FUNCTIONS OF CONNEXIN 46 IN LENS AND SOLID TUMORS DURING HYPOXIA

by

SAMUEL A. MOLINA

B.S., Michigan State University, 2005

AN ABSTRACT OF A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Graduate Biochemistry Group
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2012

Abstract

Eukaryotic cells possess a unique way to communicate with each other by passing metabolites and small molecules through protein pores that connect adjacent cells. Although there are many types and families of protein pores, connexins comprise a unique family. Six connexin monomers assemble into a hemichannel, which is transported to the cell membrane. An opposing cell membrane containing compatible connexin hemichannels is located and connected, forming an intercellular dodecameric protein complex. This results in a protein channel that connects two separate cytoplasmic compartments to each other. This type of channel is known as a gap junction.

Connexin expression and function is commonly tissue specific. Of the 21 known human connexins, less than half are currently well characterized. Three connexins are expressed in the lens, connexin 43 (Cx43), 46 (Cx46), and 50 (Cx50). Of these three, Cx46 and Cx50 both have major functions in the mature lens. Cx46 functions as a major gap junction channel, which maintains mature lens homeostasis, while Cx50 possesses growth control properties in the lens. Cx46 expression is modulated in breast and bone tumors, and during ischemia.

It is hypothesized that Cx46 provides resistance to hypoxia mediated cell death by prolonging survival. In this study, Cx46 expression was detected in human Y79 retinoblastoma cells. Decreasing the expression of Cx46 in nude mice carrying Y79 xenografts slowed early stage tumor growth. Y79 cells in culture survive for over 72 hours in 1% oxygen *in vitro*. C46 was upregulated in cultured lens cells when grown under hypoxia. Human lens epithelial cells, rabbit N/N1003A lens cells, and Y79 cells proliferated in 1% oxygen until Cx46 expression was depleted by use of siRNA. Protection from hypoxia-induced cell death was provided by transfection with the C-terminus of Cx46. We further determined that the promoter activity of Cx46 was increased in 1% oxygen. These results indicate that Cx46 would increase in response to hypoxia and suggest a role for Cx46 in protection from hypoxia. The studies demonstrate a novel function for Cx46 in cell survival during hypoxia.

FUNCTIONS OF CONNEXIN 46 IN LENS AND SOLID TUMORS DURING HYPOXIA

by

SAMUEL A. MOLINA

B.S., Michigan State University, 2005

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Graduate Biochemistry Group
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2012

Approved by:

Major Professor
Dolores J. Takemoto

Abstract

Eukaryotic cells possess a unique way to communicate with each other by passing metabolites and small molecules through protein pores that connect adjacent cells. Although there are many types and families of protein pores, connexins comprise a unique family. Six connexin monomers assemble into a hemichannel, which is transported to the cell membrane. An opposing cell membrane containing compatible connexin hemichannels is located and connected, forming an intercellular dodecameric protein complex. This results in a protein channel that connects two separate cytoplasmic compartments to each other. This type of channel is known as a gap junction.

Connexin expression and function is commonly tissue specific. Of the 21 known human connexins, less than half are currently well characterized. Three connexins are expressed in the lens, connexin 43 (Cx43), 46 (Cx46), and 50 (Cx50). Of these three, Cx46 and Cx50 both have major functions in the mature lens. Cx46 functions as a major gap junction channel, which maintains mature lens homeostasis, while Cx50 possesses growth control properties in the lens. Cx46 expression is modulated in breast and bone tumors, and during ischemia.

It is hypothesized that Cx46 provides resistance to hypoxia mediated cell death by prolonging survival. In this study, Cx46 expression was detected in human Y79 retinoblastoma cells. Decreasing the expression of Cx46 in nude mice carrying Y79 xenografts slowed early stage tumor growth. Y79 cells in culture survive for over 72 hours in 1% oxygen *in vitro*. C46 was upregulated in cultured lens cells when grown under hypoxia. Human lens epithelial cells, rabbit N/N1003A lens cells, and Y79 cells proliferated in 1% oxygen until Cx46 expression was depleted by use of siRNA. Protection from hypoxia-induced cell death was provided by transfection with the C-terminus of Cx46. We further determined that the promoter activity of Cx46 was increased in 1% oxygen. These results indicate that Cx46 would increase in response to hypoxia and suggest a role for Cx46 in protection from hypoxia. The studies demonstrate a novel function for Cx46 in cell survival during hypoxia.

Table of Contents

Table of Contents v
List of Figures ix
List of Tables xi
Acknowledgements xii
Dedication xiii
Preface xiv
Chapter 1 - Literature Review 1
Eukaryotic Multicellular Communication 1
Indirect Cell-to-Cell Communication – Paracrine and Endocrine Signaling 1
Direct Cell-to-Cell Communication – Juxtacrine Signaling: Cell Surface Proteins and Cellular Junctions 2
The Connexin Protein Family 3
Connexin Classification and Nomenclature 3
Transcription and Expression of Connexin Genes 4
Connexin Protein Structure 8
Biosynthesis and Trafficking of Connexins 10
Connexins Form Functional Transmembrane Protein Channels 13
Regulation of Connexin Function 16
Cellular Functions of Connexins in Various Tissues and Associated Diseases 16
Functional Role of Connexins in the Lens 19
Signals and Metabolites that Traverse Connexin Channels 23
Ischemia, Hypoxia and Effects Thereof 24
Hypoxia Inducible Factors Regulate the Cellular Hypoxic Response 25
Cellular Changes Common to Hypoxic Insults 27
Prolonged Cellular Adaptation Strategies to Hypoxia in the Solid Tumor and Lens 29
Model Systems Used for the Study of Hypoxic Effects of Connexin 46 – The Lens and the Solid Tumor 30
Lens as a Physiological Model for Cellular Hypoxic Response 30
The Y79 Solid Tumor as a Model of Connexin Biology in Cancer 31

Aim of Dissertation 32
References 33
Chapter 2 - The Anti-Tumor *In Vivo* Function of Connexin 46 in a Human Y79 Retinoblastoma Xenograft Tumor Model 43
Abstract 43
Introduction 43
Materials and Methods 46
Cell Culture 46
Hypoxia and Cell Viability Assay 46
Y79 siRNA Knockdown and Viability Studies 47
Transplantation of Human Y79 Retinoblastoma Cells into Nude Mice 47
Western Blot 48
Statistical Analyses 49
Results 50
Cx46 Expression in Y79 Retinoblastoma Cells in vitro 50
Transfection with Anti-Cx46 siRNA Negatively Affects Y79 Hypoxic Cell Growth in vitro 51
in vivo Proof-of-Concept and Dosage Pilot Study 56
Cx46 siRNA Suppresses Y79 Tumor Growth in vivo 59
Discussion 63
Acknowledgements 68
References 69
Chapter 3 - The Promoter of Human Connexin 46 is Oxygen Sensitive 72
Abstract 72
Introduction 72
Materials and Methods 77
Cell Culture and Hypoxic Treatments 77
Construction of GJA3 Promoter 77
Promoter-Linked Luciferase Studies 77
5' Rapid Amplification of cDNA Ends 78
Results 79

In silico Analysis of the Genomic Structure of GJA3 79
Luciferase-based Promoter Analysis under Hypoxia..... 81
5'-RACE Identifies Multiple GJA3 mRNA Species 83
DNA Sequence Analysis..... 86
Discussion..... 86
Acknowledgements..... 88
References..... 89
Chapter 4 - The Carboxy Terminal Tail Domain is Essential for Cx46 Function in Lens Cells.. 93
Abstract..... 93
Introduction..... 94
Materials and Methods..... 95
Cell Culture..... 95
Cloning and Transfection..... 96
Whole Cell Homogenate (WCH) Preparations..... 97
Western Blotting and Antisera..... 97
Immunofluorescent Labeling and Fluorescent Microscopy 97
siRNA Transfection 98
Statistical Analyses 98
Results..... 99
siRNA Mediated Knockdown of Endogenous Cx43 Results in Increased Cx46 Protein Levels in HLEC 99
Overexpression of Cx46 Causes a Decrease in Cx43 Protein Levels in N/N1003A Rabbit Lens Cells..... 101
Cx46 is Predominantly Localized to the Intracellular Compartments in N/N1003A and HLEC Lens Cells 103
The Carboxy-Terminal Tail Domain of Cx46 is Necessary and Sufficient for Induction of Cx43 Degradation 111
Discussion..... 114
Acknowledgements..... 117
References..... 118
Chapter 5 - Summary and Conclusions of Work Presented 122

Abbreviations Used.. 125

List of Figures

Figure 1.1 Schematic Overview of Multicellular Signaling 2
Figure 1.2 The Topology of Pannexin, Innexin, and Connexin Protein Families 10
Figure 1.3 The Life Cycle of Connexin Proteins 12
Figure 1.4 Connexins Form Functional Membrane Channels from Two Connexon Hemichannels 14
Figure 1.5 A Schematic of the Vertebrate Lens and Connexin Expression Profile 20
Figure 1.6 Schematic of HIF-Mediated Oxygen Sensing 27
Figure 2.1 Y79 Cells Express Cx46 and Thrive Under Hypoxic Conditions 50
Figure 2.2 Measured Volumes (mm^3) of Individual Xenograft Tumors Used in this Study 52
Figure 2.3 Y79 Cells Exhibit Normal Growth Characteristics in 1% Oxygen 53
Figure 2.4 Optimization of siRNA-Mediated Knockdown of Cx46 in Y79 Cells 54
Figure 2.5 Western Blot of siRNA Knockdown in Y79 Cells *in vitro* 54
Figure 2.6 Effects of Anti-Cx46 siRNA Treatment on Y79 Cell Viability and Proliferation Under 20% and 5% Oxygen 56
Figure 2.7 Results of Anti-Cx46 siRNA Dosing and Proof-of-Concept *in vivo* Study 57
Figure 2.8 Intratumor Cx46 siRNA Suppresses the Growth of Y79 Tumors *in vivo* 59
Figure 2.9 Gross Examination of Excised Y79 Xenografts 61
Figure 2.10 Tumor Volume of Cx46 siRNA, Non-Silencing siRNA, and No siRNA/Sham Treatment Groups in Mice Transplanted with Y79 Cells 62
Figure 2.11 Decreased Expression of Cx46 with a Reciprocal Increase in Cx43 Protein Expression in Y79 Tumors of Mice treated with Cx46 siRNA 64
Figure 2.12 Quantitative Protein Expression Densitometry of Cx46 and Cx43 Protein Levels in Representative Tumors Shown in Figure 2.11 65
Figure 2.13 Decreased Y79 Tumor Cell Burden in Mice Treated with Cx46 siRNA 67
Figure 3.1. Predicted Transcription Factor Binding Sites in the Human Connexin 46 Promoter as Analyzed with SA Biosciences TF Prediction Web Software 75
Figure 3.2. Mouse Cx46 mRNA is Present in the 17 Day Old Adult and the E14.5 Developing Embryo 76
Figure 3.3 Genomic, Transcript and Tested Promoter Schematic 79

Figure 3.4. Schematic of the Human Cx46 Promoter 80
Figure 3.5 Non-normalized Cx46 Promoter Response to 21% and 1% Oxygen in Cell Culture 82
Figure 3.6. The Cx46 Promoter Responds Quickly and Efficiently to 1% Oxygen 83
Figure 3.7. 5'RACE RNA Quality and PCR Control Reactions 84
Figure 3.8. Electrophoretic Analysis of 5'-RACE Products Produced by Using HLEC Total RNA and Cx46-Specific Primers 85
Figure 4.1 siRNA-Mediated Knockdown of Cx43 Results in Increased Cx46 Protein Levels .. 100
Figure 4.2 Overexpression of Cx46 Causes a Reduction in Cx43 Protein Levels in Rabbit Lens Epithelial N/N1003A Cells 102
Figure 4.3 Intracellular Localization of Rat Cx46-EGFP in Mouse N2A Cells 104
Figure 4.4 Intracellular Localization of Rat Cx46 Protein Mutants Labeled with EGFP in Human Lens Epithelial Cells 105
Figure 4.5 TPA-Induced Changes in Localization of Cx43 and Cx46 in HLECs 106
Figure 4.6 Cx46 Protein is Predominantly Localized to the Intercellular Compartments in Lens N/N1003A Cells 108
Figure 4.7 Protein Sequence Alignment of Rat Cx43, Cx46, and Cx50 and Schematic of Cx46 Mutants 110
Figure 4.8 The C-Terminal Tail Domain of Cx46 is Required for Cx43 Degradation 112
Figure 4.9 Effect of Cx46, Cx50 and the Cx46-Tail Deletion Mutant on Cx43 Protein Levels in N/N1003A Cells 113
Figure 4.10 Western Blot of the Expression Pattern of Various Cx46 Tail Deletion Mutants... 114
Figure 5.1 Proposed Regulatory Mechanism of Action between Cx43 and Cx46 in the Lens .. 124

List of Tables

Table 1.1 Connexin Gene Families 4
Table 1.2 Expressed Connexins in Human 8
Table 1.3 Known Connexin Channel Forming Interactions 15
Table 2.1 Calculated Volumes (mm^3) of Individual Xenograft Tumors Used in this Study 58

Acknowledgements

I wish to thank my major professor, Dr. Dee Takemoto, for all that she has taught me over the few years in her lab. I really have learned many life lessons, lessons about being a life science researcher and professor, and what it's like being a California Democrat. I will surely miss having discussions of various topics with you. I give many thanks to the many people in the Department of Biochemistry front office (Crystal Sapp, Rebecca Darkow-Rodriguez and Melinda Bainter, et al.) who have made my graduate career run smoothly. Thanks to my department head, Dr. Michael Kanost, and to my committee members Dr. John Tomich, Dr. Michal Zolkiewski, and Dr. Sherry Fleming for their support, ideas, criticisms, mentorship, and collegiality. Special thanks to Michal for letting me take up office and lab space for my final year of training. I would like to thank my friends here at KSU that have made my graduate life enjoyable and memorable. Friends come and go in the university setting, so I cherish the long lasting friendships we have developed throughout the years. Lastly, I thank my family for the love and support when times were tough, for being there whenever I needed them, and for their unwavering encouragement and understanding of my educational and personal life goals.

Dedication

I dedicate this work to my loving family whom has always been my rock. I love you all.

Preface

"While it has been billions of years since the earth adopted oxygen as the substrate of life and hundreds of years since the scientific discovery of O_2, our understanding of biological responses to hypoxia and their medical significance has greatly increased only within the last several decades. The rapid progress being made on so many fronts is remarkable and exciting. However, many challenges remain, the most obvious one being how to apply our growing knowledge of oxygen biology to the benefit of humankind, especially those who are suffering from chronic diseases such as cancer and cardiovascular diseases."

–Zhang and Semenza, 2008. (1)

Chapter 1 - Literature Review

Eukaryotic Multicellular Communication

Eukaryotic multicellular organisms communicate via a wide range of modalities. Diffusion works best in small multicellular organisms like insects, which do not have active transport mechanisms. The eukaryotic invertebrate *C. elegans* does not have a circulatory system but shares much of the same physiology of higher eukaryotes. In this organism, cell-to-cell communication is promoted by a family of cell adhesion and integral membrane proteins called the innexins. Innexins are believed to be the invertebrate version of connexins, both of which form functional gap junctions to exchange signals and metabolites (2, 3). Invertebrates possess both innexins and pannexins, while vertebrates possess both pannexins and connexins (3, 4). Connexins, claudulins, occlaudulins, adherens, and other tight junction proteins are all integral membrane proteins (5). Only two protein families form passive intercellular channels in vertebrates, the connexins and the pannexins. Pannexins and innexins are structurally similar to connexins, but differ in their primary amino acid sequences, thus making them different families of proteins (3). Connexin signaling is important for endocrine signaling throughout the body (6, 7). The focus of this thesis is on connexin signaling and function, especially during hypoxia.

Indirect Cell-to-Cell Communication – Paracrine and Endocrine Signaling

Two forms of cellular signaling are important for vertebrate communication, near-range and long-range signaling (Figure 1.1). Signals generated by one cell type that are received extracellularly by a nearby but different type of cell are of the paracrine signal type. Signals created by one cell type but received extracellularly by a different cell type that is in a distant location is an example of endocrine signaling. Both types of signals must be released from a cell and traverse the extracellular milieu, be taken up by and then distributed by a transport system similar to the lymph or circulatory system to be effective. Extracellular signals can be recognized by extracellular receptors, actively taken up by transporters, or diffuse freely into the cell and bind intracellular receptors, which activate or inhibit cellular responses. Pannexins are reported to participate primarily in paracrine signaling through open hemichannels (8, 9). This is distinct from the connexins, which often do not form open or functional hemichannels at the cell membrane (10, 11).

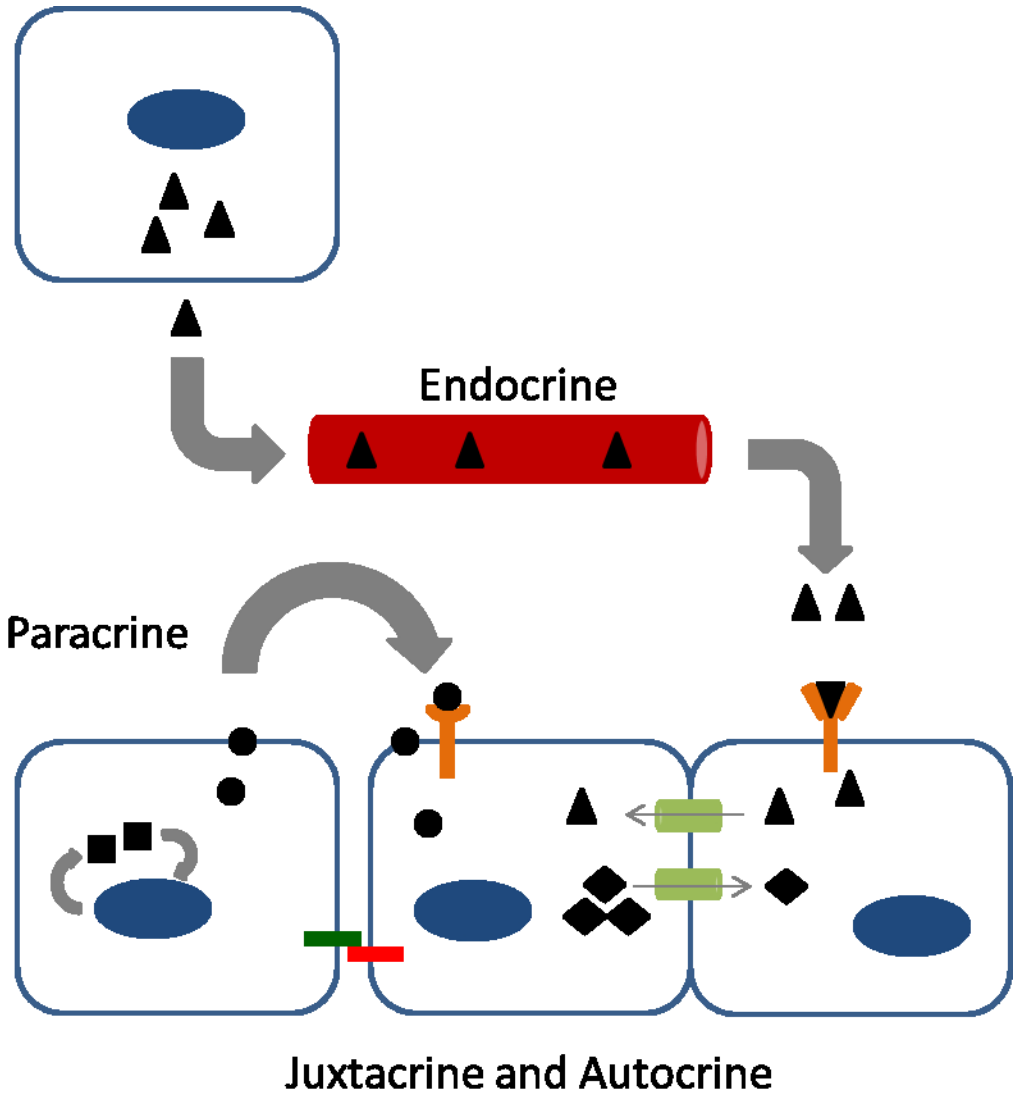

Figure 1.1 Schematic Overview of Multicellular Signaling
Multicellular organisms use a variety of signaling mechanisms. Endocrine signals are produced in one cell type then transported to a distant cell type via the bloodstream or a lymph system, and are often received in a receptor-mediated fashion. Paracrine signals are produced by cells and affect nearby cells of different types. Autocrine signals produced by one cell affect the same cell or the same neighboring cell type. Juxtacrine signals can be of the autocrine type, but generally involve physically interacting membrane proteins, which cause signal to be transduced within the cell. Gap junctions generally participate in autocrine and juxtacrine signaling.

Direct Cell-to-Cell Communication –

Juxtacrine Signaling: Cell Surface Proteins and Cellular Junctions

Direct cell-to-cell communication can occur by two distinct mechanisms, autocrine signaling and juxtacrine signaling (Figure 1.1). Autocrine signaling occurs when a cell produces a signal that acts upon the same cell type locally. These signals can be released into the

extracellular matrix by transport protein systems, by vesicle release, or can pass to the adjacent cell through gap junction channels. Autocrine signaling is often used in conjunction with gap junction channels (6, 12). Juxtacrine signals are produced or received by cells that are in direct contact with each other. Examples of juxtacrine signaling include the Notch signaling pathway, in which two cell membrane-localized receptors must physically interact to pass the signal; mechano-sensitive cell membrane channels, which release ions in response to stress; and, the signals that traverse gap junctions, such as calcium, in the heart. One can realize the importance of gap junctions in relaying juxtacrine signals when observing heart activity. Gap junctions, comprised of Cx43, can pass calcium signals that modulate heartbeat. Juvenile $Cx43^{-/-}$ mice do not survive much past birth and the individual muscles of the heart cannot beat in a coordinated manner, which leads to death (13).

The Connexin Protein Family

Connexin Classification and Nomenclature

Connexin classification and nomenclature is comprised of a gene name based on protein similarity, and the molecular weight of the connexin protein as predicted by its DNA sequence. Connexin proteins are divided into four categories, alpha, beta, gamma, and delta connexins. Each connexin gene is named for its subfamily and similarity, e.g. the human Connexin 46 gene is named *GJA3*, which is short for gap junction alpha 3. GJA3 protein has a predicted weight of 46 kDa and is called hCx46 in the literature. Mouse Cx46 is denoted as mCx46 and *gja3*. GJB3, for example is a beta connexin; GJC3 a gamma connexin; GJD1 a delta connexin. Alpha and beta connexins share high amino acid homology with other alpha and beta family members, respectively. Gamma and delta connexins are comprised of connexins that do not share high homology with alpha or beta or each other. The epsilon group of connexins contains only Cx23. Identification of all known connexin genes was accomplished by the availability of high quality nucleic acid sequence data. The nomenclature is consistent with the declarations of the International Gap Junction Conference in Elsinore, Denmark, 2007 (14) and the HUGO database for gap junction proteins (http://www.genenames.org/genefamilies/GJ) as of January 2012. The connexin gene families are given in Table 1.1.

Alpha	Beta	Gamma	Delta	Epsilon
Cx37	Cx25	Cx30.2	Cx31.9	Cx23
Cx40	Cx26	Cx45	Cx36	
Cx43	Cx30	Cx47	Cx40.1	
Cx46	Cx30.3			
Cx50	Cx31			
Cx59	Cx31.1			
Cx62	Cx32			

Table 1.1 Connexin Gene Families
There are 5 gene families of connexins based on amino acid sequence similarity. Data taken from the NCBI database, which can be found at http://www.ncbi.nlm.nih.gov/.

Transcription and Expression of Connexin Genes

The rodent genome contains 20 confirmed, distinct and expressed connexin genes (15). The human genome contains 21 confirmed connexin genes and an unusual intron-less Cx43 pseudogene. General transcription processes are assumed to control the expression of all connexins. Connexin expression is highly regulated in a spatial-temporal manor during many stages of physiological need including embryonic development (15-17), cellular health and tissue maintenance (15, 18-21), wound healing (22-24), and cancer progression (15, 19, 25-27). Connexin gene expression is limited by the transcription factors that are expressed in a given cell and is reviewed below. Connexins can be controlled individually by proteins involved in the cell cycle and often influence the control of the cycle as well (5-7, 28). Each tissue, and more specifically each cell type, can express a different combination of connexin genes to achieve the observed effect. Each channel type has distinguishable electrophysiological characteristics and mixed connexin gap junctions can be distinguished from homomeric channels (29-31).

Cx32 and Cx43 are two of the most ubiquitously expressed connexins. Common and confirmed transcription factors that contribute to the expression of connexins in most tissues are SP1/SP3 and Activator Protein 1. Cx43 is regulated by cAMP response elements and the Wnt and Ras-Raf-MAPK pathways (15). Since each connexin is expressed in only a subset of tissues, they are regulated by cell type specific transcription factors. Examples include the cardiac Nkx2-5 TF that regulates the cardiac expression of Cx40, Cx43 and Cx45; activation and repression of Cx43 and Cx40 expression by the cardiac T-box TFs; GATA-4 that influences

heart, lung, liver, gonad, and gut specific gene expression, and does this with Cx40; liver-specific HNF-1, which controls the expression of Cx32 and Mst-1 that aids in general organ development and influences Cx32 expression; Sox10 and Egr2/Krox20, which strongly regulate Cx31 and Cx32 in neuronal cells; estrogen, thyroid and parathyroid hormones that influence Cx43 expression in skeletal muscle; neuron-restrictive silencing factor that controls Cx36 in neurons; ciliary neurotrophic factor and related receptor control of Cx43 and Cx30 in the central nervous system (15); sheer stress in endothelial cells, which causes both a transient and long lived Cx40 mRNA up-regulation (32). Table 1.2 illustrates the known roles of each connexin in human physiology based on confirmed information found in the NCBI databases as of January 2012.

The GJA3 gene, which encodes Cx46 protein, has a unique gene structure. The average human gene contains 8-10 exons and 6-9 introns. Roughly 5% of all introns in the genome are longer than 200,000 bp and 10% of all introns are larger than 11,000 bp (127). The connexin family of genes generally contains 2 exons and a single intron. The sole connexin 46 intron contains ~17,500 bp while exon 1 contains only the 5' UTR, exon 2 contains the end of the 5' UTR, the protein coding sequence, and the 3' UTR.

Connexin Gene	Connexin Name	Tissue Location	Ascribed Function	Related Disease
GJA6P	**Pseudogene**	X-linked, Not Expressed	Unknown	Unknown
GJE1	**Cx23**	Buccal cells - Ear	Unknown	Nonsyndromic hearing loss
GJB7	**Cx25**	Placenta[1]	Unknown	Unknown
GJB2	**Cx26**	Inner Ear; hepatocytes, astrocytes, pancreas, testis[2]	Ion homeostasis	Pre-lingual deafness
GJB6	**Cx30**	Inner Ear; Skin/Follicles; Intestine; astrocytes, skin, kidney, mammary gland[2]	Ion homeostasis	Deafness; Hidrotic ectodermal dysplasia 2 (alopecia); upregulated in gastric cancer
GJC3	**Cx30.2**	Inner Ear; pancreas, kidney, skeletal muscle, liver, placenta[1]; Neurons, conductive myocytes, testis[2]	Ion homeostasis	Nonsyndromic hearing loss
GJB4	**Cx30.3**	Skin; Thymocytes; Inner Ear	Unknown	Erythrokeratoderma without ataxia; hearing impairment
GJB3	**Cx31**	Inner Ear; Skin; Thymocytes; Neurons, testis[2]	Proper epidermal keratinization; Neurite outgrowth	Nonsyndromic deafness with neuropathy; Erythrokeratodermia variabilis
GJB5	**Cx31.1**	Skin[2], Upper aerodigestive tract; Placenta	Epidermis development; placental labyrinthine layer morphogenesis, placental spongiotrophoblast	Gastric cancer

			differentiation	
GJD3	**Cx31.9**	Vascular smooth muscle cells; Cardiac tissue	GJIC	Unknown
GJB1	**Cx32**	Gastric tissue, Endothelial cells; oligodendrocytes, pancreas, testis and ovary, mammary gland, liver, kidney[2]	Increases intercellular adhesion; maintenance of vascular function; Regulates cytokine expression	CNS inflammation; X-linked Charcot-Marie-Tooth disease; Gastric adenocarcinoma
GJD2	**Cx36**	CNS; Retina; Inferior olive of brainstem; Pancreatic beta cells; adrenal gland	Passage of positively charged molecules	Refractive errors; myopia; Juvenile myoclonic epilepsy;
GJA4	**Cx37**	Cardiac tissue; Platelets; Kidney glomeruli, ovary[2]	Myoendothelial nitric oxide release; Platelet reactivity	Increased risk of atherosclerosis and myocardial infarction; Increased ischemic damage spread; Tumor cell proliferation
GJA5	**Cx40**	Cardiac tissue; Kidney glomeruli	Unknown	Atrial fibrillation
GJD4	**Cx40.1**	Pancreas, kidney, skeletal muscle, liver, placenta, heart[1]	Unknown	Unknown
GJA1	**Cx43**	Ubiquitous; Cardiac; Lens, Skeleton	Electrical conductance; Tissue homeostasis; Cancer activity	Occulodentodigital dysplasia; Heart malformations; Associated with cancer progression
GJC1	**Cx45**	Smooth muscle; Myofibroblasts; Colon;	GJIC; muscle contraction; synaptic	Colorectal cancer silences GJC1 expression

		Retina; Glomeruli, Bone	transmission; vasculogenesis; visual perception	
GJA3	**Cx46**	Lens; Bone	Tissue homeostasis and lens clarity	Zonular pulverulent cataract type 3
GJC2	**Cx47**	CNS; astrocytes and oligodendrocytes	Central and peripheral myelination, GJIC	Pelizaeus-Merzbacher-like disease-1; lymphedema
GJA8	**Cx50**	Lens; germ cells[2]	Growth regulation; GIJC; Ion homeostasis	Zonar pulverulent cataracts; nuclear progressive cataracts; cataract-microcornea syndrome
GJA9	**Cx59**	Retina; testis, skeletal muscle[1]	Unknown	Distortion of visual perception
GJA10	**Cx62**	Retina; skeletal muscle, heart[1]	Unknown	Distortion of visual perception

Table 1.2 Expressed Connexins in Human
Data taken from NCBI database, [1]Sohl et al., 2003 (33) and [2]Bedner et al., 2011 (34). Notice the varied distribution of connexins in human tissues and the general attribution of ion or tissue homeostasis. Other non-channel functions of connexins remain to be identified.

