardiomyocyte DNA synthesis do not require the activity of TGFβRI, as they were not prevented by SB431542. These data also strengthen the notion that inhibition of DNA synthesis by Cx43 and Cx43CT follow similar pathways.

To specifically target the activity of TGFβRII, which acts as a co-receptor for TGFβRI, a kinase deficient TGFβRII was overexpressed which has been shown to be very effective in inhibiting endogenous TGFβRII in a dominant negative fashion {Sheikh et al., 2004}. The data generated showed that the

inhibitory effects of Cx43 on DNA synthesis do not require the activity of TGFβRII as they were not affected by overexpression of the kinase-deficient receptor.

The next step was to directly inhibit downstream mediators (Smad2/3) involved in the TGFβ1 signaling pathway. Studies with HL-1 cells suggested the possibility that Cx43 may influence Smad2/3 activity, and Smad2/3-dependent gene expression even independently of TGFβRI {Dai et al., 2007}. To inhibit Smad2, DNSmad2, a mutated Smad2 which acts in a dominant negative fashion, was overexpressed {Uemura et al., 2005}. As also observed with the other inhibitors of TGFβ1-associated signal transduction, overexpression of DNSmad2 resulted in increased cardiomyocyte DNA synthesis, validating its effectiveness. These data showed that Cx43-mediated inhibition of DNA synthesis does not require Smad2 activity as it remained unaffected by DNSmad2 overexpression.

These observations are in apparent contrast with those by Dai et al {Dai et al., 2007} who reported activation of Smad2 by Cx43 in HL-1 cells. These differences may reflect differences between cells used (the mouse atrial HL-1 cell line versus rat primary ventricular cardiomyocytes). Also, as these authors did not examine effects on DNA synthesis it is possible that Smad2 activation, and or Cx43 expression, resulted in the same endpoints (DNA synthesis) in their system.

The finding in this thesis that blocking Smad2 increased baseline cardiomyocyte DNA synthesis indicated that Smad2 mediates TGFβ1 induced

inhibition of DNA synthesis in cardiomyocytes. On the other hand, Smad2 activity was not a pre-requisite for the Cx43 mediated inhibition. There could be other proteins or other signaling pathways that could be mediating Cx43 induced inhibition of DNA synthesis.

Taken together these findings showed that Cx43-mediated inhibition of cardiomyocyte DNA synthesis does not require downstream activation of signals associated with the TGFβ1 pathway. They do not, however, exclude the possibility that TGFβ1 signaling may lie upstream of Cx43-mediated inhibition. Some evidence in support of the latter was provided by our finding that TGFβ1 blocked the FGF-2-induced Cx43 phosphorylation at S262 (Fig.II-10)

It was previously shown that TGFβ1 inhibits the FGF-2 induced cardiomyocyte proliferation {Kardami, 1990}. In fact, inhibition of TGFβ1 signaling potentiates FGF-2 induced cardiomyocyte proliferation {Sheikh et al., 2004}. The exact mechanism by which TGFβ1 prevents FGF-2 (mitotic) signaling in cardiomyocytes has not been characterized. The data from this thesis suggest that Cx43 may act as a point of cross-talk between a mitotic (FGF-2) and anti-mitotic (TGFβ1) pathway. By promoting its phosphorylation at S262, FGF-2 can cancel Cx43-mediated inhibition of DNA synthesis, in agreement with data shown here and previously reported {Doble et al., 2004}. By preventing the FGF-2-induced phosphorylation at S262, TGFβ1 can maximize the growth inhibitory activity of Cx43, again as shown here and in

{Doble et al., 2004}, and thus prevent FGF-2-induced cardiomyocyte DNA synthesis.

It is not known as yet how TGFβ1 blocks Cx43 phosphorylation at S262. One can speculate that it might prevent the FGF-2-induced PKCε activation, which mediates both Cx43 phosphorylation at S262 and the mitotic stimulation of cardiomyocytes. Some evidence for antithetical signaling between TGFβ1 and PKCε was provided suggesting that lack of PKCε (in PKCε-null mice) allowed development of TGFβ1-dependent fibrosis {Meier et al., 2007}. TGFβ1 was suggested to inhibit FGF-2 induced corneal endothelial layer cell proliferation via inhibition of PI3/AKT pathway that mediates proliferation {Lu et al., 2006}. Akt also phosphorylates Cx43 {Park et al., 2007} and this may potentially be a pre-requisite step before the phosphorylation of Cx43 at S262 by FGF-2/PKCε as we had shown that other sites are phosphorylated before the S262 phosphorylation. Alternatively, TGFβ1 may alter protein-protein interaction involving the C-tail of Cx43, effectively masking the S262 site and preventing it from becoming phosphorylated.