Connexin Protein Structure

All connexin proteins have a common 9 domain structure. Each connexin contains an amino-terminal leader domain (AT), four intermembrane alpha helix domains (TM1-4), two extracellular loop domains (EL1-2), one intracellular loop domain (IL) and a carboxyl-terminal tail domain (CT). EL1 and EL2 from each connexin protein form the 12 nodules of the docking face of the hexameric hemichannel. Each extracellular loop contains at least one disulfide bridge proposed to stabilize the docking interface, highlighted by blue circles in Figure 1.2 (6). The cysteines of the extracellular loops are highly conserved for the type of channels that gap junctions and the related pannexins and innexins form. The amino-terminal domain contains a secretory signal sequence cleavage site predicted to occur between residues 41 and 42 ($AA_{41}/E_{42}D$) by using both neural network and hidden Markov model modes of prediction trained on eukaryotes on the SignalP webserver (35). This signal is not predicted on the

SecretomeP webserver, which predicts likelihood of a protein being secreated by non-classical secretion, such as with growth factors (36). This would be expected of connexins since they are integral membrane proteins. Connexins retain their signal sequences and are not cleaved upon insertion into the ER membrane, unlike other membrane proteins such as cadherins or G-protein coupled receptors (GPCRs). The current hypotheses for the retention of the signal sequence is that it is used to alter gap junction gating and coupling in either a physical or electrostatic manner, or is used to direct post-translational insertion into the ER/Golgi membrane (37, 38). Interestingly, when both alpha and beta (Cx43 and Cx32, respectively) connexins were expressed *in vitro* using cRNAs as the source template in a liver-dervived microsome supplemented reticulocyte assay system, the signal sequences were recognized and, dependent on the signal recognition particle, subsequently cleaved by signal peptidase. However, when these connexins were isolated from tissue sources the signal peptide was retained suggesting that connexins can follow the classical eukaryotic secretion system but fail to do so *in vivo* because of an unknown signal peptidase protection event (37, 39). To date, the molecules needed for proper folding and membrane insertion of connexins remain to be identified.

Connexins and innexins are non-glycosylated membrane proteins unlike the pannexins, which contain glycosylation consensus sequences and are glycosylated. It is proposed that glycosylation prevents gap junction formation in the pannexin family (6). Although connexins pass through the endoplasmic reticulum and the Golgi apparatus, they are not glycosylated but are phosphorylated during the normal life cycle (40). Post-translational modifications that affect hemichannel and gap junction activity include phosphorylation, pH/electrostatic and redox effects, and S-nitrosylation (7, 41, 42). The majority of modifications are made to the CT domain of the connexins. The CT domain harbors the most sequence variability between each connexin and provides a way of molecular signal transduction and gating, which is different from the voltage gating properties that all connexins possess.

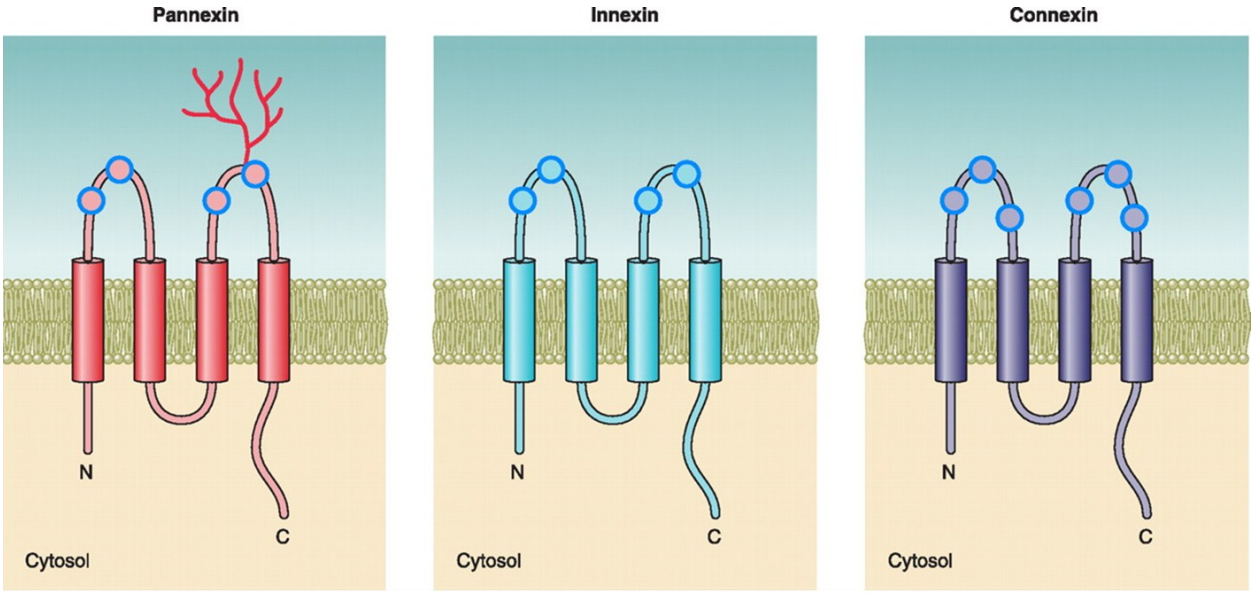

Figure 1.2 The Topology of Pannexin, Innexin, and Connexin Protein Families
The topology of the connexin family is highly homologous to other protein families with similar function. Pannexins, innexins, and connexins share a common membrane topography. The 3 families of proteins that form channels permeable to second messengers and metabolites are integral, tetra-span membrane proteins. All members of the 3 families have their NH_2 and COOH termini within the cytoplasm, and 2 extracellular loops that extend into the extracellular space. These loops contain 2 (pannexins and innexins) or 3 (connexins) highly conserved Cys residues (circles). Pannexins, but not innexins and connexins, feature a N-glycosylated branching on the second extracellular loop. Reprinted with permission from APS: Bosco D, Haefliger J-A, Meda P. Connexins: Key Mediators of Endocrine Function. Physiol Rev 91: 1393–1445, 2011; doi:10.1152/physrev.00027.2010.

Biosynthesis and Trafficking of Connexins

Connexins follow a unique maturation process for a transmembrane protein. All connexins do not have a protein secretion signal sequence nor do they have membrane anchor sequences, and therefore do not follow the same pathway as a typical type III polytopic transmembrane protein that does have these features. Connexins are generally synthesized by ribosomes on the endoplasmic reticulum (ER) and directly inserted into the ER membrane. However, unlike most integral membrane proteins, the connexins are oligomerized in the late compartments of the Golgi apparatus, termed the trans golgi network (TGN) (40, 43). VanSlyke, Naus, and Musil (40) demonstrated that both alpha and beta connexins are synthesized as conformationally stable proteins within the ER but only oligomerize into a connexon once transported and localized to the TGN, prior to vesicle transport. They also conclude that the Charcot–Marie–Tooth –X-linked (CMTX) disease causing mutants of Cx32 have an underlying defect in protein folding, and therefore do not exit endoplasmic reticulum associated degradation

(ERAD) quality control and do not subsequently oligomerize and traffic to the plasma membrane. Exogenous overexpression of both alpha and beta connexins individually mediate an ER associated connexon forming pathway alternative to that of the TGN pathway commonly seen in connexin research (40, 44). New connexons that pass quality control are packaged into vesicles and are transported to the edges of gap junction plaques and lipid rafts by microtubule-assisted vesicle transport (45, 46). However, Cx26 can follow an unknown alternative transport pathway (46). Connexins are synthesized, transported, assembled into plaques, then endocytosed and degraded by lysosomes in a matter of hours. The average cellular half-life of intact connexin proteins is 1.5-4 hours (47). Degradation pathways of connexins are dictated by interplay between phosphorylation and ubiquitination events that occur in the CT domain and lead to internalization of gap and/or annular junctions, and subsequent degradation by the proteasome and/or lysosomes, respectively, during normal cellular processes (47, 48), as illustrated in Figure 1.3.

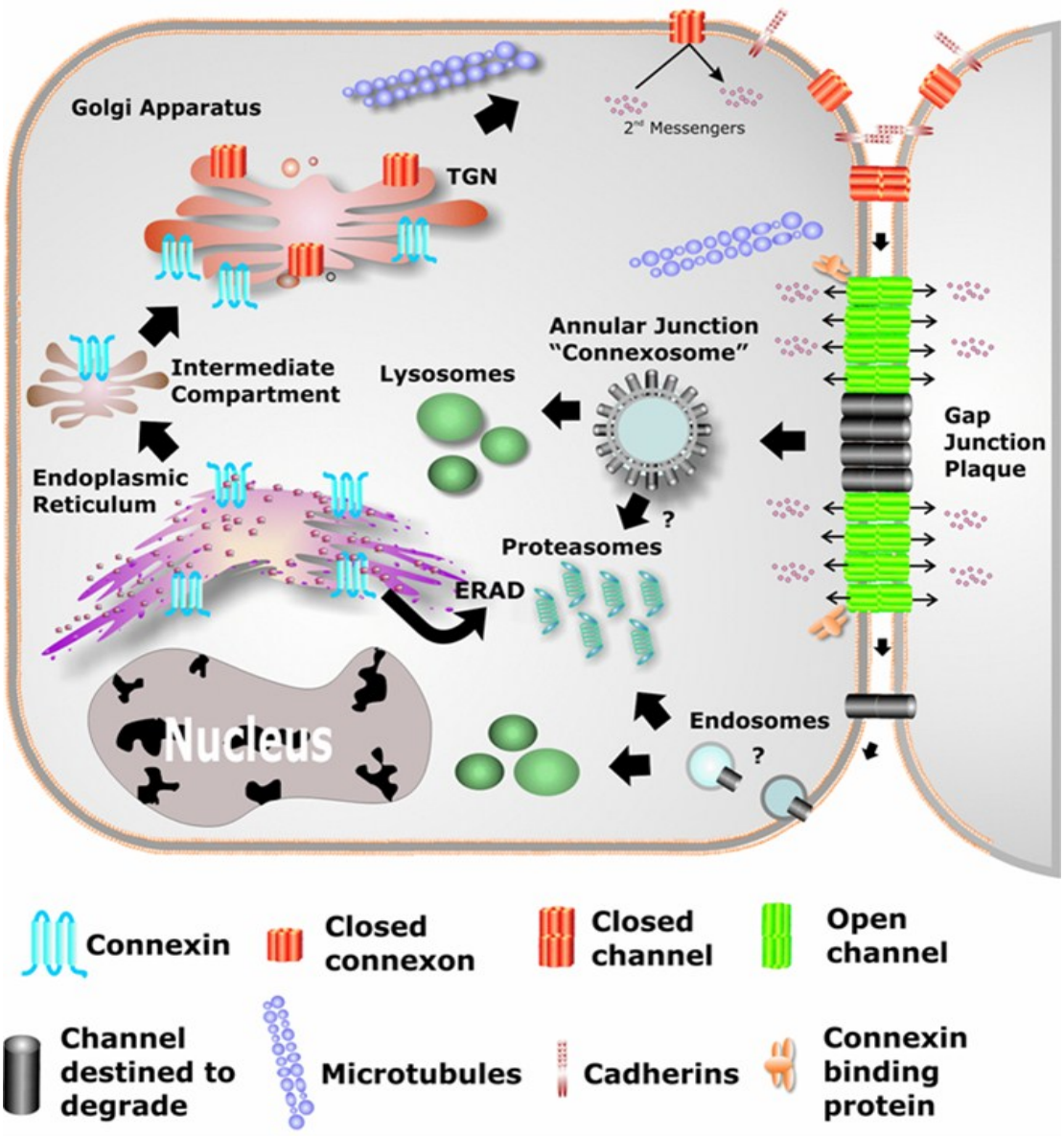

Figure 1.3 The Life Cycle of Connexin Proteins

Connexins typically co-translationally insert into the ER. If properly folded, it is expected that connexins are spared from ERAD, whereas in other cases they may be targeted for ERAD. For at least some members of the connexin family, complete oligomerization is delayed until the connexin passes through the intermediate compartment and reaches the distal elements of the Golgi apparatus, namely the TGN (trans-Golgi network). Pleiomorphic vesicles and transport intermediates are thought to deliver closed connexons to the cell surface, a process that is facilitated by microtubules. Connexons may function as hemichannels and exchange small molecules with the extracellular environment or laterally diffuse in a closed state to sites of cell–cell apposition and dock with connexons from an opposing cell. In conjunction with cadherin-based cell adhesion, gap-junction channels cluster into plaques, open, and exchange secondary messengers. New gap-junction channels are recruited to the margins of gap-junction plaques and older channels are found in the centre of the plaques. Several connexin-binding proteins have been identified, and it is likely that one or more of these binding proteins regulate plaque formation and stability, possibly by acting as scaffolds to cytoskeletal elements. Gap-junction plaques and fragments of gap-junction plaques are internalized into one of two adjacent cells as a double-membrane structure commonly referred to as an annular junction, but renamed in the present review as connexosomes. Other pathways for connexin internalization may exist where connexons disassemble and enter the cell by classical endocytic pathways. Internalized gap junctions are targeted for degradation in lysosomes, although some evidence suggests a role in proteasomal degradation. This figure and text was originally published in Biochemical Journal. Dale W. Laird, Life cycle of connexins in health and disease. Biochem. J. 2006; 394: 527–543. Reproduced with permission © the Biochemical Society.

Connexins Form Functional Transmembrane Protein Channels

Six connexin proteins are co-translationally inserted within the endoplasmic reticulum membrane then transported through the Golgi apparatus to the late TGN compartment to be fully assembled into a connexon, depending on the subfamily type. These six assembled connexins are referred to as connexons and hemichannels. Hemichannels are formed when the connexon is trafficked to the cellular plasma membrane and is not met by an opposing cell membrane or connexon-containing lipid raft (37, 40, 49, 50) such as the apical surface of polarized epithelial cells. A full gap junction channel is formed when one hemichannel is docked to an opposing hemichannel, resulting in an aqueous inner protein pore created by the number 2 and 3 transmembrane spanning helices (TM2 and TM3) of each participating connexin (44, 51-53) and as seen in Figures 1.3 and 1.4. It is assumed that water and small water soluble metabolites, including signaling molecules less than 1 kDa, flow freely through every gap junction (34, 54); signals that pass through connexins are discussed below. It is known that siRNA moves through gap junctions as well (55).

Gap junction channels can take multiple forms depending on the connexin expression pattern of the cell type. Connexons made from a single connexin protein are termed homomeric. Mixtures of two or more connexins into a single connexon are given the term heteromeric. Apposing homomeric connexons are homotypic junctions and apposing heteromeric connexons are heterotypic junctions. Annular junctions are formed when an entire 12-protein gap junction channel is internalized via a vesicle and degraded (34, 54). Heterotypic gap junctions can be formed as illustrated in Table 1.3.

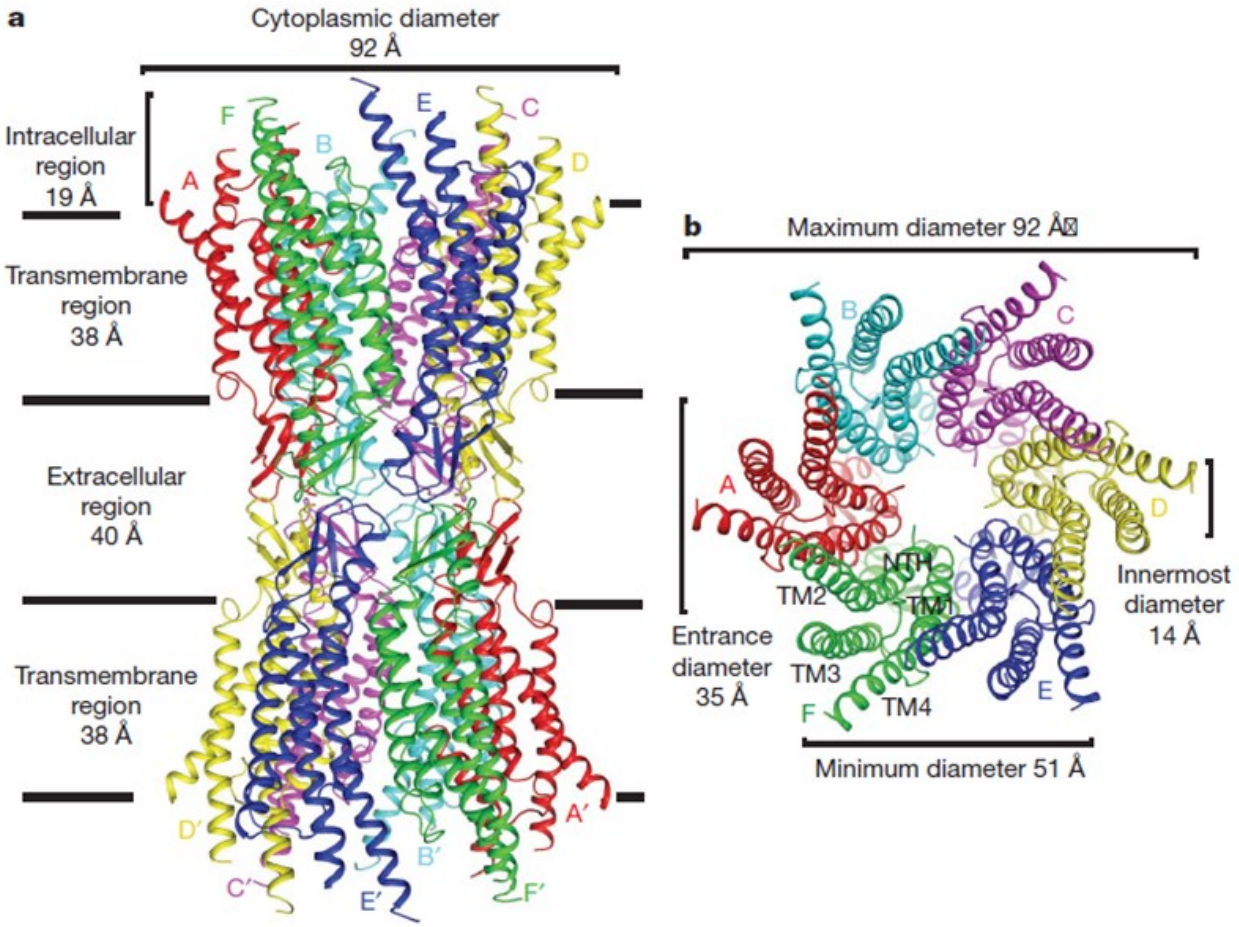

Figure 1.4 Connexins Form Functional Membrane Channels from Two Connexon Hemichannels
Six connexins are oligomerized into a connexon hemichannel. Each connexin subunit is differentially colored and labeled as in b. PDB 2zw3. Reprinted by permission from Macmillan Publishers Ltd: Nature 458, 597-602 (2 April 2009) doi:10.1038/nature07869, copyright 2009.

Connexin		Alpha							Beta							Gamma			Delta			Epsilon
		37	40	43	46	50	59	62	25	26	30	30.3	31	31.1	32	30.2	45	47	31.9	36	40.1	23
Alpha	37	X	X														X					
	40	X	X	X													X					
	43		X	X	X												X					
	46			X	X	X																
	50					X																
	59						X	X									X			X		
	62						X	X									X			X		
Beta	25								X													
	26									X	X		X		X							
	30									X	X		X		X							
	30.3											X										
	31									X	X		X									
	31.1													X								
	32									X	X				X							
Gamma	30.2															X			X			
	45	X	X	X			X	X									X			X		
	47																	X				
Delta	31.9															X			X			
	36						X	X									X			X		
	40.1																				X	
Epsilon	23																					X

Table 1.3 Known Connexin Channel Forming Interactions
Homotypic channel formation is assumed since each inter-channel interaction is unknown. Data taken from NCBI database and can be found at http://www.ncbi.nlm.nih.gov/.

Regulation of Connexin Function

Connexins can be regulated in two primary ways. The first and fastest way is through voltage-gating and electrostatic interactions (29, 53, 56, 57). The second and slowest way is through signal transduction and protein modification such as phosphorylation (58), protein docking (4, 31, 59, 60), domain structure modification (61-64), and connexon mixing between cell and channel types (31, 34, 44, 65).

Voltage-gating is the best known and easiest characteristic of gap junction channels to measure. Voltage-gating of connexons can be accomplished in a number of ways as described below. The presence of extracellular Ca^{2+} can induce changes in gap junction permeability. The pH differences between the connected cells cytoplasm can induce closure of gap junction channels as well as create charge gradients (56, 66). Gating by these mechanisms is governed by conformational changes in the pore, plug-gating, and/or loop-gating (29). pH and calcium fluxes are a general process that occurs during ischemia and hypoxia, and will be discussed below.

Phosphorylation, protein interaction and domain restructuring mainly regulate the stability and degradation of gap junction functions (47-49, 67, 68). The mixing of connexins into connexons and gap junctions has profound effects on the regulation of gap junction activity as shown when Cx32 or Cx40 are co-expressed with endogenous Cx43 in NRK cells and Cx46 and Cx50 in lens cells (31, 69). However, not all groups of connexins mix, as seen in Table 1.3. Phosphorylation is by far the most common signal that connexins receive from the cell. It is necessary for trafficking, gating, scaffolding, and degradation (38, 47, 68, 70, 71).

Cellular Functions of Connexins in Various Tissues and Associated Diseases

As described above, connexins take on primary functions as voltage-gated gap junction channels, adhesion molecules, and as mediators of cellular homeostasis. While the gating and adhesion properties of gap junctions are well known and assumed to function in healthy tissue, the effects of connexins on cellular homeostasis and growth differ with the cell type and connexin in question. For example, gap junctions comprised of Cx43 in the heart confer the necessary voltage-gated channel properties needed for the heart to generate a coordinated beating rhythm. In addition, Cx43 gap junctions in the majority of types of bone cells are necessary for proper growth control and development of bone (72-74).

Bone is a unique growth environment that is often hypoxic and unlike most other tissues. Gap junction hemichannels function in bone to release ATP and PGE_2 in response to mechanical disruption, osteocytic network communication, cellular proliferation, differentiation, and survival (74, 75). Cx40, Cx43, Cx45, and Cx46 are expressed in various bone cells. Cx43 is expressed in every bone cell type and seems necessary since disruption of Cx43 has been linked to bone hypomineralization and a delay in ossification of the skeleton, which is seen in the partial phenocopies of human oculodentodigital dysplasia (ODDD) manifested in $Cx43^{-/-}$ mice (75, 76). Cx45 is expressed in many cell types except tooth osteoblasts and osteoclasts. It is up-regulated in response to a lack of Cx43 and is also known to inhibit the function of gap junction channels formed by Cx43 (74, 75). Cx46 uniquely is expressed only in the cytoplasm and perinuclear area of osteoblasts and it fails to form functional gap junctions at the cell membrane (74, 75, 77). Interestingly, Cx43 has been shown to indirectly regulate gene transcription of many important molecules in bone development including osteocalcin, osteopontin, bone sialoprotein, alkaline phosphatase, RUNX2 transcription factor, and even the related bone connexins Cx45 and Cx46 during development (74, 76).

Cx26, Cx30, Cx30.3, Cx31are implicated in many forms of hearing loss and skin diseases (Table 1.2). The epidermis expresses Cx26, Cx30, Cx30.3, Cx31.1, Cx37, and Cx43. Follicular skin expresses Cx26, Cx30, Cx30.3, Cx31, Cx43, and Cx45. Keratinocytes express Cx26, Cx30, Cx31, Cx37, Cx40, and Cx43 (22, 78). Expression is dependent on the layer of skin in which the connexins are being expressed, which again indicates cell-type specific functions of the connexins. Cx26 and Cx30 share similar functions in skin and have been implicated in controlling the proliferation, differentiation, and migration of keratinocytes. Aberrant expression of these connexins results in slower wound closure and skin diseases caused by hyper-proliferative cells with epidermal thickening and psoriasis (22). Cx43 is known to be down-regulated during wound closure and inhibition of Cx43 function speeds wound recovery time as seen with the pre-clinical candidate antisense oligodeoxynucleotide that targets Cx43 in skin epidermis and dermis tissues (24). Additionally, knockdown of Cx43 protein expression results in Cx26 and Cx30 expression being increased, indicating functional redundancy in GJIC. But loss of Cx43 retained functional discrepancy in reducing cellular proliferation and migration (24, 78). Many parallels have been made between the function of connexins in the outer epidermal skin and in the lens, both tissues being devoid of blood vessels and lymph.

Mutant Cx30 gap junction channels cause hidrotic ectodermal dysplasia and play a critical role in hair follicle homeostasis (22). Cx30.3 and Cx31 mutations are implicated in erythrokeratodermia variabilis and related skin disorders. Mutations in Cx32 cause the neuronal sensory disease X-linked Charcot-Marie-Tooth disease by disrupting signals passed between Schwann cells and neurons. In general, many mutations in disease causing connexin genes are causative of phenotypes in cell culture models, showing trafficking disorders, impaired gap junction activity, dominant negative effects on heterotypic junction activity, and the formation of leaky hemichannels (22).

At least nine different connexins are expressed in the various tissues of the kidney: Cx26, Cx30.3, Cx31, Cx32, Cx37, Cx40, Cx43, Cx45, and Cx46 (79, 80). Cx26 and Cx32 are co-expressed in the proximal tubule. The co-expression pattern is similar to that in the liver, stomach, and pancreas (79). Cx26, Cx30, Cx32, Cx30.3, and Cx37 are considered the tubular connexins, while the remainders are part of the vasculature. The only known phenotype associated with the tubular connexin knockout mice is prevalence of salt sensitive hypertension in $Cx30^{-/-}$ mice (79). In the renal vasculature, Cx40 plays a major role in the negative-feedback loop of renin secretion and detection of blood pressure. Only Cx43 can modulate renin secretion correctly as demonstrated by knock-in studies (34). In the case of $Cx40^{-/-}$ mice, severe hypertension is attributed to the function of Cx40 as a scaffolding protein rather than a functional gap junction. However correct Cx40 gap junction function is needed for proper renin secreting cell development and to turn off renin production (79).

There are many CNS disorders caused by mutations in connexins. Cx32 and Cx47 are implicated in the majority of nervous disorders, which all have defects in sensory defects. In glial cells, structural and homeostatic functions have been ascribed to Cx32, Cx43, and Cx47. Interestingly, loss of Cx47 gap junctions in $Cx47^{-/-}$ mice leads to repression of oligodendrocyte differentiation, development, and proliferation due to the loss of scaffolding function and subsequent aberrant transcription factor accumulation under non-inducing conditions (81). Two known astrocyte connexins, Cx43 and Cx30, aid in the recovery of stimulus-induced ion flux by relocating potassium from the extracellular space into the cell via hemichannels (34, 81). Again, connexins are demonstrated to have a variety of functions in every tissue examined to date.

The knowledge of tissue specific connexins and sequencing of disease-associated connexin genes provides evidence that connexins are diverse proteins whose channel function is

not always as important as the scaffolding, structural, or other intracellular functions. Evidence of this comes from functional redundancy and cell-specific ablation studies as discussed above. One specific tissue which expresses three types of connexins, the vertebrate lens, allows for the deciphering of well-defined roles in the function of each expressed connexin. These roles have been assigned through the use of genetically modified mouse strains with various knockouts and knock-ins of the lens connexin genes complimented by *in vivo* phenotype analysis, and *in vitro* cell biology and electrophysiology studies.

Functional Role of Connexins in the Lens

Three connexins are fully expressed in the mammalian lens tissue and each plays a specialized role. Figure 1.5 illustrates that the expression of Cx43, Cx46 and Cx50 changes with the stage of differentiation of different regions of the lens. Through the use of gene knockout and knock-in technology, connexins in the mouse lens have been extensively studied. Without this technology, insights into the circulatory system of the lens would not have been made. The lens is a naturally avascular and hypoxic tissue. It must remain hypoxic and devoid of vasculature to maintain clarity and function. The mature fiber cells of the lens, the oldest of the lens cells and the most centrally located, remain alive and functional by maintaining a low level of anaerobic respiration. Although the mature fiber cells utilize glycolysis for cellular fuel, the low level of ATP production is only enough to maintain the G_0 cell cycle phase, or essentially senescence. Senescence seems to be required to allow the mature fiber cell to function properly and remain transparent. The transition between lens epithelial to fiber cell differentiation is marked by changes in protein expression and protein modification as well as phenotypic changes such as elongation and reorganization of gap junction plaques (82).

In the outer epithelial cells, where cell division and elongation occur, Cx43 is preferentially expressed along with other transporting proteins, for example, the Na^+ K^+ pump, aquaporin 1, volume regulatory transporters, K^+ Cl^- co-transporter, stretch-activated cation channel, GLUT1 glucose transporter, and the Na^+ Ca^{2+} exchanger. pH is regulated by the Na^+ H^+ exchanger and the Cl^- HCO_3^- exchangers. Many animals with knockouts of these various channels still maintain clarity or size of the lens despite having specific channel function defects. However, knockouts of the lens connexins exhibit lens defects. Although the $Cx43^{-/-}$ mouse model is lethal at birth, the lenses and various other primary tissues and cells have been studied

to see the effect that loss of Cx43 has on the tissues that express it. $Cx43^{-/-}$ mice do not live beyond birth due to severe heart defects. However, their lenses appear normal. In the lens, the most notable difference in function was a lack of proper epithelial adhesion and communication, because there were gaps and vacuoles present in histological samples of the knockout lenses (82). The other lens connexins, Cx46 and Cx50, cause remarkable cellular changes in the lens when their functions are perturbed.

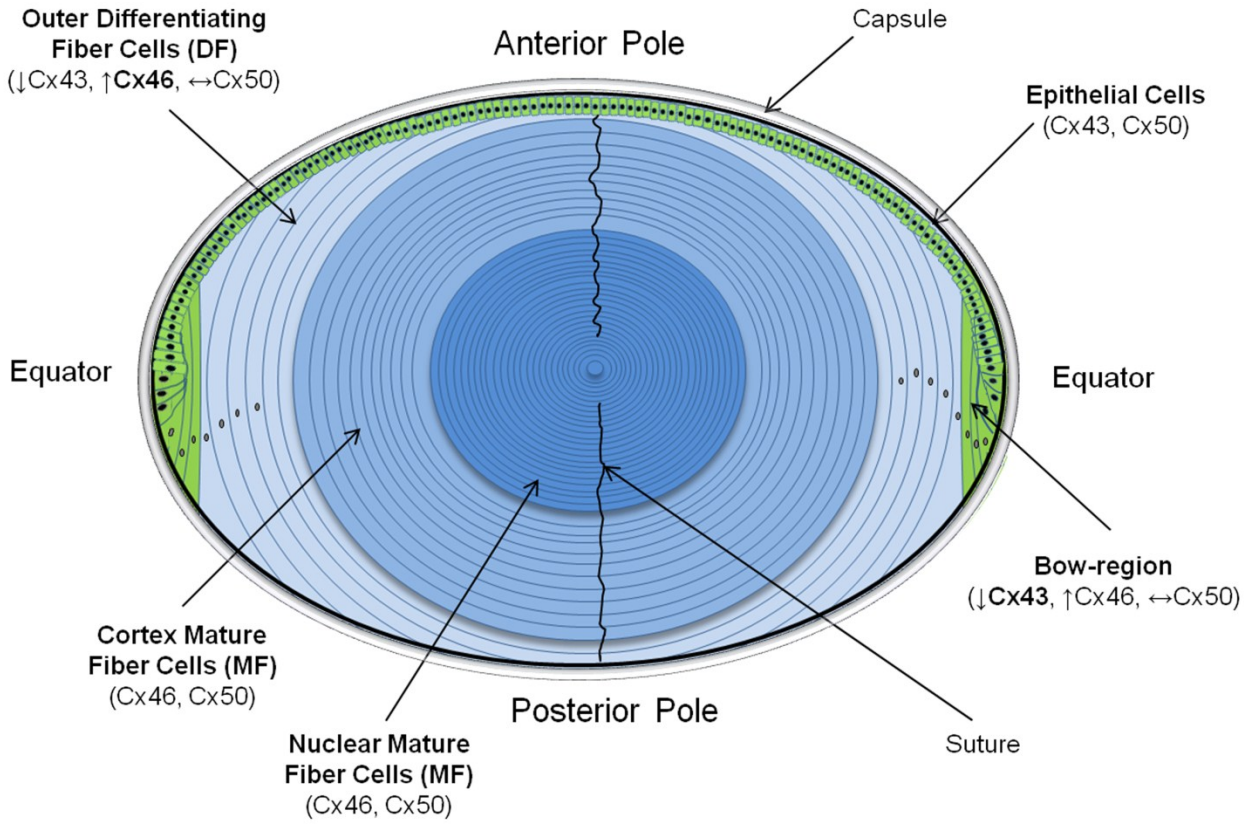

Figure 1.5 A Schematic of the Vertebrate Lens and Connexin Expression Profile
Connexins are expressed throughout the lens. Cx43 is expressed predominately in the outer epithelial layer where the majority of the lens metabolism occurs. It is also the region that has the most access to oxygen. As the epithelial cells migrate around the lens to the equator, they begin to differentiate and express different proteins like crystallins, Cx46, and Cx50. Cx43 expression decreases with differentiation and stops all together when fibers being to elongate. Cx46 and Cx50 are required to maintain lens transparency and homeostasis. The hypoxic nucleus is devoid of organelles, protein turnover and nuclei. Original figure published in Das et al., Curr Eye Res. 2011 Jul; 36(7):620-31.

Cx46 is expressed early in lens fiber cell development and is strongly expressed and maintained in the mature fiber cells of the lens nucleus. Cx46 expression begins to appear as the transition between outer differentiating and inner differentiating cells begins and continues into

the nucleus. In the same region where Cx46 is being up-regulated and Cx43 is down-regulated, the lens becomes hypoxic. Oxygen tensions in this region are 5% (~40 mm Hg) at the lens surface to less than 1% (~8 mm Hg) oxygen in the lens nucleus. The high metabolic oxygen demand in the lens nucleus is thought to be responsible for the lack of oxidative damage to lens core proteins since the majority of available oxygen in the lens nucleus is used for energy production (82). Cx46 is not expressed in the epithelium but forms heterotypic gap junctions with both Cx43 and Cx50 between epithelial cells and differentiating fiber cells. Cx50 does not interact with Cx43 in a heterotypic manner (34, 67, 69, 82, 83). $Cx46^{-/-}$ mice display severe punctate cataract formation early in life, bone development retardation, and general bone malformations (74, 75). Various mutations within the Cx46 genes of clinical patients have been directly linked to the cause of congenital cataracts but bone has not been analyzed in any clinical patients to date (62, 84-86).

Cx50 also affects lens clarity and regulates lens differentiation, growth, and size. Cx50 is expressed throughout the lens in each cell type and confers cell growth regulation qualities (82, 87). It was observed that $Cx50^{-/-}$ mice possessed a smaller lens with fewer cells, but the cells were larger than wild type mice lens cells. $Cx50^{-/-}$ mice also had nuclear cataract formation, albeit generally less severe than $Cx46^{-/-}$ mice (87). The growth properties conferred to fiber cells from Cx50 was highlighted in a study about connexin function using mouse knock-in models (69, 82, 88).

Although gene transcription and protein translation rates decline as the lens cells mature, Cx46 and Cx50 remain as functional membrane channels well into the mature region of the lens. Cleavage of connexins in the lens probably lead to stability of the functional, pH, and Ca^{2+}-gated channel functions of Cx46 and Cx50 (61). This would allow for the junctions to remain at the plasma membrane, unaffected by global protein changes in the cytoplasm since the carboxy-terminal domains are clipped, unable to provide scaffolding functions (89, 90) and would not sense molecular signals related to degradation (82, 91).

Major intracellular degradation occurs in the same differentiating fiber cells as the disappearance of Cx43, appearance of Cx46, and stabilization of gap junction plaques by carboxy-terminal domain clipping. Evidence suggests that the creation of the organelle-free zone in the mature fiber cells relies on the same cellular pathways that are involved in apoptosis. These pathways lead to nuclear disorganization, organelle dismantling, and cellular scaffold

destruction. Interestingly, with the maturation of the lens fiber cells, the lens becomes increasingly hypoxic, metabolism changes to favor the anaerobic type, and all organelles and advanced cellular structures are degraded with the exception of cytoskeletal components, the crystallins, and gap junction plaques (64, 67, 82, 91-93). Similar clipping of connexins has been noted in cardiac tissue, where constant electrical coupling is crucial and CT domain clipping concurs with gap junction plaque remodeling (63, 94, 95).

Indeed, some apoptotic proteins are used in lens cell fiber differentiation as shown by Weber and Menko (91, 96). The authors showed that there is a delicate balance between apoptosis and defined organelle destruction that is regulated by the Bcl family of proteins and cytochrome *c* release from the mitochondria (96), indicating that fiber cell differentiation in the absence of sufficient oxygen is basically selective and controlled cellular apoptosis, as only incomplete destruction of organelles in the lens nucleus leads to cataract (91). The authors termed this type of controlled-apoptotic differentiation as apoptosis-related Bcl-2- and caspase-dependent (ABC) differentiation (96). Similar differentiation processes take place in a few different cell types, such as in keratinocytes and erythroid cell differentiation, and monocyte differentiation into macrophages (91). What effect connexins have on this differentiation pathway is unknown at this time. It could be hypothesized that in the lens, Cx50 must maintain heterotypic gap junctions with Cx46 in order for differentiation and growth to occur, because only $Cx50^{-/-}$ mice display reduced fiber cell differentiation and growth, and Cx43 and Cx50 do not form heterotypic junctions (as discussed above). Functional channels formed by Cx46 and Cx50 could pass selective molecules needed to drive organelle destruction or maintain cellular homeostasis during differentiation. Another hypothesis could be that Cx50 homotypic or Cx46 heterotypic junctions are necessary for correct cytoskeletal maintenance due to their protein scaffolding abilities. Many questions remain regarding lens cell differentiation as a whole.

Work by Mathias, Gong, and White showed that only Cx46 is responsible for gap junction coupling in the mature fibers, and that since the CT domains of both connexins are clipped rendering Cx50 nonfunctional, it is Cx46 that supplies the much needed metabolite and waste circulation to the lens nucleus in conjunction with high-affinity metabolite transporters known to be up-regulated and maintained in mature fibers (82). By knockout analysis of $Cx50^{-/-}$ mice, Cx50 appears to play a role in regulating lens differentiation and growth before the CT domain becomes cleaved, so the functional consequence of cleaved Cx50 in the lens nucleus is

irrelevant to lens development at the point in which Cx46 takes on functional significance. However, without functional Cx46, lenses lose homeostasis and develop cataracts. A direct correlation has been made between lens epithelial cell differentiation and growth and presence of Cx50 homotypic gap junctions (82, 97, 98). Much evidence points to connexins playing a central role in the development of a naturally avascular and functionally unique hypoxic tissue.

Signals and Metabolites that Traverse Connexin Channels

The abundance of cellular second messengers and ions and the number of connexins expressed in each tissue makes the possible gap junction selectivities almost limitless. A few important metabolites and signaling molecules are known to traverse connexin channels, such as bicarbonate, Mg^{2+}, Na^{+}, K^{+}, Ca^{2+}, second messengers such as IP_3, ATP, AMP, cAMP and cGMP, and small metabolites including glucose and glutamate (38, 47, 78, 99, 100). Charge and size selectivity of the pore depends on the type of connexins that constitute the connexon, but in general the pores are believed to be at least 12 Å in diameter (99). For example, homomeric Cx45 has a pore diameter that only allows molecules smaller than 0.3 kDa to pass, compared to homomeric Cx43 channels that can pass molecules up to 1.2 kDa in size (74). The diameter of channels of homomeric Cx46 has not been measured. Given the varying roles that connexins play in physiology, it is easy to speculate that the mixing of connexins gives distinctive juxtacrine channels that spread process-specific molecules, and that this spreading of molecules can dictate cellular responses in other nearby cells, possibly at the sub-organ/tissue level.

There have been attempts to decipher which connexins can create heteromeric connexons and/or heterotypic gap junctions. Such studies are facilitated by the use of connexin chimeras (29, 30, 38, 99). These chimeras often contain individual domain replacements, offering insights into the importance of each domain in the overall connexin gap junction function. The charged residues of the extracellular loops and various residues in TM3 seem most important in Cx43 connexon mixing and gap junction formation (44, 52). Cx43 connexons are less selective than Cx26 or Cx40, and this allows for higher cAMP flux between coupled N2A and HeLa cells (100). By using a molecular simulation and analysis of limited, but available, connexin structural data, Maeda and Tsukihara (38) observed that as a consequence of the biochemical and mutagenic techniques used to confer connexin gap junction transport ability, the techniques themselves induced structural changes in the extracellular loops and TM helices, such that the

disulfide bridges that stabilize the docking face could not form correctly. This ultimately led to discrepancies in published data and confusion as to which connexins actually connected and retained selective gap junction permeability. Therefore, the questions regarding the types of solutes that can pass through the various mixings of gap junctions *in vivo* in tissue or *in vitro* in cell cultures in any physiological state still remain controversial and unresolved.

As outlined above, the connexin protein family is diverse, redundant, but specialized. Important and detailed roles of each connexin are being uncovered. However specific attributes are likely to be cell type and tissue type dependent. This thesis identifies and confirms an additional non-channel function of Cx46 in diseased tissue similar to the hypoxic lens. Results suggest that Cx46 provides functional protection from hypoxia.

Ischemia, Hypoxia and Effects Thereof

Cellular processes, such as differentiation, regulation of metabolism, and proliferation, result from the presence or absence of cellular oxygen. The most amount of cellular energy created in metazoans is through oxidative phosphorylation. When oxygen becomes limited, metazoans have a secondary, less efficient glycolytic energy production system, which meets the minimum requirement for cellular fuel and metabolites. Glycolysis is efficient and has been used in some form since the beginning of unicellular life (6). It is no surprise that glycolysis has survived through evolution of life to present day organisms (101).

Ischemia, a less than adequate delivery of oxygen and nutrients to tissues, contributes to major medical problems in human health and disease. Ischemic events occur during stroke, heart disease and infarction, diabetes, lung diseases, vascular diseases, solid tumor growth, and high altitude. In each of these health states, hypoxia, or low oxygen, is common to all diseased or affected tissues. Hypoxia, however, is also a fundamental physiological stimulus that takes place during development and tissue growth in vertebrates. Lack of an adequate cellular oxygen supply, defined as hypoxia, creates a demand that far exceeds the availability of oxygen for oxidative phosphorylation to be efficient. Cells begin to sense this change in oxygen and other environmental clues such as the buildup of metabolic waste and the increased demand for cellular fuel (102, 103).

The lack of oxygen and the buildup of waste, in turn, up-regulate some response genes needed to correct the dire situation, such as transporters, proteolytic enzymes, and kinases, such

as in the case of PC12 cells (adrenal neuron pheochromocytoma) in response to treatment with hypoxia plus high extracellular calcium (104). Other examples of the effects of hypoxia are cellular apoptosis and necrosis, as a result of irreversible metabolic changes within the cell. The change in availability of cellular oxygen is sensed by a redundant system of oxygen-dependent hydroxylating enzymes and associated transcription factors that comprise the hypoxia-inducible factor (HIF) response to hypoxia in all cell types. Much is known about this pathway (Figure 1.6) and is reviewed below.

Hypoxia Inducible Factors Regulate the Cellular Hypoxic Response

The HIF system comprises a core set of transcription factors and the proteins that modulate their activity. The master regulator protein of the HIF system is a heterodimeric transcription factor named hypoxia-inducible factor 1 (HIF1), with both α and β monomers being constitutively expressed in most cell types. There are two other known human homologs of HIF-1α, named HIF-2α and HIF-3α. These transcription factors are all modulated in much the same manner. HIF alphas are regulated by the cellular ubiquitin-proteasome system, which follows the ubiquitination pathway.

The von Hippel-Lindau protein (VHL) is the dihydroxy-HIF recognition protein subunit of an E3 ubiquitin ligase. For VHL to identify and bind to HIF, an iron, oxygen, and TCA cycle metabolite-dependent hydroxylation reaction must occur on HIF. The prolyl-4-hydroxylase domain (PHD) enzymes facilitate two oxygen-dependent reactions with one molecule of oxygen; one reaction involves the transfer of one oxygen atom to regulatory prolines of HIF (P402 and P564 on human 1α and similar in 2α/3α) (105), and the other atom to split α-ketoglutarate to succinate and carbon dioxide (101, 106) (Figure 1.6). Once HIF is hydroxylated on both regulatory prolines, VHL recognizes and binds to it, facilitating the transfer of ubiquitin chains and ultimately destruction by the proteasome under normal oxygen concentrations. The K_m values for the family of oxygen-dependent PHDs are near 100 μM, well above the cellular concentrations of physiological oxygen (4-40 μM or 3-6% or ~25 mm Hg), indicating the sensitivity and efficiency of catalysis utilizing cellular oxygen tension as a sensor (106).

A second and equally important mode of HIFα subunit regulation is through the iron and oxygen-dependent hydroxylation of asparagine-803 in the carboxy-terminal trans-activating domain (CTD) of alpha subunits, catalyzed by the protein FIH (factor inhibiting HIF-1) (105) as

shown in Figure 1.6. In the absence of molecular oxygen such as during hypoxia or in the presence of divalent metal chelators or competing divalent metals, the hydroxylation reactions cannot continue, which results in VHL not being able to bind HIF, leading to cytoplasmic accumulation of the alpha subunit. Accordingly, if FIH cannot hydroxylate the HIF CTD, transactivation of response genes can be induced more efficiently. These two hydroxylating mechanisms offer a graded response to oxygen and necessary cellular metabolites and cofactors, which surely provide a delicate balance between metabolism and cellular health under various oxygen tensions (101, 105-107). The HIF-1β subunit, also known as aryl hydrocarbon receptor nuclear translocator (ARNT), is constitutively expressed and not regulated by oxygen or metabolites, leading to dimerization of the alpha and beta subunits and translocation to the nucleus (105, 106).

HIFs dimerize by utilizing their PAS and bHLH protein domains on the alpha and beta monomers, respectively, while leaving the amino-terminus of each monomer available for DNA binding and the carboxy-terminus of the alpha monomer for binding to the transactivation coactivator protein p300/CBP. The HIF alpha/beta heterodimer binds to and activates DNA transcription at defined sites of 5'-XRCGTG-3' where R is a purine (A or G, in sequence terms) and X is any purine or pyrimidine. The alpha subunit binds 5'-XRC-3', while the beta subunits bind 5'-GTG-3' (101, 103, 105, 108) (Figure 1.6). HIF is considered the master regulator of hypoxia response genes and is necessary for correct embryonic development as well as recovery from anemia and growth of new tissue (101, 109, 110).

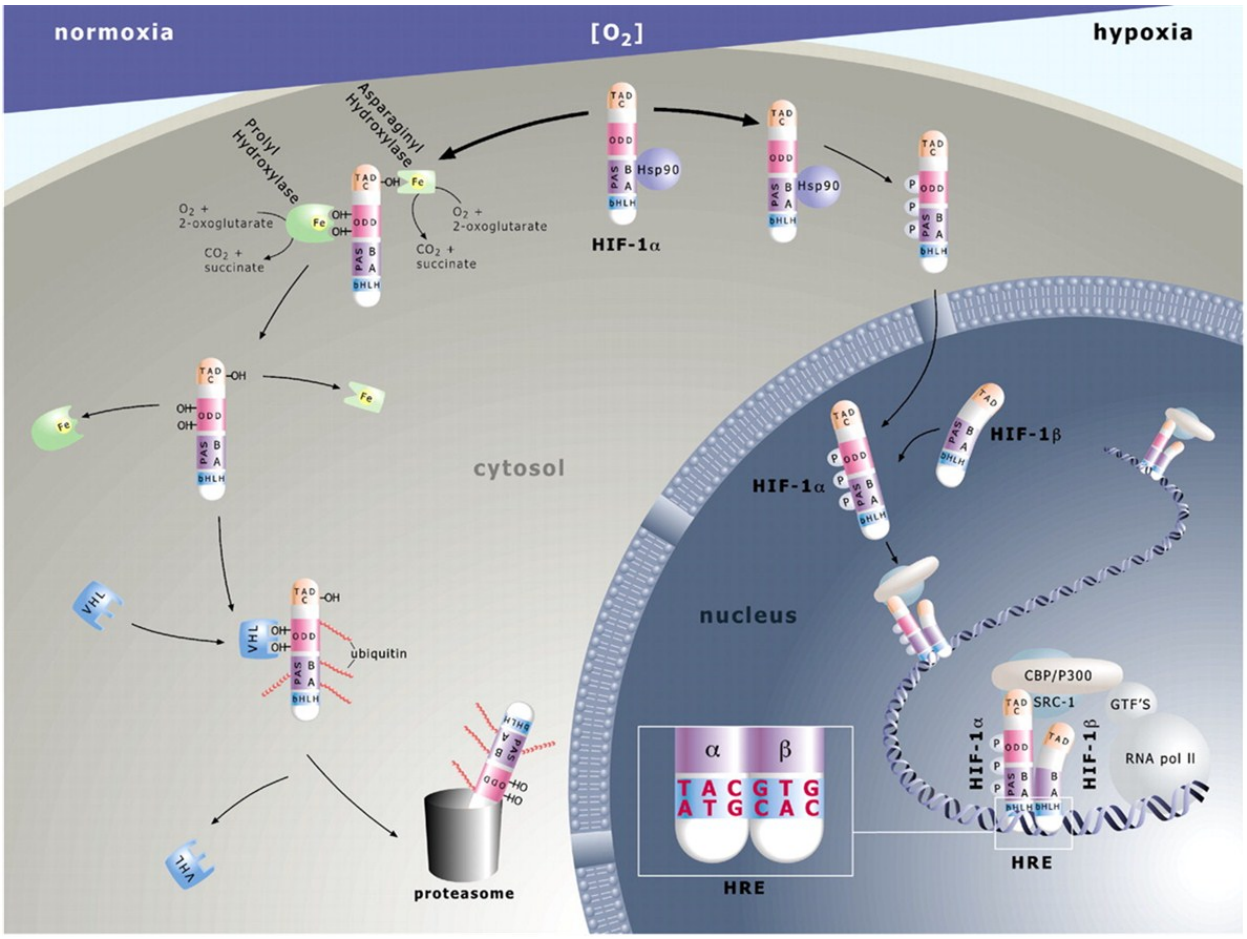

Figure 1.6 Schematic of HIF-Mediated Oxygen Sensing
HIF-1α and the HIF-β subunits are constitutively expressed under normal physiological conditions. Under normal tissues oxygen tension conditions, the alpha subunit is degraded very rapidly upon its expression via specialized oxygen and α-ketoglutarate intermediate-regulated iron-dependent hydroxylases. Enzymatic hydroxylation of specific proline and arginine residues by the oxygen-regulated hydroxylases provides a binding platform for the von Hippel-Lindau tumor suppressor E3 ubiquitin ligase (VHL). When oxygen tension is sufficient, HIF-1α is degraded via the proteasome very efficiently. Insufficient oxygen, iron, or α-ketoglutarate leads to HIF-1α stabilization and dimerization with the β subunit. This leads to nuclear import and gene transcription of proteins with hypoxia sensitive promoters. Prolonged hypoxia results in accumulation of HIF in the nucleus leading to transcription from gene promoters which contain non-standard hypoxia response elements, like connexin 46. Permission to modify and reprint obtained from Oxford Journals. Original figure: Cardiovasc Res 2006; 71:642-651. © Oxford University Press

Cellular Changes Common to Hypoxic Insults

To date, there are at least 70 confirmed HIF gene targets in the human genome. Each of these genes plays a role as part of two well defined categories of functions: 1) to increase the flux of catabolic, energy-rich metabolites to be used in ATP production, and for reducing molecule generation, and 2) to increase tissue oxygen penetrance and delivery (105). Fandrey, Gorr, and Gossmann (105) offer an interesting perspective on cell specific differences. They postulate that,

since a graded response to oxygen is sensed the same in all cells, it is the shear amount of hydroxylating enzymes produced by the cell under hypoxic stresses that dictates cell type specific differences in the overall response. This is logical, because some cells that operate better under low oxygen tensions, such as bone chondrocytes, differentiating neural crest cells, or differentiating fiber lens cells, respond to hypoxia differently than cells that respond transiently or do not respond at all, e.g. erythropoietin secreting kidney cells or cardiac and vascular tissue. This also dictates which proteins are up- and down-regulated during hypoxia and the sensitivity of gene regulation in these cells.

All cells need to be characterized more extensively under hypoxic growth conditions to be able to assign proteins a specific function during hypoxia. Since cells that are destined to function normally under hypoxic conditions express cell type specific maintenance proteins to cope with the inefficient metabolic respiration and toxic metabolic waste accumulation, functions assigned to proteins have the possibility of possessing other functions in a different cell type under different conditions. For example, adipose tissue was found to up-regulate some 3000 genes during mouse studies of sleep apnea, a condition where transient decreases in global oxygen supply occur during sleep (111). This is in stark contrast to the known 70 genes that are regulated by HIF directly as mentioned above. One obvious question raised about this discrepancy is what function these other 2900+ gene products have in relation to hypoxia? Another question that could be raised is how much HIF transcription factor is needed to transcribe from non-canonical HREs in promoters? Other studies have implicated that adipose cells that rely on hypoxia to maintain function respond to outside stimuli differently that what would be expected, indicating that adipose tissue is unique because it requires hypoxia to be resistant to inflammatory signals (112). Nonetheless, cells from different tissues with different functions respond differently to a lack of metabolic oxygen.

The mitochondria rely on NADH and $FADH_2$ as electron energy shuttles from the tricarboxylic acid cycle (TAC), and also rely on O_2 as the final proton acceptor in the efficient production of H^+ gradients and ATP (101). As the amount of available metabolic oxygen decreases, the TAC continues to provide the reducing equivalents needed for electron transport. Subsequently, as time without oxygen progresses, unpaired electrons accumulate and escape the electron transport chain, ultimately becoming reactive oxygen species (ROS) like oxide anions (e.g. $\cdot O_2^-$) and peroxides (e.g. H_2O_2). At the same time ROS production is increasing, HIF

activity is increasing which leads to controlling the expression of TAC enzyme isoforms and glycolytic enzymes that modulate the flux and metabolism of necessary metabolites needed for anaerobic respiration (113, 114). Additionally, cells that must respond to prolonged hypoxia induce mitochondrial autophagy as a means of protection against damage by reactive oxygen species (ROS) (115). This is manifested in the hypoxic differentiating lens cells in which the creation of the organelle-free zone that maintains lens clarity is concurrent with increasing and prolonged hypoxia, as described above.

ROS are very damaging to the cell because of their inherent high reactivity and ambiguous substrate preferences. The lipids, nucleic acids, and proteins that comprise the cell can become extremely damaged by ROS which results in the death of the cell through either apoptosis or necrosis. Thus, it is important for cells to cope with ROS stresses that are above the normal physiological levels produced from the electron transport chain. Cells have a number of options to cope with this stress encoded in the genome and through the availability of antioxidants. Enzymes like superoxide dismutase and antioxidants like glutathione can be produced in cells in response to ROS. Cells can also express transporter proteins and gap junctions to increase the intracellular availability of antioxidants (a shared metabolite process) or to decrease the intracellular damage (as a dilution process) as well as express different mitochondrial proteins that increase the efficiency of electron transport under hypoxia (an induced process) (116, 117). ROS production is not the only damaging process that occurs during cellular hypoxia as changes in intracellular pH play a particular role in regulating the cells response to hypoxia. The pH regulation of gap junctions in particular, was described above.

Prolonged Cellular Adaptation Strategies to Hypoxia in the Solid Tumor and Lens

Otto Warburg first noted that an increase in glycolysis was associated with solid tumor growth and termed this metabolism “aerobic glycolysis” which in present day is referred to as Warburg metabolism (114). Increased and prolonged glycolytic activity is associated with cancer progression and clinical tests of cancer use this phenomenon to diagnose solid tumors by using fluorodeoxyglucose and PET imaging techniques (114, 118). Changes in metabolic activity, as described above, occur chronically in many cancer cell types. Specific changes in cell behavior have been attributed to the type of HIFs that are expressed during that change.

HIFs have been implicated as being both a cause and a marker of the aggressive solid tumor phenotype (106, 119) and are discussed below.

HIF1 is generally described as the master regulator of the normal hypoxic response and in neonatal development, whereas HIF2 appears to be active in the prolonged hypoxic response such as in disease and tumors (114, 118-123). When HIF-1α escapes the ubiquitination process and subsequently forms functional HIF1, it translocates to the nucleus where it binds hypoxia response elements (HREs) and activates transcription of target genes. Transactivation of CBP/p300 occurs prior to efficient gene transcription through the carboxy-terminal transactivation domain (CTD) of HIF-1α and -2α. Interestingly, HIF-3α does not contain this transactivation domain and may serve as a transcriptional repressor of HRE responsive genes (120). Unfortunately, HIF3 and the HIF-3α gene locus, in particular, has not been well studied, but evidence indicates that it does act as negative transcriptional regulator having negative impact on hypoxic renal cell growth, proliferation, stem cell development, and in inhibition of corneal neovascularization (120, 124-126).

HIF2 is increasingly found to be linked to hypoxic tumor progression. Semenza (101) and Kaelin (106) in their literature reviews, observed that many cellular growth and proliferation pathways, such as Myc, Ras, AKT/PKB, mTOR/ PI3K, and Notch, are up-regulated as a result of solid tumorigenesis. Additionally, support for growth pathway up-regulation is garnered from studies of v-SRC and various oncogenic viruses, which when introduced into cells in culture lead to the up-regulation of HIF and the overall growth response. HIF1 and HIF2 have been shown to functionally interact with Myc at the promoter level, indicating that HIF2 has the ability to both repress expression of Myc-activated genes and activate the expression of Myc-repressed genes through replacement of HIF1-Myc, or creation of HIF2-Myc transcriptional complexes (118).

Model Systems Used for the Study of Hypoxic Effects of Connexin 46 – The Lens and the Solid Tumor

Lens as a Physiological Model for Cellular Hypoxic Response

The lens is an ideal model for hypoxia due to persistent oxygen deprivation, limited cellular apoptotic differentiation pathway, and the cellular adaptation to nutrient deprivation and low intracellular pH (82, 91). As mentioned above, the lens naturally expresses three distinct connexin proteins, Cx43, Cx46, and Cx50. Each of these connexins has been ascribed functions

within the lens. Cx43 provides a means of circulation through the growing and dividing epithelia. Cx50 regulates the size and volume of lenses throughout development. Cx46 provides the main circulatory function of antioxidants and metabolites throughout the developing and in particular the mature lens fibers. Knockout of Cx50 results in low growth and maturation potential of the lens and contributes to cataract formation. Cx46 knockout produces severe cataracts because of lens protein modifications like oxidation, truncation, and insolubility. Since each connexin has a known function in the lens, one can use the lens to tease out effects in connexin expression levels and contribution to the survivability of the lens tissue, especially as this relates to hypoxia.

Connexin 46 seems to be important for the correct function of other lens proteins under physiologically stressful growth conditions. The lens is quite hypoxic in nature and when Cx46 function is disrupted, the effects of hypoxia can be seen as cataract formation. Our hypothesis is that Cx46 is needed under hypoxic conditions to help circulate necessary metabolites, such as sugars, ADP/ATP, NADH/NADPH, and ions, and to provide a means to dilute the damaging molecules produced during hypoxic insult, such as ROS, peroxides, and reduced antioxidants.

Interestingly, the lens displays controlled apoptosis and subsequent cellular senescence upon full differentiation. The mechanisms behind the formation of the organelle free zone (OFZ) are unknown, but there is a causal link between the OFZ and the type of ubiquitin linkage seen in the lens. Oxygen tensions correlate well with the onset and creation of the OFZ as well as Cx46 stabilization. This correlation drives the reasoning that hypoxia and possibly signal molecule dilution through gap junctions are the initiating signal.

The Y79 Solid Tumor as a Model of Connexin Biology in Cancer

The Y79 cell line was first isolated from a 2.5 year old child with a maternal family history of retinoblastoma (128). Retinoblastoma as a diagnosis affects roughly 3.6 children per million children under the age of 15 in the United States per year alone (129). Retinoblastoma as a cancer exhibits many of the same features as other solid tumors in the body. Solid tumors generally have the same three physiological features of nutrient and growth factor deprivation, low oxygen content, and low pH due to the rapidly dividing cells and unorganized growth (130). New vasculature is produced once hypoxic growth conditions have been reached within the solid retinoblastoma tumor, typically at a size above 3.5 mm^3 (128).

Vasculature is needed to provide nutrients and growth factors, maintain tissue and cellular pH, and to remove toxic metabolites that are produced due to the hypoxic environment and Warburg glycolysis (130-131). The main functions of lens gap junctions are to circulate these metabolites and other molecules to the epithelia. This function would most likely be present in tissues lacking sufficient vasculature.