Control of cardiomyocyte proliferation plays an important role in cardiac embryonic and neonatal development and also under conditions of cardiac repair post-injury. Studies have shown the presence of resident cardiac stem cells and also local population of amplifying cells with characteristics of immature cardiomyocytes that are capable of proliferation {Anversa, 1998; Beltrami et al., 2003; Leri et al., 2008; Matsuura et al., 2004; Oh et al., 2003}. Even certain populations of adult cardiomyocytes may be capable of entering

the cell cycle {Srinivas et al., 2009}. As reviewed in the introduction all these cell populations can be stimulated by FGF factors to enter the cell cycle. It is highly likely that expression and phosphorylation of cardiomyocyte Cx43 under the influence of the local environment of cytokines and growth factors, plays an important role in modulating DNA synthesis all these situations. The finding presented here could apply in a scenario of myocardial injury where increase in TGFβ1 could potentially prevent the FGF-2 induced phosphorylation of Cx43 leading to inhibition of FGF-2 induced proliferation of responsive myocyte populations. Thus the studies presented in this thesis suggest that by controlling the phosphorylation of Cx43 at S262 one could regulate cell proliferation in the heart and potentially facilitate or prevent cardiac regeneration.

IV.C. CONCLUDING REMARKS-FUTURE STUDIES

The studies presented here show that Cx43, as well as its phosphorylation at S262 play important roles both in promoting increased cardiomyocyte resistance to injury and also in permitting stimulation of DNA synthesis. Both of these activities are mediated by PKCε which interacts with and phosphorylates Cx43 at S262. Thus it would appear that Cx43 is an important mediator of FGF-2-PKCε activities in cardiomyocytes.

It was found that removal of the channel forming domain, in Cx43CT, eliminated the cytoprotective properties of moderate Cx43 overexpression, but preserved the ability for inhibition of DNA synthesis as well as for reversal of that inhibition via (simulated) phosphorylation at S262. Thus Cx43-mediated cytoprotection likely requires membrane targeting and channel/hemi-channel formation, while the Cx43-mediated inhibition of DNA synthesis appears to be independent of intercellular communication or membrane targeting.

As my studies were conducted using *in vitro* models it would be important to validate these findings using *in vivo* models. One such possibility would be to create transgenic mouse models with conditional, cardiomyocyte-specific expression of S262A-Cx43 to determine if hearts from these mice would be capable of raising their resistance to injury in response to ischemic preconditioning or FGF-2 administration. After induction of S262A-Cx43 overexpression, isolated hearts from these animals could be examined *ex vivo* (in a Langendorff perfusion setting) for their ability to develop a protected

phenotype against ischemia-reperfusion induced tissue damage, cell death, and contractile dysfunction, in response to FGF-2, or ischemic preconditioning. Myocardial infarction, furthermore, would be expected to result in greater tissue damage in the S262A-Cx43 overexpressing mice.

Another transgenic mouse model could be created with conditional, cardiomyocyte-specific, overexpression of S262D-Cx43, which simulates constitutive phosphorylation at S262. These mice would be subjected to myocardial infarction, as well as transient (2-7 days) activation of S262D-Cx43 overexpression in myocytes. It would be expected that, as observed *in vitro*, S262D overexpression would lift the Cx43-mediated inhibition of DNA synthesis in cardiomyocytes, and result in an enhanced regenerative response.

Several questions remain to be addressed. Understanding the effect of Cx43 phosphorylation at S262, and other PKC target sites on subcellular localization, interaction with other proteins, susceptibility to proteolytic degradation, and overall turnover would provide a more complete picture as to the potential mechanism of action of Cx43 and its phosphorylation in the heart.

Finally, it would be important to elucidate the signalling mechanisms by which TGFβ1 inhibits both the FGF-2 induced (i) Cx43 phosphorylation at S262 and (ii) cell proliferation. As a first step, one could examine whether TGFβ1 prevents the FGF-2-induced activation of PKCε, and/or alters the interaction of Cx43 with PKCε or other proteins. One possible explanation as to how TGFβ1 prevents Cx43 phosphorylation at S262 is that it causes the

interaction of Cx43 with another protein or protein complex resulting in the masking of the S262 site, which becomes inaccessible to PKC. Proteomics-based analyses of Cx43 protein-protein interaction in the presence and absence of TGFβ1 stimulation would be a first step in addressing this possibility.

CHAPTER VI: REFERENCES

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