The retina is a rather complex tissue to where 10 neuronal cell types comprise the full tissue, each having a specific function. The cell layers comprising the mammalian retina include the inner limiting membrane (Müller cells), the nerve fiber layer (axons of the ganglion cell nuclei), ganglion cell layer (ganglion cell bodies), inner plexiform layer (synapses between the bipolar cell axons and the dendrites of the ganglion and amacrine cells), inner nuclear layer (bipolar cell bodies), outer plexiform layer (tips of rods and cones, and the Fiber layer of Henle), outer nuclear layer (cell bodies of rods and cones), external limiting membrane (segmentation layer), a photoreceptor layer of rods and cones, and the retinal pigment epithelium (132-134, Wikipedia). Connexin expression in these tissues is difficult to assign due to species-specific differences present in the literature. Five major connexins are expressed in the mature human retina: Cx36, Cx43, Cx45, Cx50 and Cx57/62. Cx43 dominates the literature while few other studies in the retina have focused on other connexins. Interestingly, retinoblastoma can occur from any of the cell types and the Y79 cell line has not been identified as originating from any one layer in the retina but instead in which a cell type that loses the RB protein first (134).

Aim of Dissertation

The aim of this dissertation is to uncover the regulatory mechanism in which Cx46 provides resistance to cell death caused by hypoxia and/or ischemia.

References

1. Zhang H, Semenza GL. The expanding universe of hypoxia. J Mol Med (Berl). 2008 Jul;86(7):739-46.

2. Phelan P. Innexins: Members of an evolutionarily conserved family of gap-junction proteins. Biochim Biophys Acta. 2005 Jun 10;1711(2):225-45.

3. Panchin YV. Evolution of gap junction proteins--the pannexin alternative. J Exp Biol. 2005 Apr;208(Pt 8):1415-9.

4. Herve JC, Bourmeyster N, Sarrouilhe D, Duffy HS. Gap junctional complexes: From partners to functions. Prog Biophys Mol Biol. 2007 May-Jun;94(1-2):29-65.

5. Ceelen L, Haesebrouck F, Vanhaecke T, Rogiers V, Vinken M. Modulation of connexin signaling by bacterial pathogens and their toxins. Cell Mol Life Sci. 2011 Sep;68(18):3047-64.

6. Bosco D, Haefliger JA, Meda P. Connexins: Key mediators of endocrine function. Physiol Rev. 2011 Oct;91(4):1393-445.

7. Burra S, Jiang JX. Regulation of cellular function by connexin hemichannels. Int J Biochem Mol Biol. 2011;2(2):119-28.

8. MacVicar BA, Thompson RJ. Non-junction functions of pannexin-1 channels. Trends Neurosci. 2010 Feb;33(2):93-102.

9. D'hondt C, Ponsaerts R, De Smedt H, Vinken M, De Vuyst E, De Bock M, et al. Pannexin channels in ATP release and beyond: An unexpected rendezvous at the endoplasmic reticulum. Cell Signal. 2011 Feb;23(2):305-16.

10. Sosinsky GE, Boassa D, Dermietzel R, Duffy HS, Laird DW, MacVicar B, et al. Pannexin channels are not gap junction hemichannels. Channels (Austin). 2011 May-Jun;5(3):193-7.

11. D'hondt C, Ponsaerts R, De Smedt H, Bultynck G, Himpens B. Pannexins, distant relatives of the connexin family with specific cellular functions? Bioessays. 2009 Sep;31(9):953-74.

12. Potolicchio I, Cigliola V, Velazquez-Garcia S, Klee P, Valjevac A, Kapic D, et al. Connexin-dependent signaling in neuro-hormonal systems. Biochim Biophys Acta. 2012 Aug;1818(8):1919–1936.

13. Reaume AG, de Sousa PA, Kulkarni S, Langille BL, Zhu D, Davies TC, et al. Cardiac malformation in neonatal mice lacking connexin43. Science. 1995 Mar 24;267(5205):1831-4.

14. Willecke K, Nielsen MS. Synopsis of the international gap junction conference in elsinore, denmark august 5-9, 2007. Cell Commun Adhes. 2007 Nov-Dec;14(6):251-7.

15. Oyamada M, Oyamada Y, Takamatsu T. Regulation of connexin expression. Biochim Biophys Acta. 2005 Dec 20;1719(1-2):6-23.

16. Wolosin JM, Budak MT, Akinci MA. Ocular surface epithelial and stem cell development. Int J Dev Biol. 2004;48(8-9):981-91.

17. Shaham O, Smith AN, Robinson ML, Taketo MM, Lang RA, Ashery-Padan R. Pax6 is essential for lens fiber cell differentiation. Development. 2009 August 01;136(15):2567-78.

18. Linhares VL, Almeida NA, Menezes DC, Elliott DA, Lai D, Beyer EC, et al. Transcriptional regulation of the murine Connexin40 promoter by cardiac factors Nkx2-5, GATA4 and Tbx5 Cardiovasc Res. 2004 Dec 1;64(3):402-11.

19. Anderson CL, Zundel MA, Werner R. Variable promoter usage and alternative splicing in five mouse connexin genes. Genomics. 2005 Feb;85(2):238-44.

20. Juneja SC. mRNA expression pattern of multiple members of connexin gene family in normal and abnormal fetal gonads in mouse. Indian J Physiol Pharmacol. 2003 Apr;47(2):147-56.

21. Bierhuizen MF, van Amersfoorth SC, Groenewegen WA, Vliex S, Jongsma HJ. Characterization of the rat connexin40 promoter: Two Sp1/Sp3 binding sites contribute to transcriptional activation Cardiovasc Res. 2000 Jun;46(3):511-22.

22. Scott CA, Tattersall D, O'Toole EA, Kelsell DP. Connexins in epidermal homeostasis and skin disease. Biochim Biophys Acta. 2012 Aug;1818(8):1919-1936.

23. Lai-Cheong JE, Arita K, McGrath JA. Genetic diseases of junctions. J Invest Dermatol. 2007 Dec;127(12):2713-25.

24. Chin KY. Connexins, a new target in wound treatment. J Wound Care. 2011 Aug;20(8):386-90.

25. Sentani K, Oue N, Sakamoto N, Anami K, Naito Y, Aoyagi K, et al. Upregulation of connexin 30 in intestinal phenotype gastric cancer and its reduction during tumor progression. Pathobiology. 2010;77(5):241-8.

26. Long AC, Bomser JA, Grzybowski DM, Chandler HL. All-trans retinoic acid regulates cx43 expression, gap junction communication and differentiation in primary lens epithelial cells. Curr Eye Res. 2010 Aug;35(8):670-9.

27. Burr DB, Molina SA, Banerjee D, Low DM, Takemoto DJ. Treatment with connexin 46 siRNA suppresses the growth of human Y79 retinoblastoma cell xenografts *in vivo*. Exp Eye Res. 2011 Apr;92(4):251-9.

28. Vinken M, Decrock E, De Vuyst E, Ponsaerts R, D'hondt C, Bultynck G, et al. Connexins: Sensors and regulators of cell cycling. Biochim Biophys Acta. 2011 Jan;1815(1):13-25.

29. Bargiello TA, Tang Q, Oh S, Kwon T. Voltage-dependent conformational changes in connexin channels. Biochim Biophys Acta. 2012 Aug;1818(8):1807-1822.

30. Hoang QV, Qian H, Ripps H. Functional analysis of hemichannels and gap-junctional channels formed by connexins 43 and 46. Mol Vis. 2010 Jul 15;16:1343-52.

31. Beyer EC, Gemel J, Seul KH, Larson DM, Banach K, Brink PR. Modulation of intercellular communication by differential regulation and heteromeric mixing of co-expressed connexins Braz J Med Biol Res. 2000 Apr;33(4):391-7.

32. Vorderwulbecke BJ, Maroski J, Fiedorowicz K, Da Silva-Azevedo L, Marki A, Pries AR, et al. Regulation of endothelial connexin40 expression by shear stress. Am J Physiol Heart Circ Physiol. 2012 Jan;302(1):H143-52.

33. Sohl G, Nielsen PA, Eiberger J, Willecke K. Expression profiles of the novel human connexin genes hCx30.2, hCx40.1, and hCx62 differ from their putative mouse orthologues. Cell Commun Adhes. 2003 Jan-Feb;10(1):27-36.

34. Bedner P, Steinhauser C, Theis M. Functional redundancy and compensation among members of gap junction protein families? Biochim Biophys Acta. 2012 Aug;1818(8):1971-1984.

35. Bendtsen JD, Nielsen H, von Heijne G, Brunak S. Improved prediction of signal peptides: SignalP 3.0. J Mol Biol. 2004 Jul 16;340(4):783-95.

36. Bendtsen JD, Jensen LJ, Blom N, Von Heijne G, Brunak S. Feature-based prediction of non-classical and leaderless protein secretion. Protein Eng Des Sel. 2004 Apr;17(4):349-56.

37. Martin PE, Evans WH. Incorporation of connexins into plasma membranes and gap junctions. Cardiovasc Res. 2004 May 1;62(2):378-87.

38. Maeda S, Tsukihara T. Structure of the gap junction channel and its implications for its biological functions. Cell Mol Life Sci. 2011 Apr;68(7):1115-29.

39. Falk MM, Kumar NM, Gilula NB. Membrane insertion of gap junction connexins: Polytopic channel forming membrane proteins. J Cell Biol. 1994 Oct;127(2):343-55.

40. VanSlyke JK, Naus CC, Musil LS. Conformational maturation and post-ER multisubunit assembly of gap junction proteins. Mol Biol Cell. 2009 May 1;20(9):2451-63.

41. Defamie N, Mesnil M. The modulation of gap-junctional intercellular communication by lipid rafts. Biochim Biophys Acta. 2012 Aug;1818(8):1866-1869.

42. Retamal MA, Yin S, Altenberg GA, Reuss L. Modulation of Cx46 hemichannels by nitric oxide. Am J Physiol Cell Physiol. 2009 June 1;296(6):C1356-1363.

43. VanSlyke JK, Musil LS. Analysis of connexin intracellular transport and assembly. Methods. 2000 Feb;20(2):156-64.

44. Martinez AD, Maripillan J, Acuna R, Minogue PJ, Berthoud VM, Beyer EC. Different domains are critical for oligomerization compatibility of different connexins. Biochem J. 2011 May 15;436(1):35-43.

45. Lauf U, Giepmans BN, Lopez P, Braconnot S, Chen SC, Falk MM. Dynamic trafficking and delivery of connexons to the plasma membrane and accretion to gap junctions in living cells. Proc Natl Acad Sci U S A. 2002 Aug 6;99(16):10446-51.

46. Fort AG, Murray JW, Dandachi N, Davidson MW, Dermietzel R, Wolkoff AW, et al. *In vitro* motility of liver connexin vesicles along microtubules utilizes kinesin motors. J Biol Chem. 2011 Jul 1;286(26):22875-85.

47. Leithe E, Sirnes S, Fykerud T, Kjenseth A, Rivedal E. Endocytosis and post-endocytic sorting of connexins. Biochim Biophys Acta. 2012 Aug;1818(8):1870-1879.

48. Kjenseth A, Fykerud T, Rivedal E, Leithe E. Regulation of gap junction intercellular communication by the ubiquitin system. Cell Signal. 2010 Sep;22(9):1267-73.

49. Hesketh GG, Van Eyk JE, Tomaselli GF. Mechanisms of gap junction traffic in health and disease. J Cardiovasc Pharmacol. 2009 Oct;54(4):263-72.

50. Laird DW. Life cycle of connexins in health and disease. Biochem J. 2006 Mar 15;394(Pt 3):527-43.

51. Ahmad S, Martin PE, Evans WH. Assembly of gap junction channels: Mechanism, effects of calmodulin antagonists and identification of connexin oligomerization determinants. Eur J Biochem. 2001 Aug;268(16):4544-52.

52. Lagree V, Brunschwig K, Lopez P, Gilula NB, Richard G, Falk MM. Specific amino-acid residues in the N-terminus and TM3 implicated in channel function and oligomerization compatibility of connexin43. J Cell Sci. 2003 August 1;116(15):3189-201.

53. Maeda S, Nakagawa S, Suga M, Yamashita E, Oshima A, Fujiyoshi Y, et al. Structure of the connexin 26 gap junction channel at 3.5 A resolution. Nature. 2009 Apr 2;458(7238):597-602.

54. Goodenough DA, Paul DL. Gap junctions. Cold Spring Harb Perspect Biol. 2009 Jul;1(1):a002576.

55. Valiunas V, Polosina YY, Miller H, Potapova IA, Valiuniene L, Doronin S, et al. Connexin-specific cell-to-cell transfer of short interfering RNA by gap junctions. J Physiol. 2005 Oct 15;568(Pt 2):459-68.

56. Eckert R. pH gating of lens fibre connexins. Pflugers Arch. 2002 Mar;443(5-6):843-51.

57. Srinivas M, Calderon DP, Kronengold J, Verselis VK. Regulation of connexin hemichannels by monovalent cations. J Gen Physiol. 2006 Jan;127(1):67-75.

58. Walter WJ, Zeilinger C, Bintig W, Kolb HA, Ngezahayo A. Phosphorylation in the C-terminus of the rat connexin46 (rCx46) and regulation of the conducting activity of the formed connexons. J Bioenerg Biomembr. 2008 Aug;40(4):397-405.

59. Herve JC, Bourmeyster N, Sarrouilhe D. Diversity in protein-protein interactions of connexins: Emerging roles Biochim Biophys Acta. 2004 Mar 23;1662(1-2):22-41.

60. Kyle JW, Berthoud VM, Kurutz J, Minogue PJ, Greenspan M, Hanck DA, et al. The N terminus of Connexin37 contains an α-helix that is required for channel function. Journal of Biological Chemistry. 2009 July 24;284(30):20418-27.

61. Zeilinger C, Steffens M, Kolb H. Length of C-terminus of rCx46 influences oligomerization and hemichannel properties. Biochimica et Biophysica Acta (BBA) - Biomembranes. 2005 12/30;1720(1-2):35-43.

62. Pal JD, Liu X, Mackay D, Shiels A, Berthoud VM, Beyer EC, et al. Connexin46 mutations linked to congenital cataract show loss of gap junction channel function Am J Physiol Cell Physiol. 2000 Sep;279(3):C596-602.

63. Maass K, Chase SE, Lin X, Delmar M. Cx43 CT domain influences infarct size and susceptibility to ventricular tachyarrhythmias in acute myocardial infarction. Cardiovasc Res. 2009 84(3):361-367.

64. Wang Z, Schey KL. Phosphorylation and truncation sites of bovine lens connexin 46 and connexin 50. Exp Eye Res. 2009 Dec;89(6):898-904.

65. Banerjee D, Das S, Molina SA, Madgwick D, Katz MR, Jena S, et al. Investigation of the reciprocal relationship between the expression of two gap junction connexin proteins, connexin46 and connexin43. J Biol Chem. 2011 Jul 8;286(27):24519-33.

66. Lau AF. c-src: Bridging the gap between phosphorylation- and acidification-induced gap junction channel closure. Sci STKE. 2005 Jul 5;2005(291):pe33.

67. Boswell BA, Le AC, Musil LS. Upregulation and maintenance of gap junctional communication in lens cells. Exp Eye Res. 2009 May;88(5):919-27.

68. Yin X, Liu J, Jiang JX. Lens fiber connexin turnover and caspase-3-mediated cleavage are regulated alternately by phosphorylation. Cell Commun Adhes. 2008 May;15(1):1-11.

69. Martinez-Wittinghan FJ, Sellitto C, Li L, Gong X, Brink PR, Mathias RT, et al. Dominant cataracts result from incongruous mixing of wild-type lens connexins. J Cell Biol. 2003 Jun 9;161(5):969-78.

70. Akoyev V, Das S, Jena S, Grauer L, Takemoto DJ. Hypoxia-regulated activity of PKCepsilon in the lens. Invest Ophthalmol Vis Sci. 2009 Mar;50(3):1271-82.

71. Su V, Nakagawa R, Koval M, Lau AF. Ubiquitin-independent proteasomal degradation of endoplasmic reticulum-localized connexin43 mediated by CIP75 J Biol Chem. 2010 Dec 24;285(52):40979-90.

72. Sanches DS, Pires CG, Fukumasu H, Cogliati B, Matsuzaki P, Chaible LM, et al. Expression of connexins in normal and neoplastic canine bone tissue. Veterinary Pathology Online. 2009 September 01;46(5):846-59.

73. Rodriguez-Sinovas A, Cabestrero A, Lopez D, Torre I, Morente M, Abellan A, et al. The modulatory effects of connexin 43 on cell death/survival beyond cell coupling. Prog Biophys Mol Biol. 2007 May-Jun;94(1-2):219-32.

74. Batra N, Kar R, Jiang JX. Gap junctions and hemichannels in signal transmission, function and development of bone. Biochim Biophys Acta. 2012 Aug;1818(8):1909-1918

75. Civitelli R. Cell-cell communication in the osteoblast/osteocyte lineage. Arch Biochem Biophys. 2008 May 15;473(2):188-92.

76. Chaible LM, Sanches DS, Cogliati B, Mennecier G, Zaidan Dagli ML Dvm Ms,P. Delayed osteoblastic differentiation and bone development in Cx43 knockout mice. Toxicol Pathol. 2011 Dec;39(7):1046-1055.

77. Koval M, Harley JE, Hick E, Steinberg TH. Connexin46 is retained as monomers in a trans-golgi compartment of osteoblastic cells. J Cell Biol. 1997 May 19;137(4):847-57.

78. Scott CA, Kelsell DP. Key functions for gap junctions in skin and hearing. Biochem J. 2011 Sep 1;438(2):245-54.

79. Kurtz A. Renal connexins and blood pressure. Biochim Biophys Acta. 2012 Aug;1818(8):1903-1908.

80. Hanner F, Sorensen CM, Holstein-Rathlou NH, Peti-Peterdi J. Connexins and the kidney. Am J Physiol Regul Integr Comp Physiol. 2010 May;298(5):R1143-55.

81. Abrams CK, Scherer SS. Gap junctions in inherited human disorders of the central nervous system. Biochim Biophys Acta. 2012 Aug;1818(8):2030-2047.

82. Mathias RT, White TW, Gong X. Lens gap junctions in growth, differentiation, and homeostasis Physiol Rev. 2010 Jan;90(1):179-206.

83. Xia CH, Liu H, Cheung D, Cheng C, Wang E, Du X, et al. Diverse gap junctions modulate distinct mechanisms for fiber cell formation during lens development and cataractogenesis. Development. 2006 May;133(10):2033-40.

84. Minogue PJ, Liu X, Ebihara L, Beyer EC, Berthoud VM. An aberrant sequence in a connexin46 mutant underlies congenital cataracts. J Biol Chem. 2005 Dec 9;280(49):40788-95.

85. Yoshida M, Harada Y, Kaidzu S, Ohira A, Masuda J, Nabika T. New genetic model rat for congenital cataracts due to a connexin 46 (Gja3) mutation. Pathol Int. 2005 Nov;55(11):732-7.

86. Chang B, Wang X, Hawes NL, Ojakian R, Davisson MT, Lo WK, et al. A Gja8 (Cx50) point mutation causes an alteration of alpha 3 connexin (Cx46) in semi-dominant cataracts of Lop10 mice. Hum Mol Genet. 2002 Mar 1;11(5):507-13.

87. Dunia I, Cibert C, Gong X, Xia CH, Recouvreur M, Levy E, et al. Structural and immunocytochemical alterations in eye lens fiber cells from Cx46 and Cx50 knockout mice. Eur J Cell Biol. 2006 Aug;85(8):729-52.

88. Gao J, Sun X, Martinez-Wittinghan FJ, Gong X, White TW, Mathias RT. Connections between connexins, calcium, and cataracts in the lens. J Gen Physiol. 2004 Oct;124(4):289-300.

89. Xiao F, Weng J, Fan K, Wang W. Detailed regulatory mechanism of the interaction between ZO-1 PDZ2 and connexin43 revealed by MD simulations. PLoS One. 2011;6(6):e21527.

90. Park DJ, Wallick CJ, Martyn KD, Lau AF, Jin C, Warn-Cramer BJ. Akt phosphorylates Connexin43 on Ser373, a "mode-1" binding site for 14-3-3. Cell Commun Adhes. 2007 Sep-Oct;14(5):211-26.

91. Wride MA. Lens fibre cell differentiation and organelle loss: Many paths lead to clarity Philos Trans R Soc Lond B Biol Sci. 2011 Apr 27;366(1568):1219-33.

92. Shui YB, Arbeit JM, Johnson RS, Beebe DC. HIF-1: An age-dependent regulator of lens cell proliferation. Invest Ophthalmol Vis Sci. 2008 Nov;49(11):4961-70.

93. Cheng C, Xia CH, Li L, White TW, Niimi J, Gong X. Gap junction communication influences intercellular protein distribution in the lens. Exp Eye Res. 2008 Jun;86(6):966-74.

94. Maass K, Shibayama J, Chase SE, Willecke K, Delmar M. C-terminal truncation of connexin43 changes number, size, and localization of cardiac gap junction plaques. Circ Res. 2007 Dec 7;101(12):1283-91.

95. Severs NJ. The carboxy terminal domain of connexin43: From molecular regulation of the gap junction channel to supramolecular organization of the intercalated disk. Circ Res. 2007 Dec 7;101(12):1213-5.

96. Weber GF, Menko AS. The canonical intrinsic mitochondrial death pathway has a non-apoptotic role in signaling lens cell differentiation. J Biol Chem. 2005 Jun 10;280(23):22135-45.

97. Gong X, Cheng C, Xia CH. Connexins in lens development and cataractogenesis. J Membr Biol. 2007 Aug;218(1-3):9-12.

98. Gong X, Agopian K, Kumar NM, Gilula NB. Genetic factors influence cataract formation in alpha 3 connexin knockout mice. Dev Genet. 1999;24(1-2):27-32.

99. Harris AL. Connexin channel permeability to cytoplasmic molecules. Prog Biophys Mol Biol. 2007 May-Jun;94(1-2):120-43.

100. Kanaporis G, Mese G, Valiuniene L, White TW, Brink PR, Valiunas V. Gap junction channels exhibit connexin-specific permeability to cyclic nucleotides. J Gen Physiol. 2008 Apr;131(4):293-305.

101. Semenza GL. Regulation of metabolism by hypoxia-inducible factor 1. Cold Spring Harb Symp Quant Biol. 2011 Jul 22.

102. Edwards LM, Ashrafian H, Korzeniewski B. In silico studies on the sensitivity of myocardial PCr/ATP to changes in mitochondrial enzyme activity and oxygen concentration. Mol Biosyst. 2011 Dec;7(12):3335-42.

103. Semenza GL. Regulation of oxygen homeostasis by hypoxia-inducible factor 1. Physiology. 2009 April 1;24(2):97-106.

104. Seta KA, Millhorn DE. Functional genomics approach to hypoxia signaling. J Appl Physiol. 2004 Feb;96(2):765-73.

105. Fandrey J, Gorr TA, Gassmann M. Regulating cellular oxygen sensing by hydroxylation. Cardiovasc Res. 2006 Sep 1;71(4):642-51.

106. Kaelin WG,Jr, Ratcliffe PJ. Oxygen sensing by metazoans: The central role of the HIF hydroxylase pathway. Mol Cell. 2008 May 23;30(4):393-402.

107. Siddiq A, Aminova LR, Ratan RR. Hypoxia inducible factor prolyl 4-hydroxylase enzymes: Center stage in the battle against hypoxia, metabolic compromise and oxidative stress. Neurochem Res. 2007 Apr-May;32(4-5):931-46.

108. Semenza GL. Oxygen-dependent regulation of mitochondrial respiration by hypoxia-inducible factor 1. Biochem J. 2007 Jul 1;405(1):1-9.

109. Formenti F, Constantin-Teodosiu D, Emmanuel Y, Cheeseman J, Dorrington KL, Edwards LM, et al. Regulation of human metabolism by hypoxia-inducible factor. Proc Natl Acad Sci U S A. 2010 Jul 13;107(28):12722-7.

110. Patterson AJ, Zhang L. Hypoxia and fetal heart development Curr Mol Med. 2010 Oct;10(7):653-66.

111. Gharib SA, Khalyfa A, Abdelkarim A, Ramesh V, Buazza M, Kaushal N, et al. Intermittent hypoxia activates temporally coordinated transcriptional programs in visceral adipose tissue. J Mol Med (Berl). 2011 Nov 16.

112. Famulla S, Horrighs A, Cramer A, Sell H, Eckel J. Hypoxia reduces the response of human adipocytes towards TNFalpha resulting in reduced NF-kappaB signaling and MCP-1 secretion. Int J Obes (Lond). 2011 Oct 18.

113. Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metab. 2006 Mar;3(3):187-97.

114. Denko NC. Hypoxia, HIF1 and glucose metabolism in the solid tumour Nat Rev Cancer. 2008 Sep;8(9):705-13.

115. Zhang H, Bosch-Marce M, Shimoda LA, Tan YS, Baek JH, Wesley JB, et al. Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. J Biol Chem. 2008 Apr 18;283(16):10892-903.

116. Fukuda R, Zhang H, Kim JW, Shimoda L, Dang CV, Semenza GL. HIF-1 regulates cytochrome oxidase subunits to optimize efficiency of respiration in hypoxic cells. Cell. 2007 Apr 6;129(1):111-22.

117. Semenza GL. Hypoxia-inducible factor 1: Regulator of mitochondrial metabolism and mediator of ischemic preconditioning. Biochim Biophys Acta. 2011 Jul;1813(7):1263-8.

118. Yeung SJ, Pan J, Lee MH. Roles of p53, MYC and HIF-1 in regulating glycolysis - the seventh hallmark of cancer Cell Mol Life Sci. 2008 Dec;65(24):3981-99.

119. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer. 2003 Oct;3(10):721-32.

120. Lu X, Kang Y. Hypoxia and hypoxia-inducible factors: Master regulators of metastasis. Clin Cancer Res. 2010 Dec 15;16(24):5928-35.

121. Shimoda LA, Semenza GL. HIF and the lung: Role of hypoxia-inducible factors in pulmonary development and disease Am J Respir Crit Care Med. 2011 Jan 15;183(2):152-6.

122. Wan C, Shao J, Gilbert SR, Riddle RC, Long F, Johnson RS, et al. Role of HIF-1alpha in skeletal development Ann N Y Acad Sci. 2010 Mar;1192:322-6.

123. Benita Y, Kikuchi H, Smith AD, Zhang MQ, Chung DC, Xavier RJ. An integrative genomics approach identifies hypoxia inducible factor-1 (HIF-1)-target genes that form the core response to hypoxia Nucleic Acids Res. 2009 Aug;37(14):4587-602.

124. Maynard MA, Evans AJ, Shi W, Kim WY, Liu FF, Ohh M. Dominant-negative HIF-3 alpha 4 suppresses VHL-null renal cell carcinoma progression. Cell Cycle. 2007 Nov 15;6(22):2810-6.

125. Milovanova TN, Bhopale VM, Sorokina EM, Moore JS, Hunt TK, Hauer-Jensen M, et al. Hyperbaric oxygen stimulates vasculogenic stem cell growth and differentiation *in vivo*. J Appl Physiol. 2009 Feb;106(2):711-28.

126. Makino Y, Uenishi R, Okamoto K, Isoe T, Hosono O, Tanaka H, et al. Transcriptional up-regulation of inhibitory PAS domain protein gene expression by hypoxia-inducible factor 1 (HIF-1): A negative feedback regulatory circuit in HIF-1-mediated signaling in hypoxic cells. J Biol Chem. 2007 May 11;282(19):14073-82.

127. Sakharkar MK, Chow VT, Kangueane P. Distributions of exons and introns in the human genome. In Silico Biol. 2004;4(4):387-93.

128. Chevez-Barrios P, Hurwitz MY, Louie K, Marcus KT, Holcombe VN, Schafer P, et al. Metastatic and nonmetastatic models of retinoblastoma. Am J Pathol. 2000 Oct;157(4):1405-12.

129. Abramson DH, Schefler AC. Update on retinoblastoma. Retina. 2004 Dec;24(6):828-48.

130. Lunt SJ, Chaudary N, Hill RP. The tumor microenvironment and metastatic disease Clin Exp Metastasis. 2009;26(1):19-34.

131. Boutrid H, Jockovich ME, Murray TG, Pina Y, Feuer WJ, Lampidis TJ, et al. Targeting hypoxia, a novel treatment for advanced retinoblastoma. Invest Ophthalmol Vis Sci. 2008 Jul;49(7):2799-805.

132. Bloomfield SA, Volgyi B. The diverse functional roles and regulation of neuronal gap junctions in the retina. Nat Rev Neurosci. 2009 Jul;10(7):495-506.

133. Cook JE, Becker DL. Gap-junction proteins in retinal development: New roles for the "nexus". Physiology. 2009 August 1;24(4):219-30.

134. Dyer MA, Bremner R. The search for the retinoblastoma cell of origin. Nat Rev Cancer. 2005 Feb;5(2):91-101.

Chapter 2 - The Anti-Tumor *In Vivo* Function of Connexin 46 in a Human Y79 Retinoblastoma Xenograft Tumor Model

This chapter was previously published as:

Diana B. Burr*, Samuel A. Molina*, Debarshi Banerjee, Derek M. Low, and Dolores J. Takemoto. "Treatment with connexin 46 siRNA suppresses the growth of human Y79 retinoblastoma cell xenografts *in vivo*" Exp Eye Res. 2011 Apr;92(4):251-9. *Co-First Author

Abstract

Tumors with a hypoxic component, including human Y79 retinoblastoma cells, express a specific gap junction protein, Connexin 46 (Cx46), which is usually only found in naturally hypoxic tissues such as the differentiated lens. The aim of this study was to investigate if Cx46 downregulation would suppress Y79 tumor formation *in vivo*. Five-week old nude mice were subcutaneously implanted with human Y79 retinoblastoma cells and treated with intratumor siRNA injections of 30 μg Cx46 siRNA (n=6), 30 μg non-silencing siRNA (n=6), or no siRNA treatment (n=6) every 2 days for a maximum of 10 treatments. Tumor volume (TV) was calculated from the recorded caliper measurements of length and width. Excised tumors were measured and weighed. Western blot analyses were performed to evaluate Cx46 and Cx43 expression in tumors which received Cx46 siRNA, non-silencing siRNA, or no siRNA treatment. Tumor histopathology was used to assess tumor features. Cx46 siRNA treated Y79 tumors had a reduced TV (287 mm^3 ± 77 mm^3) when compared to the tumors of mice receiving the negative control siRNA (894 mm^3 ± 218 mm^3; $P \leq 0.03$) or no siRNA (1068 mm^3 ± 192 mm^3; $P \leq 0.002$). A 6-fold knockdown of Cx46 and a 3-fold rise in Cx43 protein expression was observed from western blots of tumors treated with Cx46 siRNA compared to mice treated with non-silencing siRNA. Knockdown of Cx46 with siRNA had an antitumor effect on human Y79 retinoblastoma tumors in the nude mouse model. The results suggest that anti-Cx46 therapy may be a potential target in the future treatment of retinoblastoma.

Introduction

Ocular retinoblastoma is a rare, pediatric ocular tumor, caused by mutations in the retinoblastoma tumor suppressor gene (RB1), which may occur in both eyes of affected children.

Current treatment methods with chemotherapy using carboplatin, vincristine, and etoposide, cryotherapy, laser photocoagulation, and external beam radiation therapy have greatly improved patient outcome against this neoplasia without enucleation (1, 2). However, even in successful cases, significant side effects have been reported, including secondary malignant tumor development and deformities at the irradiation site, ischemic necrosis of the optic nerve, ototoxicity, and bone marrow suppression with potentially subsequent systemic septicemia (3-5). Currently, there is a need for an initial therapy and drug development for therapies, which are less toxic and can be used for extended periods to prevent or suppress tumor growth and protect the remaining unaffected eye to prolong vision in these young children.

The retina is usually highly oxygenated due to extensive vascularization, however, retinoblastoma, like many solid tumors, has a hypoxic stage where oxygen levels are approximately 1.5%, allowing tumor cells to have a growth advantage over normal tissue (6). Growth at 1% oxygen inside the early tumor is supported by the up-regulation of the regulatory transcription factor complex, hypoxia-inducible factor (HIF). Many genes, often controlled by HIF, contribute to the maintenance of hypoxia and are involved in early tumor formation and progression (7). One of these genes is the gap junction protein, connexin 46 (Cx46), which is naturally expressed in the hypoxic lens but can also be found in ischemic tissues such as wounds, infarcts, and traumatized or avascularized tissues (8). Our lab has recently identified Cx46 expression in solid tumors with a hypoxic component.

Gap junctions are membrane channels that allow intercellular communication through the passage of small molecules, ions, and metabolites. Connexins, the proteins that make up the gap junctions, are expressed in a tissue specific manner (9) and normal connexin expression is often altered in neoplasia. Connexins began to be investigated as tumor suppressors when it was observed that Cx32 knockout mice were more likely to develop liver tumor formation in comparison to their wild type littermates (10). It is suspected that many tumors have a decrease in their normal connexin expression and may also have an impaired ability to form gap junctions with connexin proteins (11). The loss of gap junction communication and gap junction proteins has been associated with the development of neoplastic and malignant progression (12-15). Several connexins have been investigated for their growth inhibitory effects, but Cx43 has the best documented effects as a tumor suppressor (11, 14-19). Several tissues, such as prostate and breast tissues, naturally express Cx43 and it is required for normal development and function.

However, in both prostate and breast cancer, expression of Cx43 is significantly downregulated (16-17). In the normal prostate samples, nearly all specimens were positive for Cx43, while nearly two thirds of prostate cancer tissues were negative for Cx43, and Cx43 was present in only 10% of poorly differentiated prostate tumors (16). While overexpression of Cx43 restored growth control in MDA-MB-435 human breast tumor cells *in vitro* (18), Cx43 upregulation suppressed tumor growth in MDA-MB-231 human breast tumors *in vivo* (11). Lastly, Cx43 knockdown with siRNA prompted development of an aggressive tumor phenotype (19). Studies of Cx43 with retinoblastoma protein (Rb1) also suggest that Cx43 suppresses tumor formation (20).

One very important feature of tumors is their adaptation to hypoxia which then favors tumor growth and survival beyond that of normal tissue. We previously reported that the hypoxia-specific gap junction protein, Cx46, is upregulated in MCF-7 breast cancer cells and human breast tumors but is not found in normal breast tissue. Downregulation of Cx46 suppressed tumor growth in xenograft MCF-7 cell tumors (21). We hypothesized that the presence of Cx46 promotes tumor growth in hypoxia. Our lab has also shown that overexpression of Cx46 is sufficient to protect a gap junction deficient cell line, neuronal 2A cells (N2A), from hypoxia-induced cell death, while overexpression of Cx43 does not offer any protection compared to wild-type cells. Furthermore, downregulation of Cx46 in lens epithelial cell lines, which naturally thrive in hypoxia *in vitro*, rendered these cells sensitive to the effects of hypoxia (21).

Retinoblastoma, as well as many other tumors, has an early hypoxic growth stage, allowing tumor cells to have a growth advantage over normal tissues. In human lens epithelial cells, a reciprocal relationship exists between the expression of the tumor suppressor, Cx43, and the hypoxia-specific Cx46. The reciprocal relationship may also be present in tumors, and therefore, the tumor suppressor effects of Cx43 may be absent, promoting tumor growth (Burr DB, et al. ARVO Abstract 1575, 2010). The purpose of this study was to determine if Cx46 is expressed in the human Y79 retinoblastoma cell line. In this study, we investigate Cx46 as a novel gap junction protein which confers resistance and protects cells from hypoxic death. We found that human Y79 retinoblastoma cells and tumors highly express Cx46 and have minimal expression of Cx43. Our hypothesis is that Cx46 downregulation, using short interfering RNAs (siRNA), will prevent or suppress Y79 retinoblastoma tumor development *in vivo*.

Materials and Methods

Cell Culture

Y79 cells, a human retinoblastoma cell line, were purchased from the American Type Culture Collection (ATCC), suspended and grown in RPMI-1640 medium (Invitrogen), supplemented with 20% premium fetal bovine serum (FBS) (Atlanta Biologicals), 50 U/mL of penicillin, 50 µg/mL streptomycin, and 50 µg/mL gentamycin (Gibco). Neuro2A cells (N2A) were grown in Low Glucose DMEM (Invitrogen) supplemented with 10% premium FBS (Atlanta Biologicals) with 50 U/mL of penicillin, 50 µg/mL streptomycin, and 50 µg/mL gentamycin (all Invitrogen). All cultures were maintained in a humidified 37ºC atmosphere of ambient air (21% O_2) and 5% CO_2.

Hypoxia and Cell Viability Assay

Hypoxia was defined as 1% O_2, 5% CO_2 and was maintained in a dual-controlled chamber (Bio-Spherix, ProOx Model C21, Redfield, NY) inserted into a temperature-controlled incubator, using N_2 as a displacement gas, at 37ºC and 100% relative humidity. Normoxic conditions were defined to be 21% O_2, 5% CO_2 at 37ºC with 100% relative humidity. Y79 cells were suspended in RPMI-1640 media, grown in 100 mm dishes, seeded at $2.25x10^5$ cells/plate, and incubated for up to 7 days in normoxia (21% O_2, 5% CO_2) or hypoxia (1% O_2, 5% CO_2), using pre-equilibrated hypoxic media for the hypoxic studies. Cell count and viability were assessed every 24 hours by removing an aliquot of the cell suspension. Cell viability and number was measured by automated trypan blue staining and counting using an Auto T4 Cellometer and associated software (Nexcelom Bioscience) set up for Y79 counting.

For the N2A hypoxia studies, N2A cells were stably transfected with pEGFP-Cx43GFP or pEGFP-Cx46GFP using Fugene 6 (Roche) followed by selection for 6-8 weeks in 1000 µg/mL G418 (Research Products International). Stable lines were maintained in the presence of 500 µg/mL G418. For the hypoxia cell viability studies, N2A lines were plated at 15,000 cells/well in an OptiLux clear bottom 96 well plate (BD Biosciences) and let attach for 6-12 hours in normoxic culture conditions. Media was then replaced with 100 µL antibiotic free growth media equilibrated to either 1% or 21% oxygen for hypoxic or normoxic assay and placed into the hypoxia or normoxia incubators, respectively. At the end of the indicated treatment period, 20 µL of CellTiter Blue Cell Viability Assay (Promega) fluorescent substrate

was added per well, mixed, incubated respectively for 2 hours, and then read on a Carry Eclipse fluorescent plate reader using the excitation/emission wavelength (slit) settings of 560 nm (10)/590 nm (20) at a 400V PMT setting. Fluorescence is directly proportional to the number of live cells.

Y79 siRNA Knockdown and Viability Studies

Optimization of the siRNA transfection protocol for Y79 cells was performed *in vitro* prior to the *in vivo* pilot/proof-of-concept study. Cells ($2.0x10^5$ Y79 cells) were plated in a total of 0.4 mL of complete RPMI media 16-24 hours prior to siRNA transfection and placed in normoxic growth conditions. Each siRNA were mixed with various amounts of HiPerfect (Qiagen), according to the manufacturers suggestions in serum-free RPMI, at ratios of 1 μg siRNA : 3 μL HiPerfect, 1:6 and 1:12. Various combinations were tried and the remaining transfections were completed using 250 nM siRNA and 15 μL of HiPerfect (as to avoid cell death) in a total of 100 μL mixture per well of a 12 well plate with a 20 minute incubation at room temperature. Transfection mixture (0.1 mL) was added to the 0.4 mL of pre-incubated Y79 cells, mixed, and allowed to transfect in normoxic conditions for 6 hours prior to the addition of 0.5 mL complete RPMI equilibrated to either 21% or 1% oxygen. For the knockdown studies, siRNA transfected Y79 cells (250 nM) were kept in normoxic growth conditions for a further 18, 42 or 66 hour incubation period (to give 24, 48 or 72 hours post-transfection time points), then harvested and lysed according to the protocol below. For the viability studies, siRNA transfected cells (250 nM) were incubated in normoxic or hypoxic conditions for a further 18, 42, or 66 hours (to give 24, 48, and 72 hour post-transfection time points). At the end of the incubation period, the cell suspension was transferred to a microcentrifuge tube and mixed 1:1 with trypan blue solution. Cell viability and number was measured by automated trypan blue staining and counting using an Auto T4 Cellometer and associated software (Nexcelom Bioscience) set up for Y79 counting.

Transplantation of Human Y79 Retinoblastoma Cells into Nude Mice

The study protocol and procedures were approved by the Kansas State University Institutional Animal Care and Use Committee. All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The human Y79 retinoblastoma cell tumor nude mouse model has been previously characterized (22).

The human Y79 retinoblastoma cell line was suspended in Iscove's culture medium (Invitrogen) supplemented with 20% FBS. Five-week old, mixed sex, homozygous Nu/Nu nude mice (strain NuFoxn1) (n=18), purchased from Charles River Laboratories (Wilmington, MA), were subcutaneously transplanted with $1x10^7$ human Y79 retinoblastoma cells in 0.5 ml total volume of a 1:1 mixture of basement membrane matrix (BD Biosciences) and Iscove's medium supplemented with FBS, in the left dorsal region. Assessment of tumor development was performed daily. Once a tumor was palpable, typically 3 to 7 days after cell transplantation, treatment was initiated via intratumor siRNA injections. Mice were divided into 3 treatment groups with 6 mice in each group. Groups received intratumor injections of 30 µg Cx46 siRNA, 30 µg of non-silencing Allstars Negative Control siRNA, or they remained sham treated. Anti-Cx46 siRNA (Target sequence: CGC ATG GAA GAG AAG AAG AAA) and negative, non-silencing siRNA (catalog #1027281) were purchased from Qiagen (Valencia, CA). Intratumor siRNA treatment was given every other day for a maximum of 10 treatments.

Mice were examined every other day prior to siRNA treatment and the length (L) and width (W) of the tumors were measured with calipers to the nearest millimeter and recorded. Tumor volume was estimated using the following formula: $TV = (LxW^2)/2$. The tumor size measured immediately prior to the first siRNA injection was considered the day 0 measurement. Once a tumor became larger than 15 mm in any dimension or 10 siRNA treatments had been administered, mice were euthanized by CO_2 inhalation followed by thoracotomy, as a secondary method. Tumors were immediately excised, measured in 3 dimensions (mm), and weighed (mg). Tumors were sectioned in half, one section was snap frozen in a cryogenic vial to be homogenized for western blot analysis, while the remaining half was immersion-fixed in 10% formalin. Formalin-fixed tumor tissues were embedded in paraffin, serially sectioned and processed for standard hematoxylin and eosin (H&E) staining. Sections were pathologically evaluated for tumor features and cellular characteristics by a board-certified veterinary pathologist.

Western Blot

Cx46 and Cx43 expression were measured by Western blot analyses and were carried out as previously described (23). Y79 cells and tumors were homogenized in RIPA buffer containing a protease and phosphatase inhibitor cocktail (Calbiochem). Homogenates were

sonicated for 10 seconds on ice, three times, and then centrifuged at 2000 x *g* for 15 minutes at 4ºC. Equal amounts of protein, determined by the Bio-Rad Protein Assay, were used. Supernatant proteins were separated on an 8% SDS-PAGE gel and transferred to nitrocellulose membranes (OPTI-TRAN, Midwest Scientific). Membranes were blocked overnight at 4ºC in 5% nonfat powdered milk in tris-buffered saline (TBS), and then were incubated with the rabbit anti-Cx46 (1:1000 dilution) or rabbit anti-Cx43 (1:7500 dilution) antibodies overnight at 4ºC, then incubated with a goat anti-rabbit-HRP secondary antibody (1:10000 dilution, Thermo Scientific Pierce, 31460). Rabbit anti-Cx46 antibody (US Biologicals, C7858-07A) and rabbit anti-C-terminal Cx43 antibody (Sigma-Aldrich, C6219) were commercially available. β-actin (Sigma-Aldrich, A5441) and α-tubulin (Sigma-Aldrich, T6074) were used as loading controls. The blots were visualized using SuperSignal West Femto substrate (Pierce). Blots were digitized and analyzed, using UN-SCAN-IT software (Silk Scientific).

Statistical Analyses

Commercial software (Origin; Microcal Software, Inc., Northampton, MA) was used for statistical analyses. Results were expressed as the mean ± SEM and differences of $P \leq 0.05$, using a one-way analysis of variance, were considered statistically significant. The number of replicates (**n)** are given with each figure.

Results

Cx46 Expression in Y79 Retinoblastoma Cells in vitro

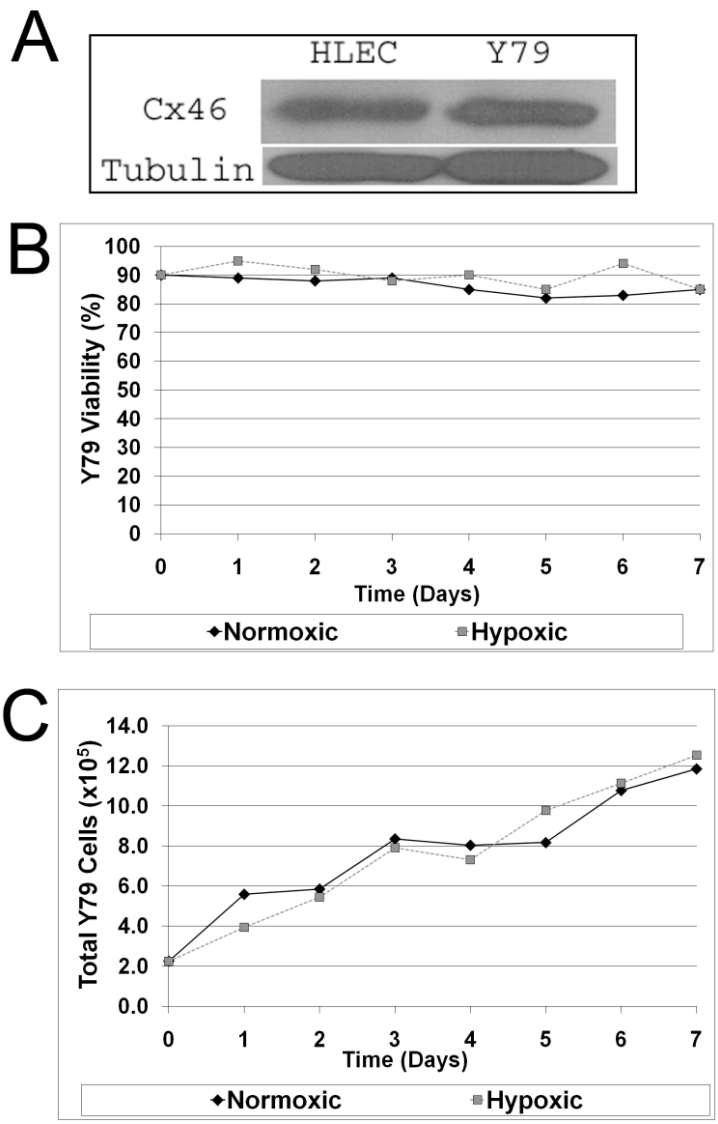

Figure 2.1 Y79 Cells Express Cx46 and Thrive Under Hypoxic Conditions
(A) Y79 whole cell lysates probed with rabbit anti-Cx46 antibody and mouse anti-tubulin antibody, demonstrating Cx46 expression in human Y79 retinoblastoma cells when compared to control human lens epithelial cells (HLEC). (B) Viability of Y79 cells under normoxia (37°C humidified ambient air, 5% CO2) and hypoxia (37°C humidified 1% oxygen displaced by N2, 5% CO2) was monitored for 7 days and recorded. (C) Total Y79 cell numbers were counted for 7 days under normoxic and hypoxic growth conditions. Data trends from three individual experiments indicate that Y79 cells express Cx46 and can survive and thrive in hypoxic conditions. Representative experimental data shown.

To investigate the effects of Cx46 in retinoblastoma growth, we had to establish that the human Y79 retinoblastoma cell line demonstrated Cx46 expression. Figure 2.1A shows that Cx46 protein is expressed endogenously in Y79 cells when compared to human lens epithelial cells (HLEC), which normally express Cx46 and naturally thrive in hypoxia. Y79 cells were then grown in both normoxic and hypoxic conditions as described in the Materials and Methods and assessed for cell viability and growth (Figs. 2.1B and 2.1C). Cells remained viable and grew in both hypoxic (5% O_2) and normoxic (21% O_2) growth conditions. After 7 days in normoxic growth conditions, there was a 5-fold increase in the number of Y79 cells with ≥80% viability. In hypoxic growth conditions, there also was a 5-fold increase in the number of Y79 cells and with ≥80% viability after 7 days. Similar results are presented in Figure 2.2. In contrast, when N2A cells are challenged with hypoxic growth conditions, these cells do not survive past 18 hours. However, only N2A cells expressing the carboxy-terminal GFP tagged wild type rat Cx46 survive in 1% oxygen. N2A cells expressing the related Cx43-GFP fusion protein do not survive hypoxia and behave similarly to un-transfected and hypoxia sensitive N2A cells (Figure 2.3). These data demonstrate that Cx46 confers resistance to hypoxia, and, that our hypoxia chamber is functional.

Transfection with Anti-Cx46 siRNA Negatively Affects Y79 Hypoxic Cell Growth in vitro

Since Y79 cells thrive in hypoxic growth conditions, we next tested if knocking down Cx46 expression levels affected the growth characteristics of Y79 cells. Once knockdown conditions were optimized (Figure 2.4), it was clear that a high amount of siRNA was required to have an effect on Cx46 protein expression levels (also observed in the *in vivo* tumor studies). Y79 cells are very resistant to many transfection methods, and this high amount of siRNA may be necessary because of low transfection efficiency (24-26). Next, the effects of anti-Cx46 siRNA were assessed by western blot (Figure 2.5) and by measuring cell count and viability (Figure 2.6).

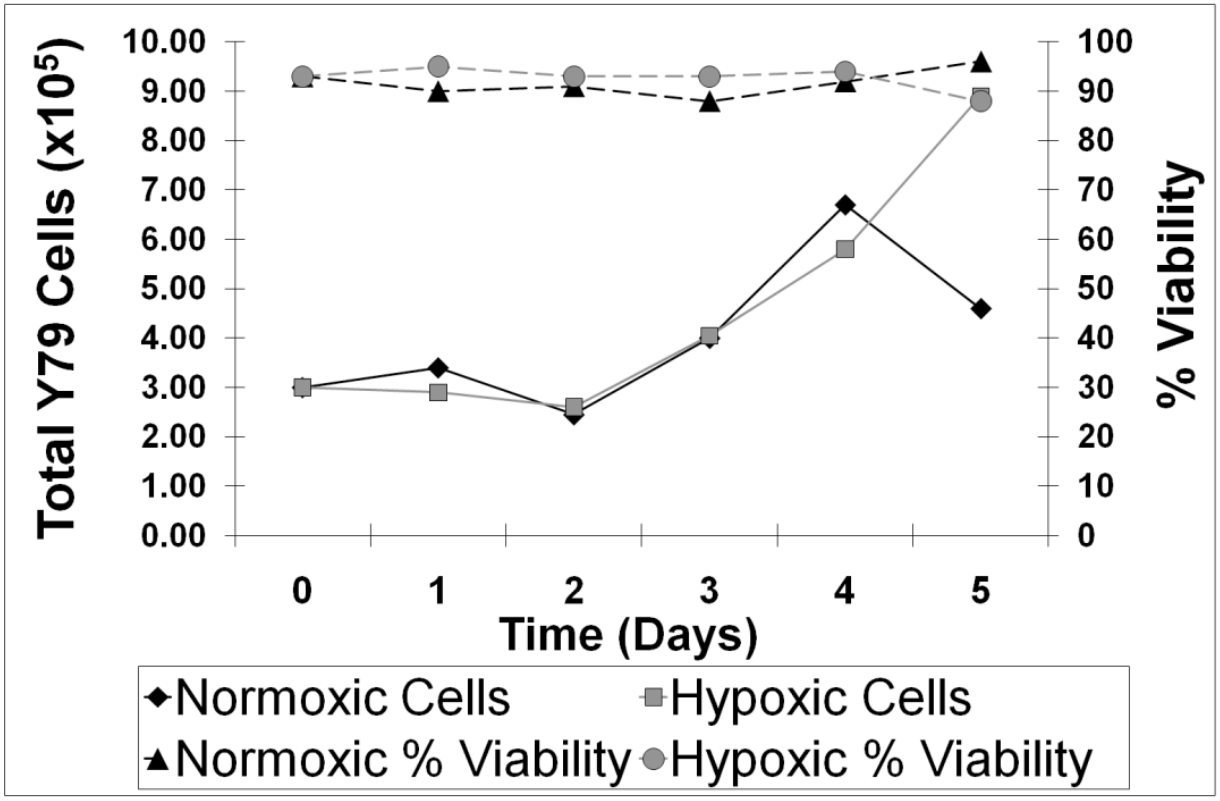

Figure 2.2 Measured Volumes (mm^3) of Individual Xenograft Tumors Used in this Study
Y79 cells exhibit normal growth characteristics in 1% oxygen. $3x10^5$ Y79 cells were placed in pre-equilibrated normoxic or hypoxic media (100mm dish) and incubated for up to 5 days in normoxia (37°C humidified 21% O_2, 5% CO_2) or hypoxia (37°C humidified 1% O_2, 5% CO_2, displaced with N_2). Cells grew after a 2 day lag period while maintaining viability under hypoxic growth conditions, indicating that Y79 cells can thrive in a hypoxic environment.

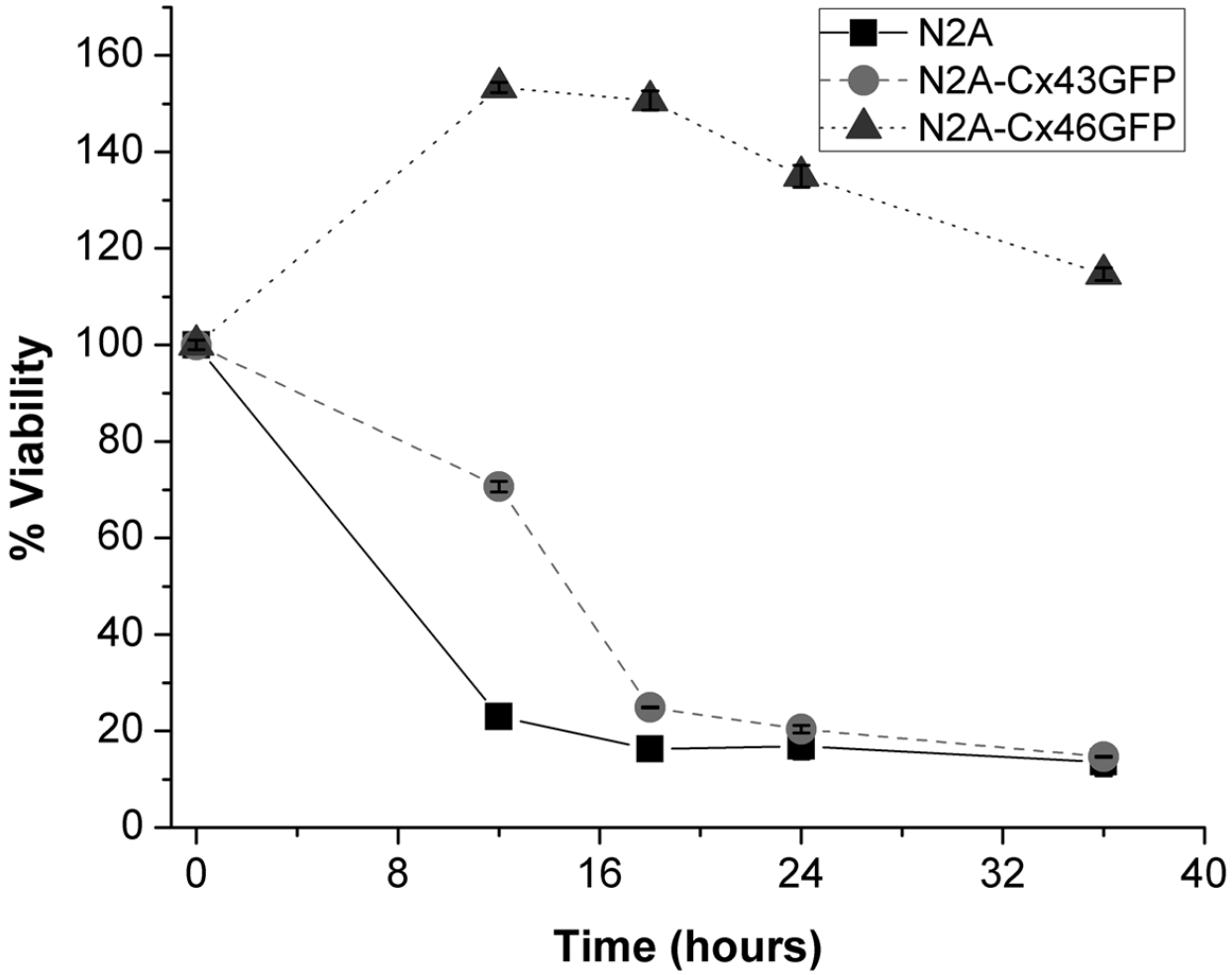

Figure 2.3 Y79 Cells Exhibit Normal Growth Characteristics in 1% Oxygen
$3x10^5$ Y79 cells were placed in pre-equilibrated normoxic or hypoxic media (100 mm dish) and incubated for up to 5 days in normoxia (37°C humidified 21% O_2, 5% CO_2) or hypoxia (37°C humidified 1% O_2, 5% CO_2, displaced with N2). Cells grew after a 2 day lag period while maintaining viability under hypoxic growth conditions, indicating that Y79 cells can thrive in a hypoxic environment.

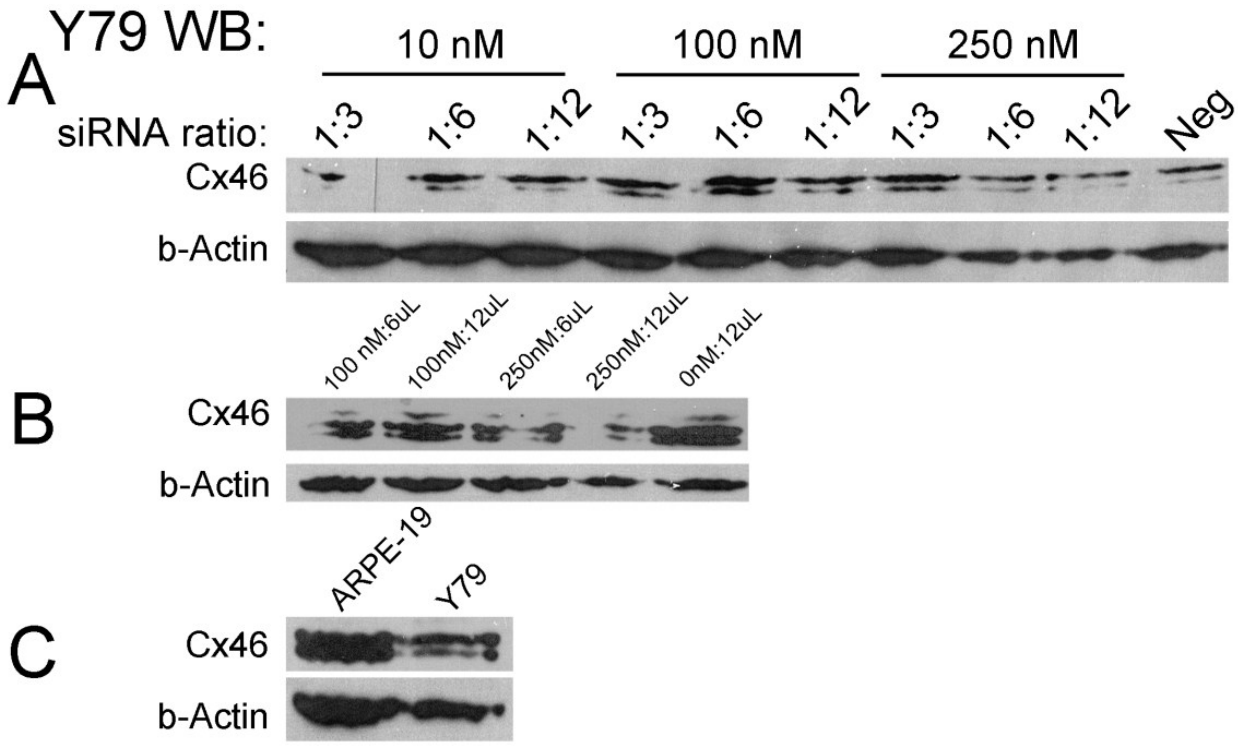

Figure 2.4 Optimization of siRNA-Mediated Knockdown of Cx46 in Y79 Cells
Optimization of anti-Cx46 iRNA transfection in Y79 cells. (A) Initial studies of 10, 100 or 250 nM siRNA with ug:uL HiPerfect ratios given. Negative control (Neg) at 100 nM, ratio of 1ug siRNA:6 uL HiPerfect. (B) Secondary optimization of siRNA transfection using nM siRNA:uL HiPerfect ratios, as given. A ratio of 250 nM siRNA:15 uL of HiPerfect used for the remaining *in vitro* siRNA assays. (C) 30 ug/lane of ARPE-19 and Y79 whole cell lysates used as Cx46 immunoblotting positive controls.

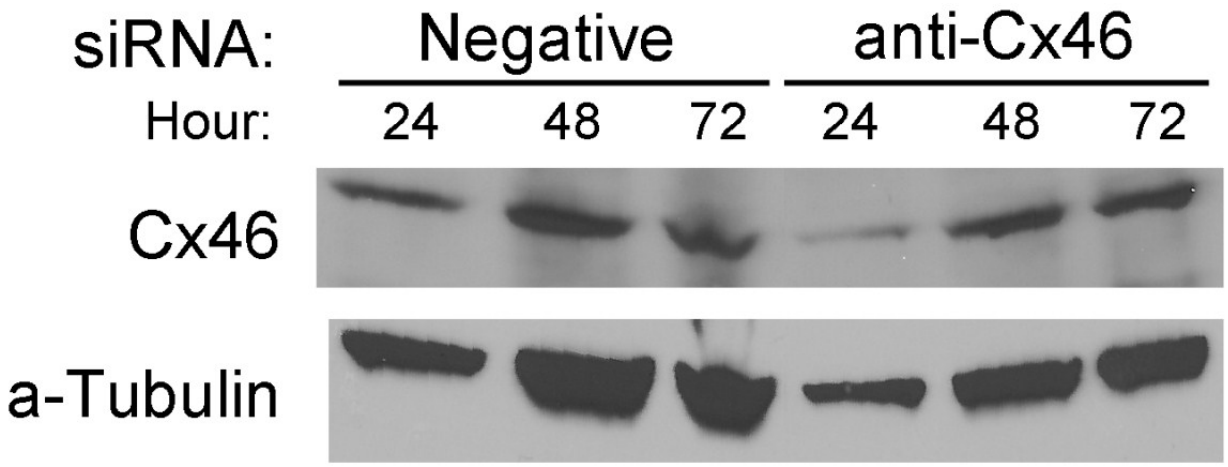

Figure 2.5 Western Blot of siRNA Knockdown in Y79 Cells *in vitro*
Y79 cells treated with 250 nM and 15 uL HiPerfect for each siRNA show were grown in normoxic conditions, lysed at the timepoints shown and western blotted for Cx46 expression. Optimal knockdown of Cx46 was shown at 24 hours.

Western blotting of Y79 cells transiently transfected with anti-Cx46 siRNA (Fig. 2.5) revealed that maximal Cx46 knockdown occurred at 24 hours post-transfection and rebounded at 48 and 72 hours. It is difficult to draw solid conclusions from the western blots based on the fact that the Y79 cell line is hard to transfect with any amount of siRNA. Increasing the HiPerfect transfection reagent increases cell death and using other lipid-based compounds did not yield better results (data not shown). Other researchers have used viral transfection to achieve a high level of transfection in Y79 cells (26). Y79 cells transfected with the negative control siRNA showed no discernible negative effects from the transfection in both normoxic (Figures 2.6A and 2.6C) and hypoxic (Figures 2.6B and 2.6D) growth conditions over 72 hours; viability remained high and cell counts increased. Cells transfected with anti-Cx46 siRNA showed no discernible effects in normoxia compared to the negative control in both viability and cell count (Figures 2.6A, 2.6C). However, when subjected to hypoxia, viability (Figure 2.6B) dropped slightly at 24 hours, recovered at 48 hours, but remained lower than the control at 72 hours. Y79 cell number in cells treated with anti-Cx46 siRNA did not increase significantly at any of the time points tested, while cell number greatly increased at 72 hr in the negative siRNA treated cells. Taken together with the western blot data, the knockdown of Cx46 levels did cause a decrease in Y79 cell count at 72 hr after siRNA transfection. However, this was much less than that observed in the normal N2A cells. This may suggest that knock down of Cx46 has additional effects for its anti-tumor activity. This could be through an increase in Cx43 once the Cx46 is decreased (see below).

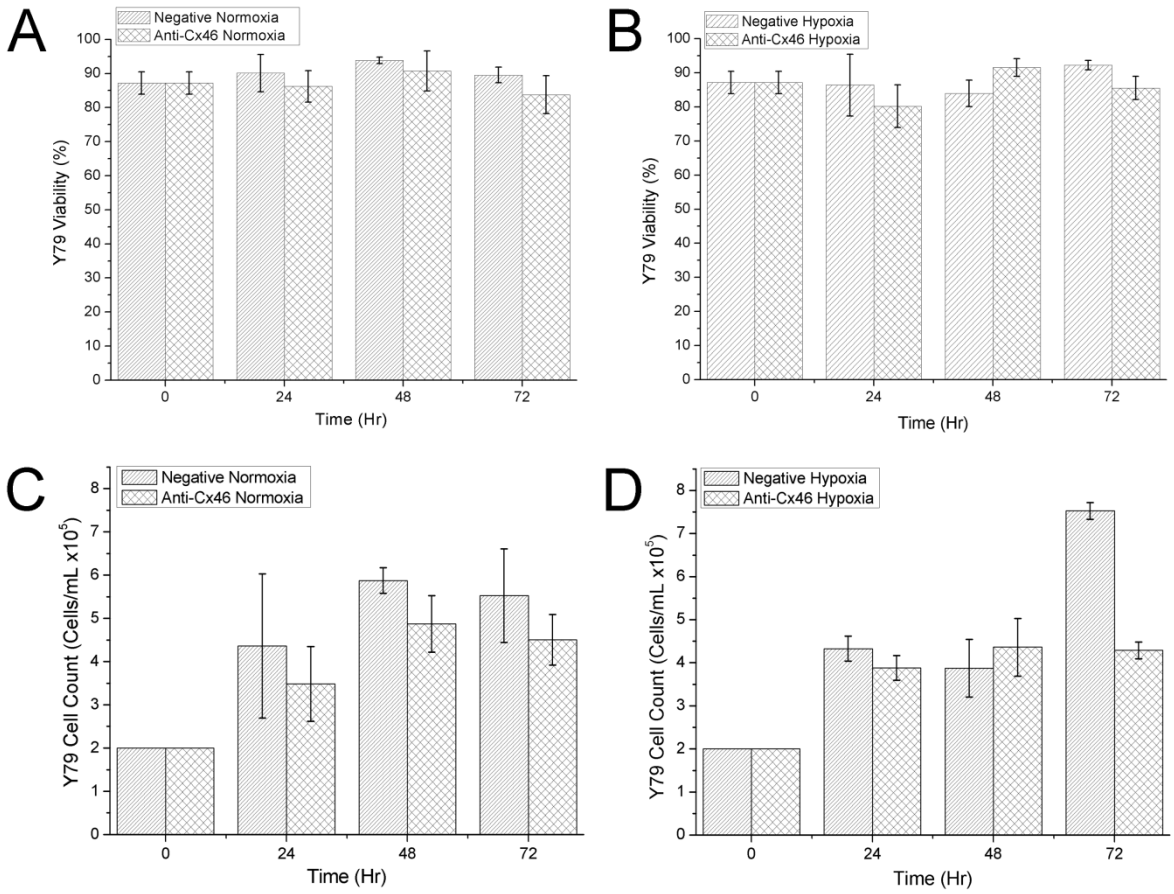

Figure 2.6 Effects of Anti-Cx46 siRNA Treatment on Y79 Cell Viability and Proliferation Under 20% and 5% Oxygen
Viability assay of siRNA knockdown in Y79 cells *in vitro*. Y79 cells treated with 250 nM and 15 µL HiPerfect for each siRNA were subjected to normoxic and hypoxic growth conditions as shown. Viability (A, B) was measured and cell counts (C, D) taken to assess the knockdown effect of Cx46 on Y79 cell growth characteristics. Each data point shown with standard error (n = 4).

in vivo Proof-of-Concept and Dosage Pilot Study

An initial proof-of-concept and dosage study was performed prior to performing a statistically correct *in vivo* study. Knowing that a high amount of siRNA was required for Cx46 knockdown in Y79 cells *in vitro*, and using previous knowledge from a related study (21), four treatment groups of two mice each were used to determine the best *in vivo* dosage amount. Three dosages of 7.5 µg, 15 µg, and 30 µg/injection of the anti-Cx46 siRNA and a single dosage of 7.5 µg/injection of negative control siRNA were tested. Figure 2.7 shows the effects of each dosage on the Y79 tumors in mice confirmed to have a tumor and Table 2.1 shows the individual

tumor data compiled from the study. The 7.5 µg and 15 µg/injection of anti-Cx46 siRNA had no effect on the growth of the Y79 xenografts, while the highest dose of 30 µg/injection showed a reduction in tumor growth and burden (Fig. 2.7). Statistical analysis of this initial study was not performed as it was a pilot study. For the primary *in vivo* study, 30 µg/injection of anti-Cx46 siRNA was chosen for the dosage amount.

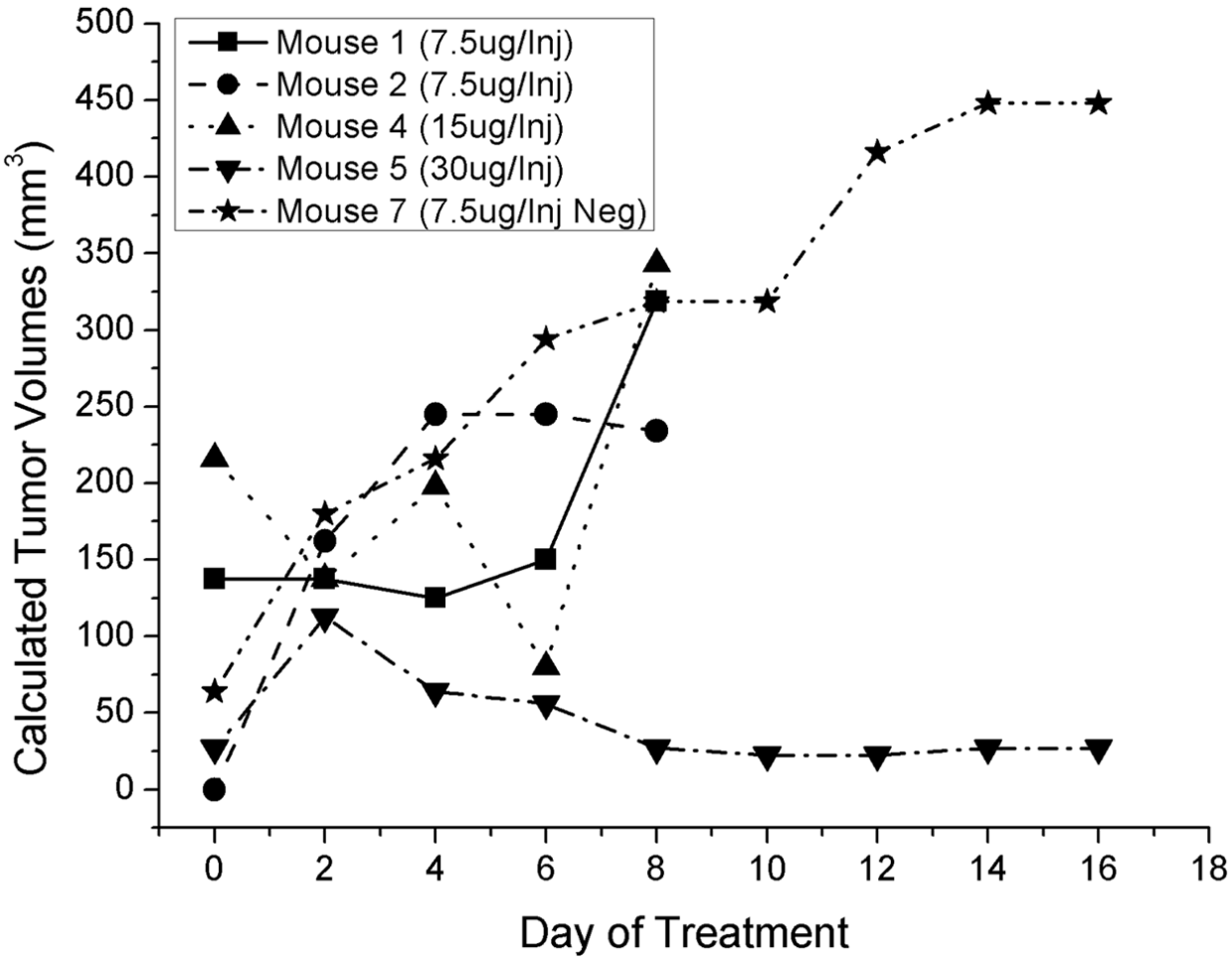

Figure 2.7 Results of Anti-Cx46 siRNA Dosing and Proof-of-Concept *in vivo* Study
Four groups of mice (n = 2) were used in the study to assess the most effective dosing regimen for the primary study. Tumors receiving anti-Cx46 siRNA were allowed to grow until they became a health concern for the mice harboring the tumors, then the mice were euthanized to prevent any further suffering. A dosage of 30 µg/Injection every other day was used for the primary study. Tabulated data for each mouse can be found in Table 2.1.

Day		1	2	4	6	8	10	12	14	16	18	20	22	24	FINAL TV	Average	WB
Anti-Cx46 siRNA																	
Cage 1																	
	1	NT	NT	NT	NT	6	56	180	288	445.5	600	936	1862 (E)	-	~~3450~~		
	2	NT	NT	NT	6	48	22.5	8	40	62.5	56	64	144	269.5 (E)	288		
	3	8	22.5	27	87.5	100	100	100	125	198	343	480 (E)	-	-	544		
Cage 2																287	
	1	6	8	6	18	87.5	48	32	32	40	32	32 (E)	-	-	80		
	2	10	48	100	125	112.5	87.5	87.5	100	100	87.5	87.5 (E)	-	-	198		*
	3	NT	NT	NT	48	100	48	56	48	108	72	48	162	320 (E)	324		
Negative Control siRNA																	
Cage 3																	
	1	22.5	48	87.5	196	196	196	256	288	364.5	445.5	486 (E)	-	-	462		
	2	NT	NT	22.5	56	87.5	22.5	22.5	18	22.5	27	87.5	144 (E)	-	~~168~~		
	3	NT	NT	NT	100	112.5	320	600	600	600	847	1470 (E)	-	-	1620		
Cage 4																894	
	1	NT	NT	NT	22.5	27	36	31.5	36	150	294	318.5	416	688.5 (E)	720		
	2	NT	NT	NT	40	144	125	216	448	729	950	1210 (E)	-	-	1144		*
	3	NT	NT	NT	27	27	27	72	36	87.5	125	137.5	294	480 (E)	520		
Sham-No siRNA																	
Cage 5																	
	1	NT	NT	NT	NT	NT	NT	NT	22.5	48	NT	125	252	367.5 (E)	450		
	2	NT	8	22.5	56	100	196	162	180	384	750	1152 (E)	-	-	1360		*
	3	NT	NT	NT	27	31.5	72	269.5	294	650	1267.5	1352	1521 (E)	-	1296		
Cage 6																1068	
	1	NT	31.5	64	112.5	100	112.5	125	294	416	800	1152 (E)	-	-	1456		
	2	NT	56	72	80	125	125	234	112.5	220.5	607.5	1296 (E)	-	-	1365		
	3	NT	NT	22.5	64	112.5	100	245	269.5	245	448	526.5	567 (E)	-	480		

Table 2.1 Calculated Volumes (mm^3) of Individual Xenograft Tumors Used in this Study
Tumor volumes for individual tumors were measured and recorded on each injection day, then calculated using TV = $[L*(W^2)]/2$. NT = Measurement not taken for that day; E = Euthanized; (~~Strikethrough~~) indicates which tumors were not included in the volume calculations and statistics due to being an outlier; (*) indicates which tumors were used in western blotting experiments.

Cx46 siRNA Suppresses Y79 Tumor Growth in vivo

The effects of Cx46 gene silencing was investigated on xenograft Y79 cell tumors *in vivo*. All experimental and control mice developed tumors at the site of injection. The gross appearance of mice 14 days post-Y79 cell implantation in untreated, control tumors, or following 5 treatments with non-silencing siRNA or Cx46 siRNA, are shown, outlined with arrows, in Figure 2.8. In control mice without siRNA treatment (Fig. 2.8A) and in mice in the non-silencing siRNA treatment group (Fig. 2.8B), large, subcutaneous, and vascularized tumors were observed where Y79 cells had been injected in their dorsal areas. Mice who received intratumor Cx46 siRNA therapy (Fig. 2.8C) had an extensive reduction in tumor size and vascularization. Images of representative excised tumors are shown in the Figure 2.9.

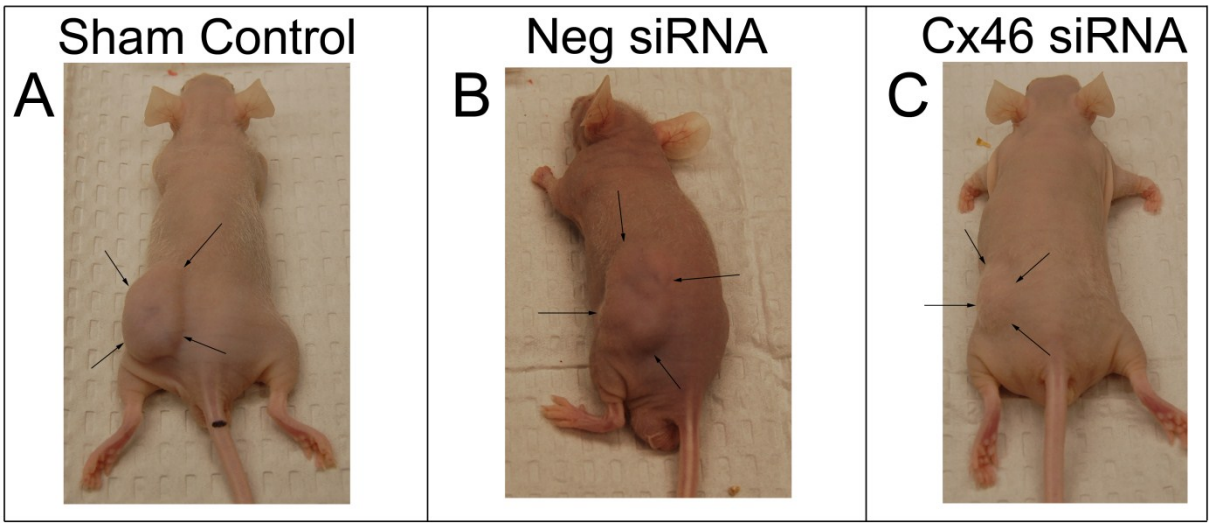

Figure 2.8 Intratumor Cx46 siRNA Suppresses the Growth of Y79 Tumors *in vivo*
Macroscopic appearance of Y79 tumors in nude mice 14 days post-transplantation of human Y79 retinoblastoma cells in (A) untreated control tumors, or following 5 siRNA treatments, with (B) 30 µg non-silencing siRNA or (C) 30 µg Cx46 siRNA. Arrows outline the representative tumors in the left dorsal region of the mice. Tabulated data for each mouse can be found in Table 2.

During the 21-day treatment period, the tumor growth rate and the final tumor volumes were greater in the untreated control and non-silencing siRNA treatment groups than in the mice treated with Cx46 siRNA (Figure 2.10). Y79 cell tumors grew rapidly in the untreated control and the non-silencing siRNA treatment groups, while the rate of tumor growth was significantly reduced in the Cx46 siRNA treatment group (Figure 2.10A). The reduction in tumor volumes of Cx46 siRNA treated mice were statistically significant ($P \leq 0.05$), when compared to non-

silencing siRNA or no siRNA treated groups. The Cx46 siRNA group began to be statistically different from the non-silencing siRNA treatment group beginning 8 days after treatment was initiated and continued to be significant throughout the study ($P \leq 0.02$). The Cx46 siRNA group began to show significance on day 6 ($P \leq 0.04$) and was also significant on day 8 ($P \leq 0.02$), day 14 ($P \leq 0.04$), and day 16 ($P \leq 0.003$) from the untreated, control group, however, days 10 and 12 were not as statistically significant ($P \geq 0.05$). Mice treated with intratumor Cx46 siRNA injections had significantly smaller final tumor volumes (287 mm3 ± 77 mm^3) when compared to untreated control mice (1068 mm^3 ± 192 mm3; $P \leq 0.002$) or to mice who received intratumor injections of non-silencing siRNA (894 mm^3 ± 218 mm^3; $P \leq 0.03$) (Figure 2.10B). Although the non-silencing siRNA group tumor volumes were lower than the untreated sham group, this was not statistically significant. The tumor weights of the treatment groups paralleled the final tumor volumes closely (Figure 2.10C). Mice in the Cx46 siRNA treatment group had significantly reduced mean final tumor weight (226 mg ± 75 mg) in comparison to mice treated with non-silencing siRNA (700 mg ± 218 mg; $P \leq 0.05$) or the untreated control mice (864 mg ± 136 mg; $P \leq 0.01$). Data for all mice used in this study can be found in Table 2.2.

Y79 tumor homogenates were analyzed by western blot and probed with anti-Cx46 and anti-Cx43 antibodies using β-actin as a loading control. Blots were digitized and analyzed as described in the Materials and Methods. Not surprisingly, Cx46 expression was markedly decreased in tumors treated with Cx46 siRNA (Figure 2.11A), however, upregulation of Cx43 protein was observed in a reciprocal manner (Figure 2.11B). Both the sham untreated control and non-silencing siRNA treatment groups demonstrated high expression of Cx46 protein (Figure 2.11A) and had low Cx43 protein expression (Figure 2.11B). A 5-fold reduction in Cx46 and 6-fold rise in Cx43 expression were observed in mice treated with Cx46 siRNA in comparison to mice who received no siRNA treatment (Figure 2.12). Similarly, mice treated with Cx46 siRNA had a 6-fold decrease in Cx46 and 3-fold increase in Cx43 expression compared to mice treated with non-silencing siRNA. The increase in the expression of the Cx43, a known tumor suppressor protein, may contribute significantly to the observed tumor reduction.

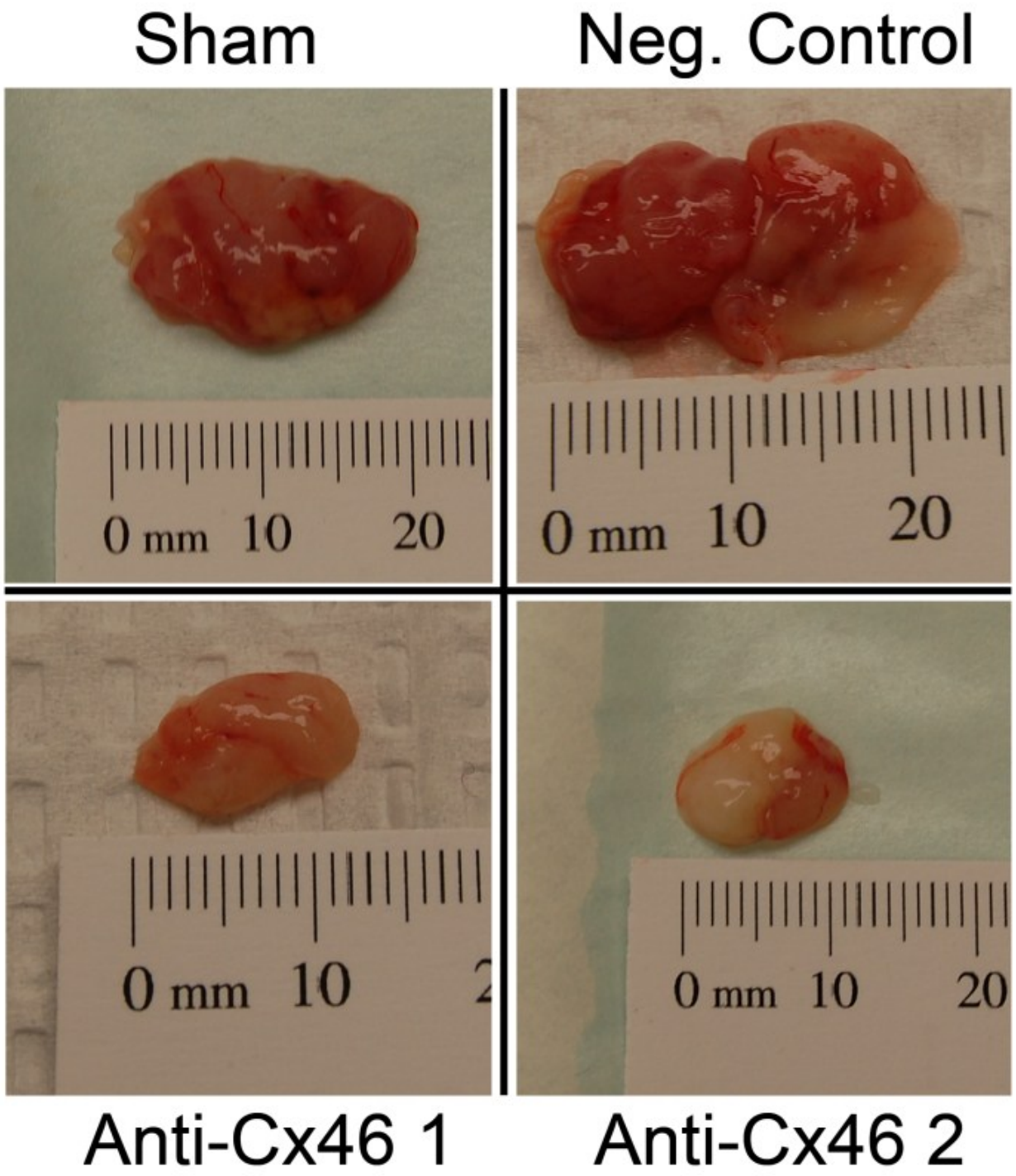

Figure 2.9 Gross Examination of Excised Y79 Xenografts
Mice were euthanized and the tumors were immediately excised, measured in three dimensions (mm), imaged and then formalin fixed. Representative tumors shown are from cage 2, mouse 1 and mouse 2 (both treated with 30 µg/mL anti-Cx46 siRNA); cage 4, mouse 2 (negative control); cage 5, mouse 2 (sham). Note the reduced amount of visible vascularization present in the anti-Cx46 siRNA treated tumors compared to either the sham or negative control tumor.

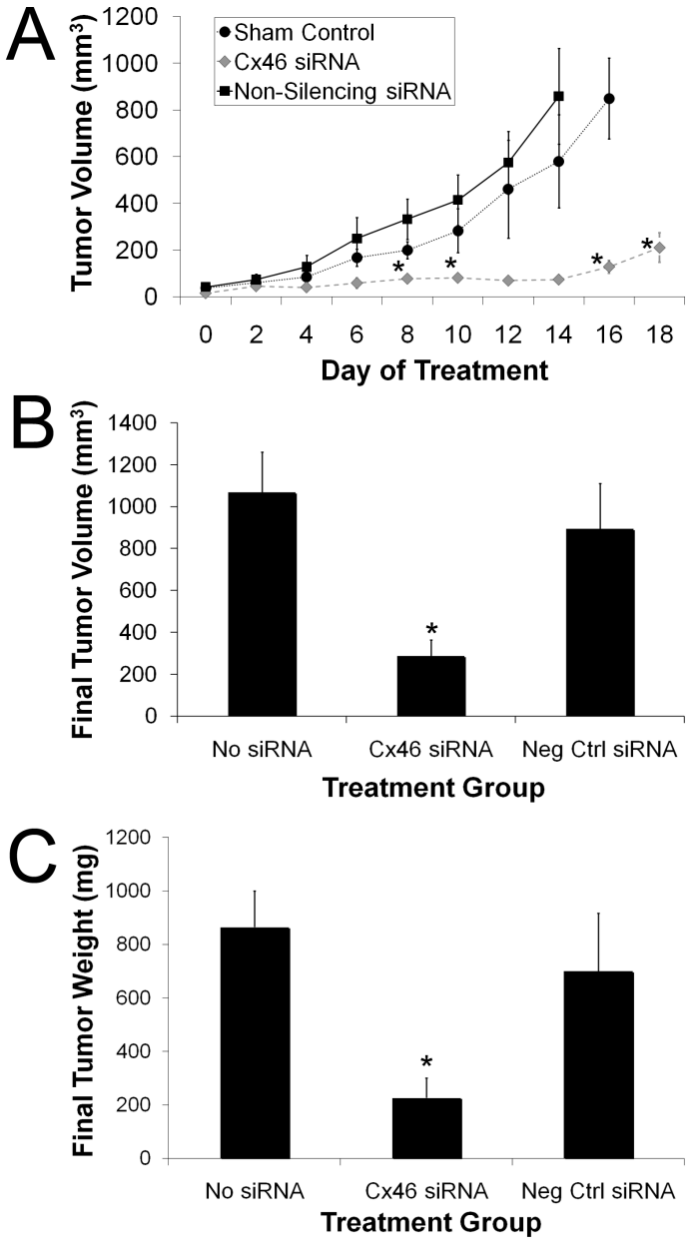

Figure 2.10 Tumor Volume of Cx46 siRNA, Non-Silencing siRNA, and No siRNA/Sham Treatment Groups in Mice Transplanted with Y79 Cells

Tumor volume of the transplanted mice was measured for a maximum of 21 days, with the administration of 30 µg/Injection Cx46 siRNA (n = 6), 30 µg/Injection non-silencing siRNA (n = 6), or no treatment (n = 6). (A) The calculated tumor volumes were averaged by treatment group and day over the course of the study (mean ± SE). The tumor volumes of Cx46 siRNA treated mice (n = 6) were statistically significant (* = $P \leq 0.05$), when compared to non-silencing siRNA treatment group (n = 6) beginning on 8 days after treatment was initiated and continued to be significant throughout the study. The Cx46 siRNA treatment group was statistically different ($P \leq 0.05$) from the untreated, no siRNA group (n = 6) beginning on day 6 and was also significant on days 8, 14, and 16. (B) Final measurements were taken on the excised tumors and the volumes were averaged by group. There was a significant reduction in final tumor volume after treatment with Cx46 siRNA (287 mm^3 ± 77 mm^3, n = 5) when compared to the non-silencing siRNA (894 mm^3 ± 218 mm^3, n = 5) or untreated (1068 mm^3 ± 192 mm^3, n = 6) groups ($P \leq 0.03$ and $P \leq 0.002$, respectively). (C) Cx46 siRNA treated mice had a decreased tumor weight. Final tumor weights of groups treated with Cx46 siRNA, non-silencing siRNA (negative control siRNA) ,or no siRNA, shown as mean tumor weight (mg) ± SE (n = 6 for each group). The weight of tumors treated with Cx46 siRNA (226 mg ± 75 mg) were significantly less than the tumors of mice treated with non-silencing siRNA (700 mg ± 218 mg, * = $P \leq 0.05$) or no siRNA (864 mg ± 136 mg, $P \leq 0.01$).

Histopathology of the tumor sections, shown in Figure 2.13, determined that mice treated with Cx46 siRNA had the least number and, therefore, the least density of Y79 cells when compared to mice treated with non-silencing siRNA or no siRNA. There were no differences in the Y79 cell density between the non-silencing siRNA treated mice and mice which received no siRNA treatment by observation of the stains, hematoxylin (purple, nuclei) and eosin (pink, cytoplasm and connective tissue) in Figure 2.13.

Discussion

A connection between gap junction intercellular communication (GJIC) and control of cell growth was established over 40 years ago (27), which resulted in a multitude of research reporting a loss or diminished ability to adjoin amongst neoplastic cells or between cancer cells and nearby normal cells (reviewed in 28). The tissue specific expression of connexins was discovered along with the finding that re-expression of specific connexins was able to reestablish control of cell growth. Additionally, recovery of GJIC alone was not always capable of normalizing cell growth (29). In other cases, re-expression of connexins was enough to restore cell growth control without restoring GJIC (30). It has been suggested that connexins, independent of GJIC, may directly control growth, possibly by altering gene expression of neoplastic cells (31), and a change from the normal expression of connexin proteins may allow tumor progression. Gap junction proteins are, therefore, often termed tumor suppressors and are downregulated in tumor development and progression.

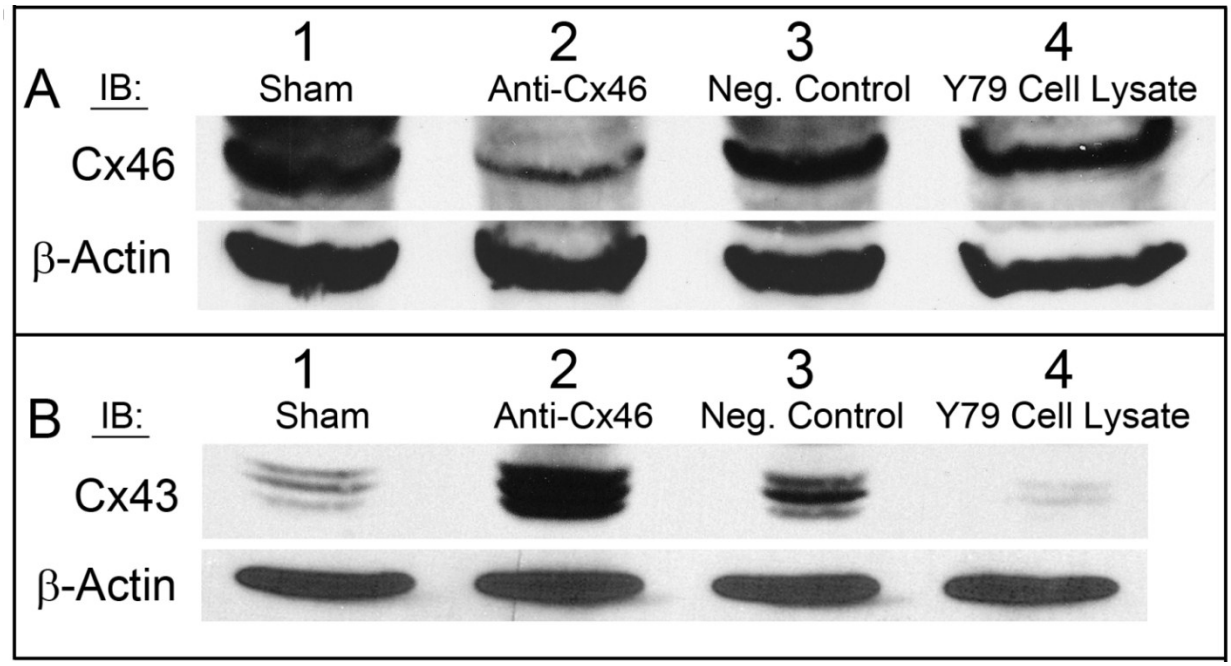

Figure 2.11 Decreased Expression of Cx46 with a Reciprocal Increase in Cx43 Protein Expression in Y79 Tumors of Mice treated with Cx46 siRNA
Conversely, tumors treated with a non-silencing siRNA or were untreated showed Cx46 upregulation and Cx43 downregulation. Equal amounts of Y79 tumor homogenate were analyzed for (A) Cx46 and (B) Cx43 where β-actin was used as the loading control. Lane 1: Tumor #14, sham treated tumor; Lane 2: Tumor #5, anti-Cx46 siRNA treated tumor; Lane 3: Tumor #11, AllStars negative control siRNA treated tumor; Lane 4: Y79 cell lysate positive control for Cx46 expression. Representative tumor western blots shown.

However, our current work shows that upregulation of Cx46 is observed in cancerous cells and may help solid tumor tissues in surviving hypoxic conditions or could cause tumor growth through the loss of Cx43. Human Y79 retinoblastoma cells express Cx46 and in contrast to connexin-deficient, wild-type neural N2A cells (Figure 2.3) Y79 cells, are able to thrive in hypoxic conditions over the course of several days while maintaining viability (Figure 2.2). Similarly, human lens epithelial cells express both Cx46 and Cx43, a related and ubiquitously expressed gap junction protein, but can only survive prolonged hypoxic conditions while actively expressing Cx46. Gene knockdown by siRNA of Cx46, but not Cx43, makes HLECs susceptible to hypoxia induced cell death (21). Although the *in vitro* siRNA data presented here for Y79 cells is not completely conclusive, there is evidence that knockdown of Cx46 protein levels contributes to stagnated growth of Y79s in hypoxic conditions at 72 hr. It could be inferred that prolonged dosing of Y79 cells with anti-Cx46 could prolong the lack of growth of Y79 cells and indeed this is what we show *in vivo*. Further evidence of Cx46-related hypoxic growth control was observed from N2A cells which lack gap junctions and can be engineered to express either

Cx43 or Cx46. In these cell lines, only the Cx46 expressing cells show delayed cellular death in 1% oxygen (Figure 2.3). Cx46 does indeed appear to play a role in cellular resistance to hypoxia. Human Y79 retinoblastoma (this manuscript), human lens epithelial, rabbit lens epithelial, MCF-7 human breast cancer (21), and the N2A-Cx46 cell line all express Cx46 and all are able to survive hypoxia (Figure 2.1; Figure 2.3). Knockdown of Cx46 in human and rabbit lens epithelial (21) and Y79 cell lines grown in hypoxic conditions clearly increases the negative effects of reduced oxygen.

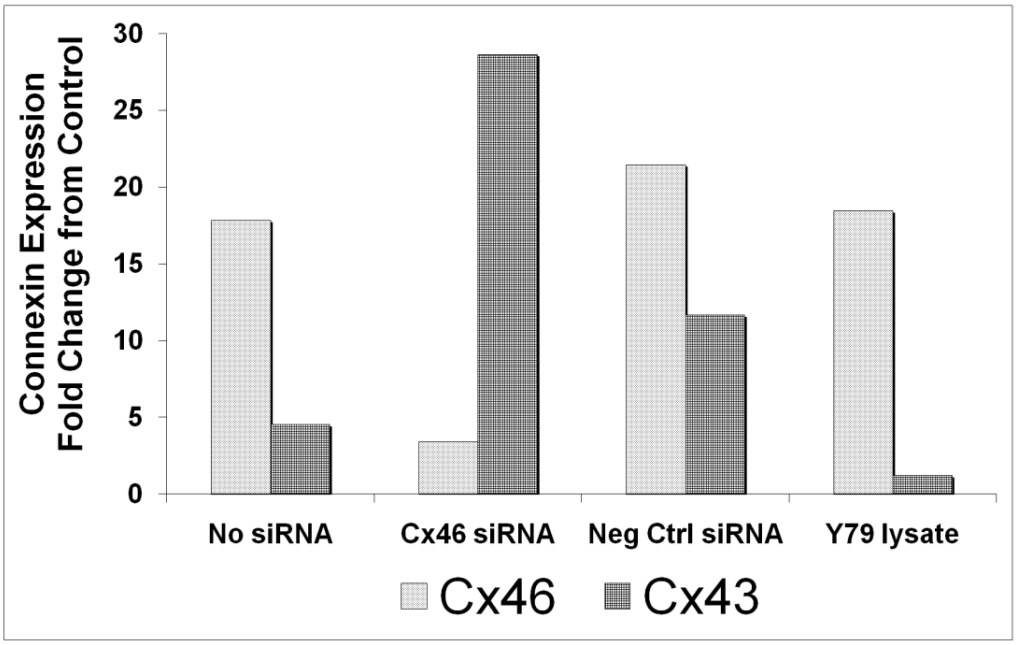

Figure 2.12 Quantitative Protein Expression Densitometry of Cx46 and Cx43 Protein Levels in Representative Tumors Shown in Figure 2.11
Blots were digitized then normalized to β-actin levels and the detected protein expression of Cx46 and Cx43 in different treatment groups was compared, using the no siRNA treatment as a control. A 5-fold reduction in Cx46 and 6-fold rise in Cx43 expression were observed in mice treated with Cx46 siRNA in comparison to mice who received no siRNA treatment. Mice treated with Cx46 siRNA had a 6-fold decrease in Cx46 and 3-fold increase in Cx43 expression compared to mice treated with non-silencing siRNA.

Although retinoblastoma is well-known as a vascularized tumor, the early tumor is hypoxic and hypoxic regions exist in the advanced tumor stage (32-33). The areas of the hypoxic tumor are increasingly refractory to conventional treatments, such as chemotherapy and radiation therapy, due to the slowly proliferating cells (32) because these therapies target rapidly dividing cells (33). With the resistant nature of hypoxic tumors and the local and systemic complications associated with current therapy protocols, the search for drugs that are non-

cytotoxic to normal cells and can effectively target cancer cells is continuous. One approach would be to target a component of the cancerous cells which confers growth and viability of the tumor specifically during hypoxic events.

We chose to evaluate *in vivo* treatments using a subcutaneous model as opposed to the eye for several reasons. Y79 cells were used for the *in vitro* studies and subsequently using the same cell line allowed us to make direct correlations between the studies. In a preliminary Fisher rat eye retinoblastoma animal model (data not presented) we intraocularly treated with intratumor injections and it was very difficult to inject on an every other day basis without an increase in eye damage due to repeated injections as well as the increased risk of death due to repeated general anesthesia. The subcutaneous xenograft tumors presented in this paper were much easier to assess and reliably measured throughout the study which was especially helpful when testing an *in vivo* treatment protocol for the first time. Subsequent studies are needed to further develop and optimize a delivery system for the siRNA drug. Alternatively, other means to specifically lower Cx46 protein levels would need to be developed, such as use of a specific modified antibody similar to current antibody treatments for macular degeneration, or a long-lasting siRNA derivative.

Previously, siRNAs have been used successfully to provide a strong and specific knockdown of gene expression (35), and are therefore ideal for the initial testing of targets. Nude mice injected with Cx46 siRNA exhibited a significant reduction in Y79 cell tumor growth, having a greater than 3-fold decrease in mean tumor volume, 21 days after initiation of treatment. Tumors treated with Cx46 siRNA had a projected knockdown of Cx46 but an unexpected increase in the expression of Cx43. While untreated tumors or tumors treated with non-silencing siRNA had an upregulation of Cx46 and subsequent downregulation of Cx43. This is the first time the reciprocal relationship between Cx46, a connexin upregulated in hypoxia, and Cx43, a known tumor suppressor, has been reported *in vivo*. Future studies would need to elucidate how loss of Cx46 results in upregulation of the Cx43 tumor suppressor. These results demonstrate that Cx46 gene silencing using siRNA has an antitumor effect in xenograft human Y79 retinoblastoma cell tumors. We hypothesize that the pro-tumor effects of Cx46 may be partially due to its ability to confer resistance from hypoxic death to tumor cells expressing Cx46. Additionally, subsequent reappearance of Cx43 may then aid cells to acquire more "mortal" properties and could render the tumor cells more susceptible to current antitumor

therapies. However, additional studies are needed to determine the details of how Cx43 and Cx46 are reciprocally controlled. Current work in our lab indicates that degradation of Cx43 is induced when Cx46 is expressed, and this degradation takes place in the proteasome (21). Additional studies on the mechanism of Cx43 reappearance upon anti-Cx46 siRNA treatment are outside the scope of this paper.

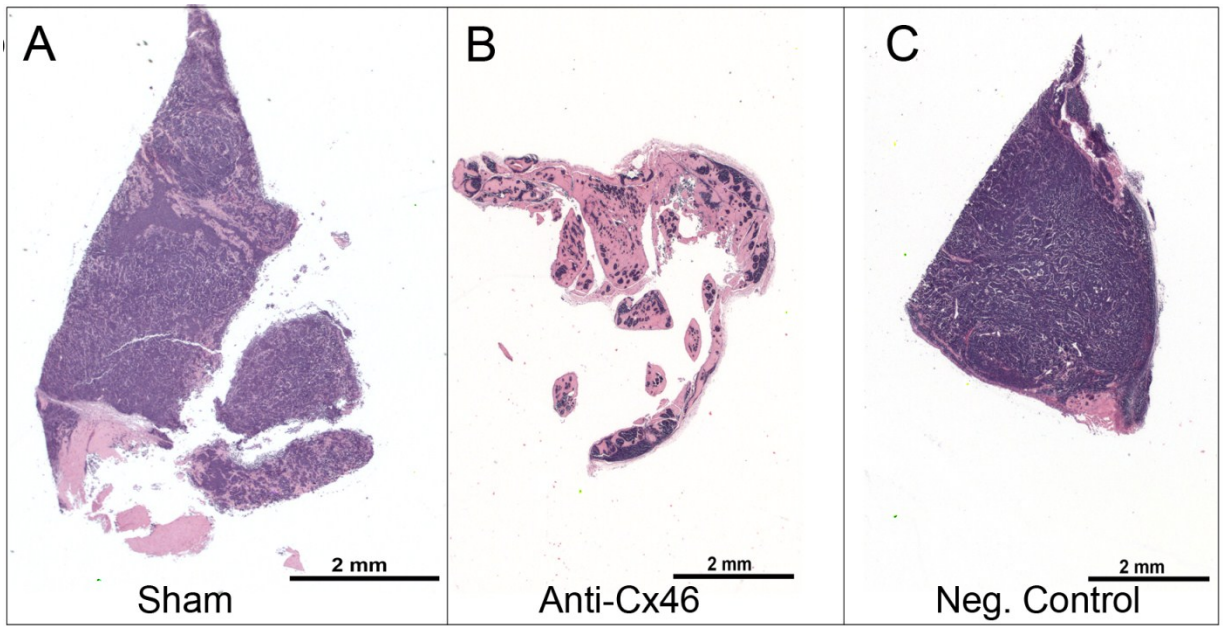

Figure 2.13 Decreased Y79 Tumor Cell Burden in Mice Treated with Cx46 siRNA
Excised tissues were fixed, embedded, sectioned and then stained with hematoxylin (purple, nuclei) and eosin (pink, cytoplasm and connective tissue). (A) Untreated sham Y79 tumor (cage 5, mouse 2); (B) Cx46 siRNA treated Y79 tumor (cage 2, mouse 2); (C) Non-silencing siRNA treated Y79 tumor (cage 4, mouse 2). The representative images were taken by a board-certified veterinary pathologist and show the microscopic appearance of the Y79 tumors isolated from the xenograft mice, treated with Cx46 siRNA, a non-silencing siRNA, or sham treated for a maximum of 21 days.

In conclusion, this study was the first to demonstrate the presence of Cx46 protein in human Y79 retinoblastoma cells. This is the second neoplastic cell line and related tumor tissue reported to express high levels of Cx46 and reciprocally low levels of Cx43 protein. Our findings reveal that local administration of anti-Cx46 siRNA can mediate effective knockdown of this target protein and suppress tumor growth *in vivo*. We observed no associated side effects with siRNA treatment in our subcutaneous Y79 retinoblastoma tumor model in nude mice. Since Cx46 is only naturally found in the hypoxic and differentiated lens, ill effects of gene silencing of this protein would likely be negligible; however, a potential yet treatable side effect

with intraocular use may be cataract development. While siRNAs are not an optimal treatment due to their short half-life and transient effects (35-36), other anti-Cx46 treatment modalities more suitable for therapy, such as modified RNAi or an antibody, can be developed in the future. The findings of this study provide an exciting new direction in drug development for the treatment of retinoblastoma. In addition, Cx46 could provide an early marker for hypoxia and these findings could have broader significance for other hypoxia-related diseases.

Acknowledgements

This work is supported by grant number NIH R01EY13421 to DJT from the National Eye Institute and partially funded by the Graduate Student Summer Stipend for DB and the Undergraduate Cancer Research Award to DML from the Johnson Center for Basic Cancer Research at Kansas State University. The authors would like to thank Kyathanahalli Janardhan at the Kansas State University College of Veterinary Medicine for the histopathological assistance.

References

1. Antoneli, C.B., Ribeiro, K.C., Steinhorst, F., Novaes, P.E., Chojniak, M.M., Malogolowkin, M., 2006. Treatment of retinoblastoma patients with chemoreduction plus local therapy: experience of the AC Camargo Hospital, Brazil. J. Pediatr. Hematol. Oncol. 28, 342-345.

2. Wong, F.L., Boice, J.D., Abramson, D.H., Tarone, R.E., Kleinerman, R.A., Stovall, M., Goldman, M.B., Seddon, J.M. Tarbell, N., Fraumeni, J.F. Jr., Li, F.P., 1997. Cancer incidence after retinoblastoma. Radiation dose and sarcoma risk. J. Am. Med. Assoc. 278, 1262-1267.

3. Jehanne, M., Lumbroso-Le Rouic, L., Savignoni, A., Aerts, I., Mercier, G., Bours, D., Desjardins, L., Doz, F., 2009. Analysis of ototoxicity in young children receiving carboplatin in the context of conservative management of unilateral or bilateral retinoblastoma. Pediatr. Blood Cancer. 52, 637-643.

4. Abramson, D.H., Beaverson, K.L., Chang, S.T., Dunkel, I.J., McCormick, B., 2004. Outcome following initial external beam radiotherapy in patients with Reese-Ellsworth group Vb retinoblastoma. Arch Ophthalmol. 122, 1316-1323.

5. Benz, M.S., Scott, I.U., Murray, T.G., Kramer, D., Toledano, S., 2000. Complications for systemic chemotherapy as treatment of retinoblastoma. Arch. Ophthalmol. 118, 577-578.

6. Vaupel, P., Kelleher, D.K., Hockel, M., 2001. Oxygen status of malignant tumors: pathogenesis of hypoxia and significance for tumor therapy. Semin. Oncol. 28, 29-35.

7. Semenza, G.L., 2007. Regulation of tissue perfusion in mammals by hypoxia-inducible factor. Exp. Physiol. 92, 988-991.

8. Harris, A.L., Locke, D., 2009. Connexins: A Guide. Springer, New York.

9. Ruch, R.J., 1994. The role of gap junctional intercellular communication in neoplasia. Ann. Clin. Lab. Sci. 24, 216e231.

10. Temme, A., Buchmann, A., Gabriel, H.D., Nelles, E., Schwarz, M., Willecke, K., 1997. High incidence of spontaneous and chemically induced liver tumors in mice deficient for connexin32. Curr. Biol. 7, 713-716.

11. Qin, H., Shao, Q., Curtis, H., Galipeau, J., Belliveau, D.J., Wang, T., Alaoui-Jamali, M.A., Laird, D.W., 2002. Retroviral delivery of connexin genes to human breast tumor cells inhibits *in vivo* tumor growth by a mechanism that is independent of significant gap junctional intercellular communication. J. Biol. Chem. 277, 29132-29138.

12. Lee, S.W., Tomasetto, C., Sager, R., 1991. Positive selection of candidate tumor-suppressor genes by subtractive hybridization. Proc. Natl. Acad. Sci. U.S.A. 88, 2825-2829.

13. Laird, D.W., Fistouris, P., Batist, G., Alpert, L., Huynh, H.T., Carystinos, G.D., Alaoui-Jamali, M.A., 1999. Deficiency of connexin43 gap junctions is an independent marker for breast tumors. Cancer Res. 59, 4104-4110.

14. Kanczuga-Koda, L., Sulkowska, M., Koda, M., Reszec, J., Famulski, W., Baltaziak, M., Sulkowski, S., 2003. Expression of connexin 43 in breast cancer in comparison with mammary dysplasia and normal mammary gland. Folia Morphol. 62, 439-442.

15. Kanczuga-Koda, L., Sulkowska, M., Tomaszewski, J., Koda, M., Sulkowska, M., Przystupa, W., Golaszewska, J., Baltaziak, M., 2005. Connexins 26 and 43 correlate with Bak, but not with Bcl-2 protein in breast cancer. Oncol. Rep. 14, 325-329.

16. Habermann, H., Ray, V., Habermann, W., Prins, G.S., 2002. Alterations of gap junction protein expression in human benign prostatic hyperplasia and prostate cancer. J. Urol. 167, 655-660.

17. McLachlan, E., Shao, Q., Wang, H.L., Langlois, S., Laird, D.W., 2006. Connexins act as tumor suppressors in three-dimensional mammary cell organoids by regulating differentiation and angiogenesis. Cancer Res. 66, 9886-9894.

18. Hirschi, K.K., Xu, C.E., Tsukamoto, T., Sager, R., 1996. Gap junction genes Cx26 and Cx43 individually suppress the cancer phenotype of human mammary carcinoma cells and restore differentiation potential. Cell. Growth Differ. 7, 861-70.

19. Shao, Q., Wang, H., McLachlan, E., Veitch, G.I., Laird, D.W., 2005. Down-regulation of Cx43 by retroviral delivery of small interfering RNA promotes an aggressive breast cancer cell phenotype. Cancer Res. 65, 2705-2711.

20. Sanchez-Alvarez, R., Paino, T., Herrero-Gonzalez, S., Medina, J.M., Tabernero, A., 2006. Tolbutamide reduces glioma cell proliferation by increasing connexin43, which promotes the up-regulation of p21 and p27 and subsequent changes in retinoblastoma phosphorylation. Glia. 54, 125-134.

21. Banerjee, D., Gakhar, G., Madgwick, D., Hurt, A., Takemoto, D., Nguyen, T.A., 2010. A novel role of gap junction connexin46 protein to protect breast tumors from hypoxia. Int J Cancer. 127, 839-848.

22. Kimura, K., Usui, Y., Hattori, T., Yamakawa, N., Goto, H., Usui, M., Okada, S., Shirato, K., Tomoda, A., 2008. Phenoxazine derivative, 2-amino-4,4α-dihydro-4α,7-dimethyl-3H-phenoxazine-3-one suppresses growth of human retinoblastoma cell line Y79 *in vitro* and *in vivo*. Oncol. Rep. 19, 3-10.

23. Akoyev V., Takemoto, D.J., 2007. ZO-1 is required for protein kinase C gamma-driven disassembly of connexin 43. Cell Signal. 19, 958-967.

24. Farber, D.B., Learner, L.E., and Viczian, A.S., 2001. Vision Research Protocols. Methods in Molecular Medicine. Springer, New York.

25. Mitra, M., Kandalam, M., Verma, R.S., UmaMaheswari, K., Krishnakumar, S., 2010. Genome-wide changes accompanying the knockdown of Ep-CAM in retinoblastoma. Mol. Vis. 16, 828-842.

26. White, J.B., Taylor, R.E., and Pittler, S.J., 2001. Reproducible high efficiency gene transfer into Y79 retinoblastoma cells using adenofection. J. Neurosci. Methods 106, 1–7.

27. Loewenstein, W.R., 1979. Junctional intercellular communication and the control of growth. Biochim. Biophys. Acta. 560, 1-65.

28. Mesnil, M., Crespin, S., Avanzo, J.L., Zaidan-Dagli, M.L., 2005. Defective gap junctional intercellular communication in the carcinogenic process. Biochim. Biophys. Acta. 1719, 125-145.

29. Mesnil, M., Krutovskikh, V., Piccoli, C., Elfgang, C., Traub, O., Willecke, K., Yamasaki, H., 1995. Negative growth control of HeLa cells by connexin genes: connexin species specificity. Cancer Res. 55, 629-639.

30. Huang, R.P., Fan, Y., Hossain, M.Z., Peng, A., Zeng, Z.L., Boynton, A.L., 1998. Reversion of the neoplastic phenotype of human glioblastoma cells by connexin 43 (cx43). Cancer Res. 58, 5089-5096.

31. Cronier, L., Crespin, S., Strale, P.O., Defamie, N., Mesnil, M., 2009. Gap junctions and cancer: new functions for an old story. Antioxid. Redox. Signal. 11, 323-38.

32. Maschek, G., Savaraj, N., Priebe, W., Braunschweiger, P., Hamilton, K., Tidmarsh, G.F., De Young, L.R., Lampidis, T.J., 2004. 2-deoxy-D-glucose increases the efficacy of adriamycin and paclitaxel in human osteosarcoma and non-small cell lung cancers *in vivo*. Cancer Res. 64, 31-34.

33. Boutrid, H., Jockovich, M.E., Murray, T.G., Pina, Y., Feuer, W.J., Lampidis, T.J., Cebulla, C.M., 2008. Targeting hypoxia, a novel treatment for advanced retinoblastoma. Invest Ophthalmol Vis Sci. 49, 2799-2805.

34. Burnier, M.N., McLean, I.W., Zimmerman, L.E., Rosenberg, S.H., 1990. Retinoblastoma. The relationship of proliferating cells to blood vessels. Invest Ophthalmol Vis Sci. 31, 2037-2040.

35. Devroe E., Silver, P.A., 2004. Therapeutic potential of retroviral RNAi vectors. Expert Opin. Biol. Ther. 4, 319-327.

36. Behike, M.A., 2008. Chemical modification of siRNAs for *in vivo* use. Oligonucleotides. 18, 305-319.

Chapter 3 - The Promoter of Human Connexin 46 is Oxygen Sensitive

Parts of this chapter have been previously published as:

Samuel A. Molina and Dolores J. Takemoto. "The role of Connexin 46 promoter in lens and other hypoxic tissues" Commun Integr Biol. 2012 March/April 5(2) p114-117.

Abstract

Gap junctions are multimeric membrane protein channels that join the cytoplasm of one cell to another. Much information about the connexins comes from electrophysiology and channel function studies, but relatively little information is known about non-channel functions of the connexins. Lens connexins allow the movement of small metabolically relevant molecules and ions to pass between lens cells and disruption of this movement contributes to cataract. Connexin 46 (Cx46) has been extensively studied in mammalian lens, where it is found along with Cx43 and Cx50. Interruption of Cx46 channel function leads to cataract formation due to dysregulation of lens homeostasis. The induced loss of Cx46 suppresses tumor growth and also upregulates Cx43 in lens cell culture and in breast and retinoblastoma tumor xenografts. Cx46 influences Cx43 degradation in these models. The upregulation of Cx46 may be due in part to the effects of increasing hypoxic conditions in these tissues. We report that the Cx46 promoter is regulated by hypoxia and that the mRNA transcript that is produced under hypoxia is similar to the transcribed product observed under normoxia.

Introduction

There are 21 genes in the human genome that encode 20 functional connexin proteins. Many connexins are expressed in a tissue specific pattern while others, like connexin 43 (Cx43), are nearly ubiquitous. Connexins are often co-expressed with other connexins in the same cell. Connexins assemble into homotypic or heterotypic hexameric assemblies termed connexons, or half gap junctions. The connexins that comprise the gap junction channel influence the size and charge of the pore as well as the type of molecules that can traverse through it (1-4). Proteins are often cell type specific and with the sheer number of possibilities of connexin gene expression in a given tissue, cells can fine tune the types of molecules they need by regulating connexin gene

expression. However, there is little known about tissue specific connexin gene regulation. Literature on this topic is limited to a small set of connexin proteins known to cause disease, such as Cx43 in the heart, Cx50 in the lens, and Cx26 in the ear (5-7). Data from developmental studies are limited as well with most analysis performed in mice for a few select connexins at a time.

Although there is much information about connexin electrophysiology and channel function little information is known about non-channel functions of the connexins. Studies of Cx46 outside of the eye are limited. Cx46 has been extensively studied in the mammalian lens, where it is found with Cx43 and Cx50 (8). These three connexins are differentially expressed in the various regions that define the lens. In the outer epithelial layer, Cx43 and Cx50 are the predominate connexins. Immature outer fiber cells express lower amounts of Cx43 and roughly the same amount of Cx50. As the fiber cells mature, they lose the ability to maintain protein expression and turnover. However, once the cells further differentiate into mature fiber cells they retain functional Cx46 and Cx50 gap junction channels (8-10). Interruption of Cx46 channel function leads to cataract formation in the lens (11-16). Cataract is the most notable defect in Cx46 null mice and Cx46 is the dominant functional gap junction in the mature region of the lens (8, 17).

Our lab has hypothesized that Cx46 is important for cellular survival during hypoxic conditions. We subsequently showed that exogenous Cx46 prolongs survival from hypoxia-induced cell death in mouse neuro2a (N2A) cells (18) and is expressed abnormally in solid tumors (18, 19). Cx46 is upregulated by hypoxia in rabbit lens epithelial cells, which express Cx46 naturally (18). We also demonstrated that Cx46 affects tumor growth in two separate human xenograft studies (18, 19). In both studies, the xenograft cells were allowed to form sizeable solid tumors then each tumor was treated with high doses of Cx46 siRNA directly injected into the tumor at various positions. We demonstrated that Cx46 was upregulated in early growth tumors and knockdown of Cx46 by siRNA resulted in slower growing tumors, either by the prevention of Cx46 upregulation or by knockdown of existing and ongoing Cx46 mRNA expression. The amount of Cx43 protein found in the tumor increased when Cx46 protein decreased (19). This finding could explain why the tumor xenografts slowed their growth when treated with anti-Cx46 siRNA. Cx43 is a known tumor suppressor and a decrease in its expression is casually linked to tumor progression and metastasis (9, 20-23). This

mechanism of tumor suppression may contribute to the slow growth of anti-Cx46 siRNA treated tumors. We then determined why and how Cx46 is upregulated in a tumor environment since it is thought to be unique to lens fiber cells.

Lens fiber cells are differentiated via the master differentiation transcription factor PAX6 which controls eye development and protein expression in all parts of the eye during development. Other eye specific transcription factors that contribute to development include PAX2, MITF, FKHL7, PITX2, PITX3, HESX1, CRX, Hedgehogs, and NRL (The Online Metabolic and Molecular Bases of Inherited Diseases: http://dx.doi.org/10.1036/ommbid.281). Analysis of mutated eye development in mice has led to the identification of the majority of transcription factors involved in the eye development. In particular, PAX6, CHX10, and PITX3 are all important in lens maturation and result in ocular malformation when mutated based on mutational analysis in mice. Cx46 expression is maintained in the mature regions of the lens. None of these transcription factors are predicted to bind to the *GJA3* promoter as seen in Figure 3.1.

Of interest to our lab is the regulation of the lens fiber cell specific connexin 46, *GJA3*. Limited data is available for this protein in regards to overall embryonic development because it is thought to be a lens-only protein. However, our lab has found Cx46 gene and protein expression in hypoxic tissues, therefore the regulation of Cx46 gene transcription is important to understand in the context of both the hypoxic lens and to understand Cx46 role in hypoxic solid tumors. Other labs have seen expression of Cx46 in adult osteoblasts and osteosarcomas (24), and placental tissue (S.J. Lye, E. Winterhager, and J. Fandrey. Deutsche Physiologische Gesellschaft 2012, Dresden, Germany).

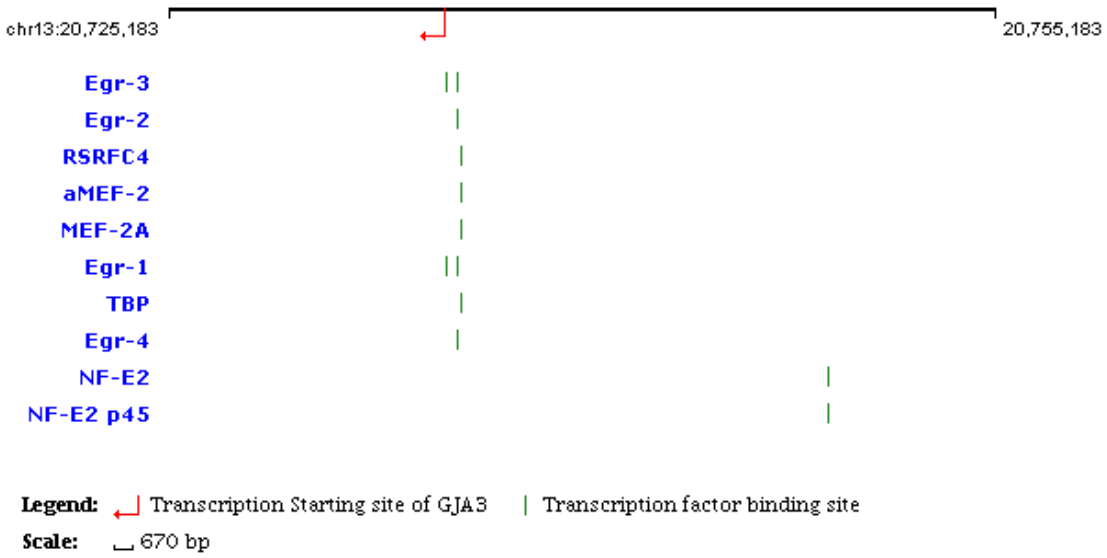

Figure 3.1. Predicted Transcription Factor Binding Sites in the Human Connexin 46 Promoter as Analyzed with SA Biosciences TF Prediction Web Software
Numbering is from chromosome ends. Red arrow indicates start and direction of transcription. Each of the predicted transcription factors lies within 1 kb upstream of the TSS. This sequence was selected for analysis in this chapter. Four major transcription protein families may bind to the Cx46 promoter: Early growth response (Egr), Myocyte enhancing factor (MEF), TATA binding protein (TBP) and Nuclear factor erythroid-derived 2 (NF-E2 or Nrf2). Image output from SA Biosciences.

No traditional promoter studies have been performed with the human Cx46 gene. The transcriptional start site of the *GJA3* gene is only predicted based upon mRNA and 5'-rapid amplification of cDNA ends (5'RACE) analysis studies in mouse cells and embryos. In a study by Anderson et al (25), two different transcription start sites were identified, each marking the alternative exons 1a and 1b (Figure 3.2). The mechanism that drives tight regulation of the connexins in all cell types is unknown. *GJA3* exons 1a and 1b did not contain the same sequences when analyzed by 5'RACE and subsequent sequence analysis. The authors suggest that connexin genes are expressed by alternative promoters. This type of regulation scheme would afford tight regulation of connexin genes in all cell types. Another feature the authors point out is the varied use of internal ribosome entry sites (IRES) in many connexin 5'UTRs, including Cx26, Cx32, and Cx43 (26). The Anderson et al. (25) work was performed with a 10-12 day old mouse embryo cDNA library. The connexin genes could be controlled differently between mouse and human cells or even between species or stages of development.

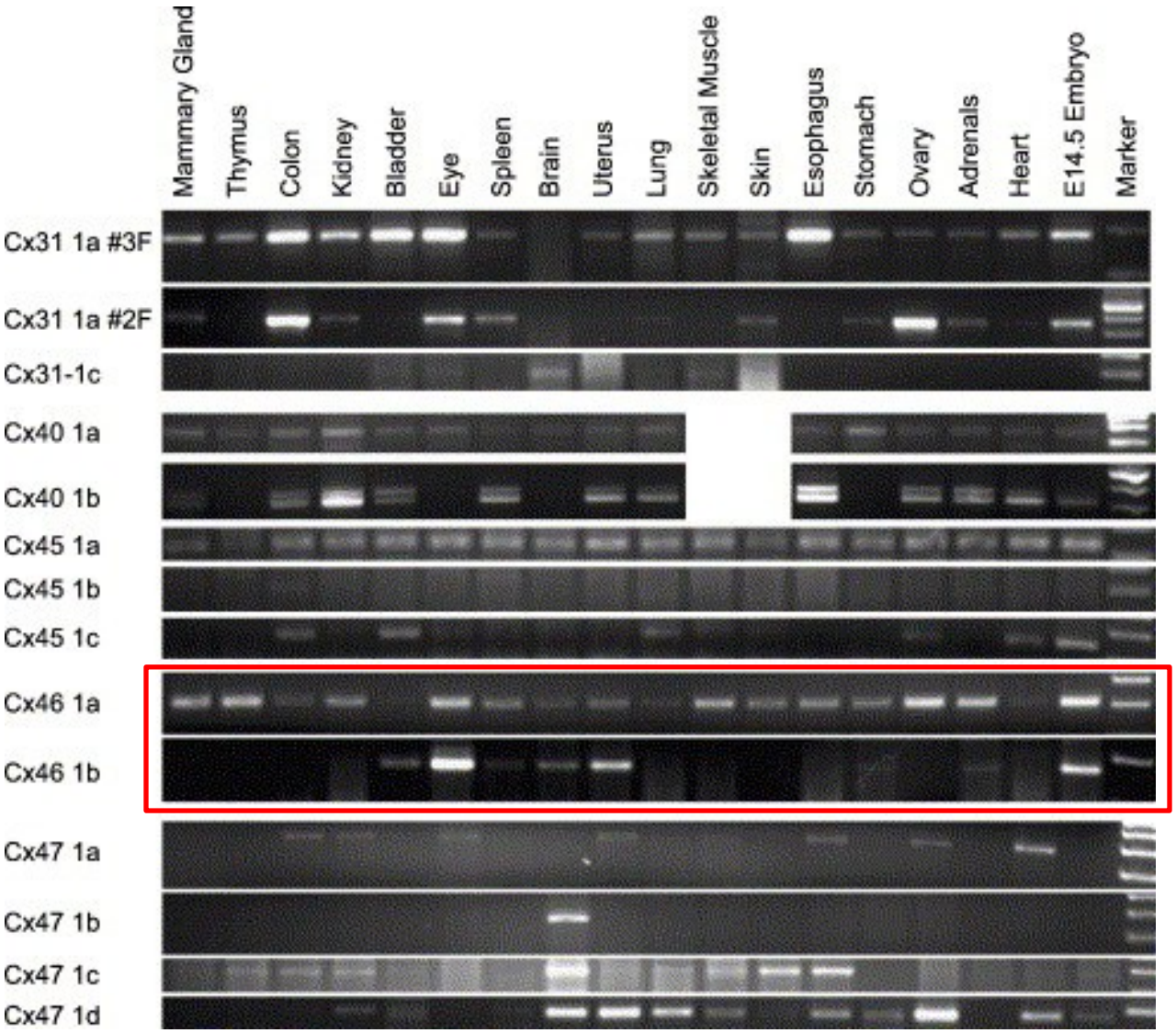

Figure 3.2. Mouse Cx46 mRNA is Present in the 17 Day Old Adult and the E14.5 Developing Embryo
This RT-PCR experiment shows that various connexin transcripts are present in a variety of tissues in the young adult mouse. The red box outlines the two detected forms of Cx46 mRNA using two separate PCR primer sets that cross exon-intron boundaries, effectively amplifying cDNA made from only mature RNA. Taken from Anderson et al. Genomics. 2005 Feb;85(2):238-44. "Variable promoter usage and alternative splicing in five mouse connexin genes" with permission from Elsevier.

Treatment with the protein kinase C activator and phorbol ester TPA induced Cx46 expression in lens cells correlating with a reduction in Cx43 and an increase in Cx46 protein levels (27). The Cx46 mRNA was most upregulated by three hours post-treatment, while the Cx46 protein levels increased up to six hours after treatment. TPA administration to lens cells caused an increase in *GJA3* mRNA, which signifies that the promoter may be regulated by trans-acting transcription factors. TPA also caused a delayed but significant Cx46 protein response,

which indicates that the newly transcribed Cx46 mRNA is most likely translated after the increased transcription. By analyzing the promoter of Cx46, we seek to understand why that specific connexin is preferred by the cell in a given growth condition, particularly hypoxia. In this chapter, the core promoter elements that allow increased transcription under hypoxic growth conditions were studied.

Materials and Methods

Cell Culture and Hypoxic Treatments

Human lens epithelial cells (HLEC), mouse neuro2A (N2A), and human Y79 retinoblastoma cells were cultured at 37°C in ambient air supplemented with 5% CO_2. HLEC and N2A cells were cultured in Low Glucose DMEM (Invitrogen) supplemented with 10% FBS (Atlanta Biologicals). Y79 cells were cultured in RPMI-1640 (Invitrogen) supplemented with 20% FBS. Hypoxia (1% oxygen) was induced by use of a ProOx Hypoxia Chamber (BioSpherix, Ltd) using N_2 as the displacement gas supplemented with 5% CO_2. The ProOx Chamber was temperature controlled by inserting it into a 37°C culture chamber.

Construction of GJA3 Promoter

1183 bp immediately upstream of the transcription start site (TSS) was synthesized (GenScript, Inc) and supplied in pUC57 by flanking EcoRV restriction sites (1195 bp total length). Since the GJA3 gene lies in the negative orientation, the promoter sequence was based upon the reverse positive strand, in other words the promoter nucleotide sequence chosen precedes the TSS in a 5'→3' direction ending at the TSS.

Promoter-Linked Luciferase Studies

Each promoter fragment that was tested was generated by PCR using the following primers: Reverse primer prhCx46-R1PCR-BamHI 5'-GCAGGATCCCAGTGTGCGCTGCGCCCGAC-3'; Forward primer pr46-810StuI-EcoRI 5'-TCAGAATTCAGGCCTCGCCCTGGCATCTCG-3'; Forward primer pr46-482AvrII-EcoRI 5'-TCAGAATTCCCTAGGTGCCCGACGCCCAC-3'; Forward primer pr46-287SphI-EcoRI 5'-TCAGAATTCGCATGCCCCGGAGTGCGT-3'; Forward primer pr46-147BsmI-EcoRI 5'-TCAGAATTCGAATGCGGACGCGGGCGAGT. The full-length (1183 bp) was removed from

pUC57 by excision with EcoRV. Directional cloning of each promoter construct into the multiple cloning site of the pMetLuc2-Reporter vector (Clontech, Inc.) was carried out with either EcoRV for the full-length or EcoRI/BamHI (New England Biolabs) for the shorter fragments. All final reporter vectors were DNA sequenced to verify correct orientation and sequence.

Control plasmids encoding either renilla or firefly luciferases under the control of either no or constitutive promoters were used for the normalization of data. Control vectors were co-transfected with each Cx46 promoter reporter vector using Fugene 6 (Roche) into white opaque flat bottom 96-well plates containing 20,000 cells per well, in triplicate. For the hypoxia control reporter vector, pTransLucent-Hif1 plasmid (Panomics) was used; for the cell positive control vector, pGL4.20 (Promega) was used. The promoter reporter negative control vector was the empty and promoter-less pMetLuc2-Reporter vector; the promoter reporter positive control vector was pMetLuc2-Control with luciferase driven by the CMV promoter.

5' Rapid Amplification of cDNA Ends

RNAs from normoxic (~21% O_2) and hypoxic (1% O_2) HLEC and Y79 cell cultures grown in T75 flasks for 7 days were harvested with the aid of the RNAeasy Mini Prep Kit (Qiagen) according to the manufacturer's directions using QiaShredder cartridges (Qiagen). All cells used were ≥75% viable on the day of harvest. RNA was quantified by using a NanoDrop (Thermo Scientific) and the quality assessed by analysis of 28S:18S RNA bands on a 1% agarose tris-borate EDTA native gel electrophoresis in the presence of 0.5 µg/mL ethidium bromide with a 0.24-9.5 Kb RNA Ladder (Invitrogen). Intact rRNA bands indicated intact total mRNA extraction.

First-strand cDNA synthesis for 5'RACE analysis was carried out according to the manufacturer's directions in the SMARTer™ RACE cDNA Amplification Kit (Clontech). Briefly, 2.75 µL of total mRNA (~500 ng/µL) was added to a reverse transcription reaction containing the 5'-CDS Primer A and the SMARTer IIA oligonucleotide (similar to an adaptor) to generate random 5'-IIA-adapted mRNA-based 5'RACE cDNA products.

2.5 µL of 5'RACE ready cDNA was used as the template for the gene specific primer PCR step. Reverse strand gene specific primers used in the 5'RACE analysis were as follows: #5 hsCx46wt_REV_NS_AgeI 5'-TCGACCGGTGATGGCCAAGTCCTCCGGTC-3'; #36

gja3RP1 5-‘AATTCCTTTCACACGTGCGGTGCT-3’; #38 gja3RP2 5’-TGATCGCCATCCCTGCAGCC-3’; #42 PCx46-exon3-5 5’-GGCTCCCGGCCGCTCTGAAAAGAATTCC-3’; #65 huCx46F2-PCR 5’-ATGGGCGACTGGAGCTTTCTGG-3’ (from Nielsen et al. 14 (6): 2470. 2003); #66 huCx46R2-PCR 5’-CTAGATGGCCAAGTCCTCCGGTCTGGC-3’ (from Nielsen et al. 14 (6): 2470. 2003). Ten µM of each reverse gene specific primer and 1x Universal Primer Mix were used to generate the 5’RACE gene specific products by three-step PCR using Phusion GC 2X Master Mix (New England Biolabs). PCR was performed with 30 cycles of {98°C 10 seconds, 61°C 5 seconds, 72°C 1 minute} and a final cycle of {72°C 1 minute, 4°C hold}.

The major gene specific 5’RACE PCR products were separated on a 0.8% agarose tris-acetate EDTA gel with 1 kb and 100 bp DNA ladders (New England Biolabs). Major products were excised, purified by spin cartridge and submitted for DNA sequencing with the T7 primer. The Universal Primer Mix primers contain a T7 priming site for direct PCR-product DNA sequencing.

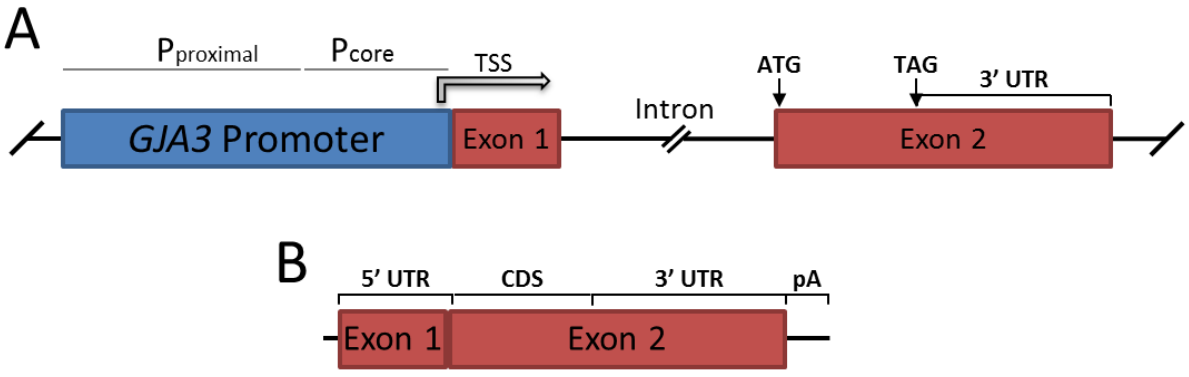

Figure 3.3 Genomic, Transcript and Tested Promoter Schematic
(A) The GJA3 promoter is proposed to have a core promoter which contains the minimal sequences needed for constant transcription and a proximal promoter which contains the regulatory sequences of the gene. (B) Predicted Cx46 mRNA sequence features based on NCBI database and genomic sequence analysis. P: Promoter; TSS: transcription start site; UTR: untranslated region; CDS: coding sequence; pA: polyadenine tail.

Results

In silico *Analysis of the Genomic Structure of GJA3*

The human gene *GJA3* has a simple but unique genomic structure (Figure 3.3). According to information obtained from the NCBI databases (Gene ID 2700; NC_000013.10, Build 37.3; NM_021954.3), the gene lies in reverse strand orientation, spanning 22,790 base

pairs (bp) located within the 13q12.11 region of genomic DNA. 13q12 also contains the genes *GJB2* and *GJB6*. The mature mRNA of *GJA3* is predicted to be 5,219 bp in length comprised of two exons containing all of the necessary information to efficiently translate the coding sequence. Exon 1 contains 155 bp of the 172 bp 5' untranslated region (UTR), while exon 2 contains the 17 bp directly upstream of the required translation-initiating ATG codon. Exon 2 also contains the entire coding sequence of Cx46 (1,308 bp = 435 residues), the 3' UTR (predicted to be 3,703 bp in length), and the polyadenylation signal. It is unclear if the sole 17,571 bp intron plays an important role in gene regulation. Very little is known about the functional regulation of the *GJA3* gene under any disease associated physiological growth conditions but species specific differences have been found between hamsters (28).

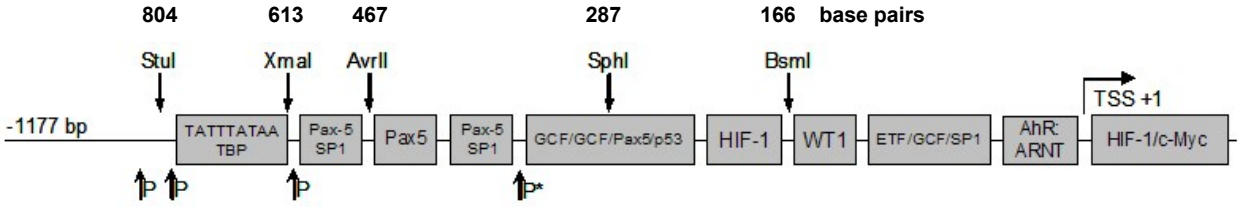

Figure 3.4. Schematic of the Human Cx46 Promoter
1,177 base pairs upstream from the predicted transcription start site (TSS) were entered into promoter and transcription factor prediction web servers. Predicted promoter sites with greater than 80% similarity to reference sequence were identified and marked with P. The P* promoter was predicted as 100% probability with two different web server programs. Transcription factors (grey boxes) were identified using the PROMO3.0 web program and the TRANSFAC 8.3 web database trained on human transcription binding sequence. Restriction sites indicate the start of each tested promoter fragment used to elucidate the regulation of Cx46 gene expression. Not to scale.

The mRNA contains a premature in-frame stop codon 15 bp upstream of the initiator Met codon. This may indicate early abortive translation to prevent unintended translation or to encode a novel internal ribosome entry site (IRES). The data presented in Figure 3.2, show abundant use of Cx46 exon 1a, but it is the exon 1b that is used for lens expression. This would be indicative of abortive transcription. Abortive transcription would provide a means of tight control of expression and fit the idea of tight regulation in oxygenated tissues. Interestingly, the 3' UTR is among the top 5% longest encoded in the human genome and spliced into mRNA (AceView: a comprehensive cDNA-supported gene and transcripts annotation, Genome Biology 2006, 7 (Suppl 1):S12).

Human transcription factor binding sites were predicted using a variety of web programs. Figures 3.1 and 3.4 both point out different TF predictions simply by using differently trained

programs. Promo3.0 and TransFac2.0 (Figure 3.4) both use blast-like sequence in conjunction with a database trained on human TF binding sequences. The early growth response (EGR 1-5) transcription factors are actively expressed under conditions of environmental stress and injury. Specific stresses include shear and mechanical stress (29), ultraviolet light, and by reactive oxygen species (ROS) (30). ROS can be generated by UV catalysis, ischemia/reperfusion and by tissue hypoxia. Myocyte enhancing factor 2 (MEF2; aMEF2 and RSRFC4 are related) is expressed in a variety of tissues including muscle, neural crest cells, endothelial cells, chondrocytes, neurons, and lymphocytes and contributes to tumorigenesis as well as epigenetic effects. Some cellular functions that result from MEF2 activity include migration, signaling, neuromuscular junction building, ion transport, metabolism and contractility (31). Nuclear factor erythroid-derived 2 45 kDa subunit (NF-E2 p45) is a transcription factor that is involved in mainly erythrocyte differentiation and maturation and is similar in function to the nuclear respiratory factor (Nrf) family of transcription factors that are upregulated and respond to cellular oxidative stress (32). Oddly, Cx46 has not been found in these other cell types to date.

The Aryl-hydrocarbon receptor (Ahr)/Aryl hydrocarbon nuclear translocator (ARNT) and hypoxia-inducible factors (HIFs 1-3) contribute to a number of biological responses under hypoxic and environmental stress growth conditions. We are interested in the function of the HIFs in control of Cx46 expression because of the naturally hypoxic environment in which Cx46 is preferentially expressed, the lens. Ahr/ARNT activity is activated when genobiotics are present in the cell, allowing direct binding to Ahr/ARNT dimers and allowing efficient transcription of genes with DREs or XREs (dioxin or xenobiotic response elements) (33) in which Cx46 has three consensus sites in the core promoter region.

Luciferase-based Promoter Analysis under Hypoxia

A luciferase-based approach was pursued as a means to understand regulatory regions located within the genetic code. Various lengths of the promoter were amplified by PCR and ligated to the promoter-less luciferase reporter vector. Each construct was tested in both N2A and HLE cells for activity. Both the 1177 bp and 625 bp constructs of the human promoter do not respond to any mouse transcription factor signals in the N2A cell line as tested (Figure 3.5), however, N2A do not naturally express mouse Cx46. Both promoter lengths exhibited low levels of transcription in N2A which may indicate differing control patterns of Cx46 expression

between human and mouse. Many of the current mRNA transcripts analyzed in the literature regarding Cx46 come from mouse tissues. When each construct was transfected into HLE cells, luciferase was efficiently produced in both normoxic and hypoxic growth conditions for 12 hours (Figure 3.5). Although the level of transcription was not high there was a notable amount of luciferase activity detected. We suspect Cx46 is transcribed at a near constant rate which is in ine with the dynamics of the gap junction life cycle.

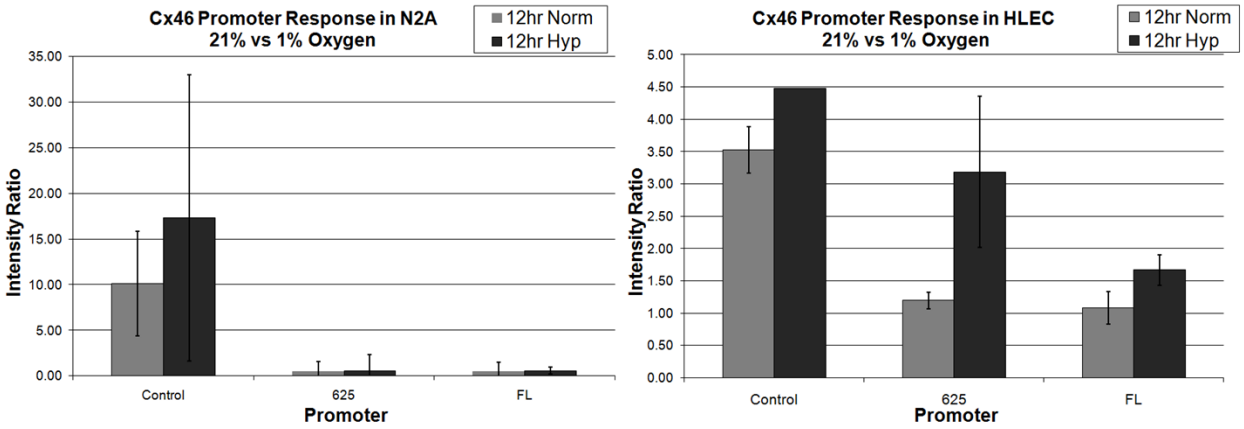

Figure 3.5 Non-normalized Cx46 Promoter Response to 21% and 1% Oxygen in Cell Culture
N2A and HLEC cells were grown to 80% confluence then transfected with luciferase gene under the control of either CMV (control) or 625 bp (half) or FL (1177 bp) of the GJA3 promoter for 24 hours. Cells were transferred to either 1% oxygen or kept in the normoxic incubator for 12 hours. N2A cells (Left) do not transcribe Cx46 mRNA as well as HLEC cells (Right) in either oxygenated or hypoxic growth conditions. Averages with standard variation shown.

Next, luciferase production was measured in HLE cells under a 1% oxygen and 5% carbon dioxide atmosphere in fresh media. Cells were near confluence when transfected to ensure connexin expression conditions. Expression conditions may or may not be affected by cell-to-cell contact, mechanical shear stress (34), cell cycle, nutrient, and/or genotoxic stress (35). When HLE cells were tested again under 1% oxygen growth conditions and luciferase activity was monitored, all tested promoter constructs responded to hypoxia when the data was normalized to the HIF reporter, as shown in Figure 3.6. The 467 bp promoter fragment showed the largest increase in activity, characteristic of a promoter burst of activity. The 287 bp fragment showed prolonged activity in 1% oxygen, so this promoter may be the minimal needed for sustained hypoxic expression. In other words, it may be the core promoter used for hypoxic expression of Cx46. The longer promoter fragments showed more restriction over all time points

tested indicating the presence of negative regulators of expression. It is possible that hypoxia-induced transcription factors could bind upstream of the promoter and block transcription in a trans-inhibitor fashion. Much of these assumptions need to be tested further to confirm this hypothesis.

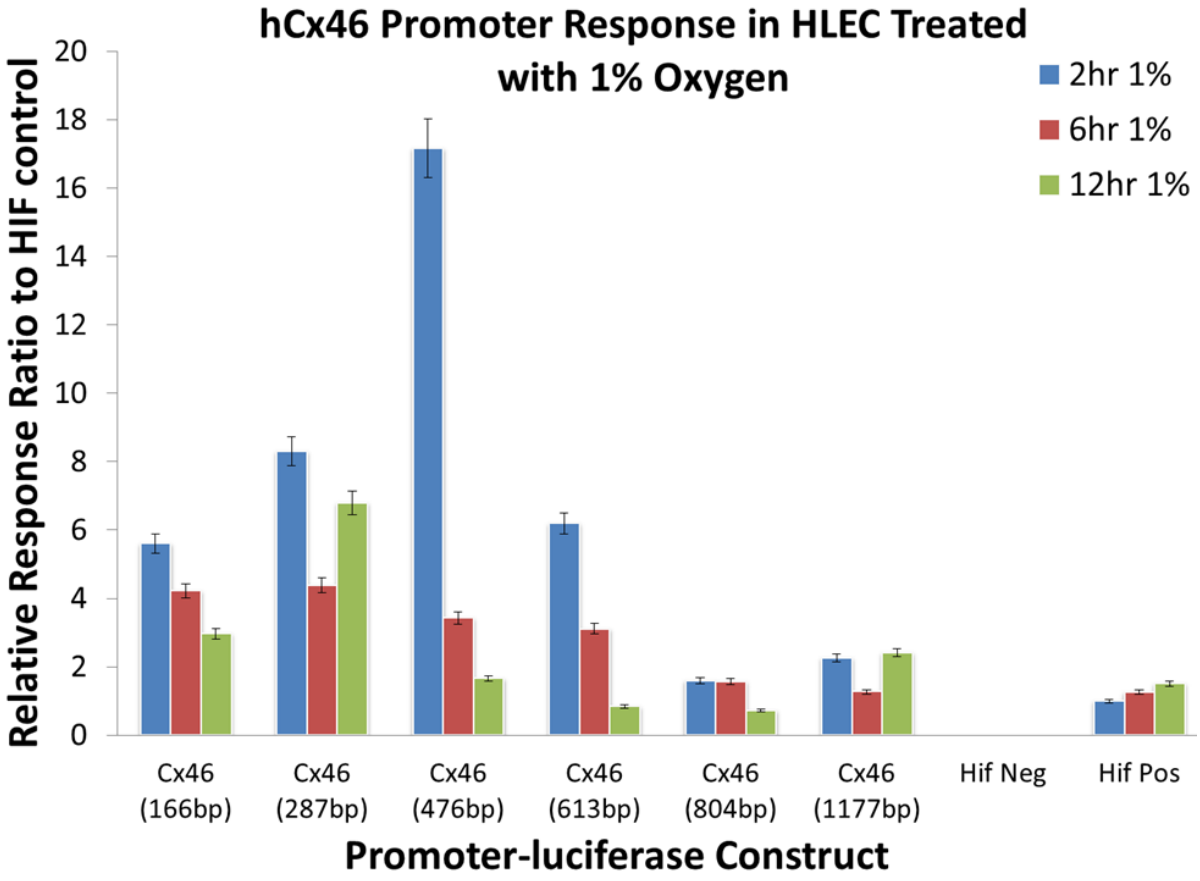

Figure 3.6. The Cx46 Promoter Responds Quickly and Efficiently to 1% Oxygen
HLE cells were transfected with each reporter vector and assayed at the given time for luciferase activity. The shorter fragments respond quicker and are more robust than the longer fragments (>613 bp) indicating the control regions of the promoter.

5'-RACE Identifies Multiple GJA3 mRNA Species

The advantage of using 5'-RACE for identifying the transcription start sites of genes is that the true transcription start point under any given growth condition can be identified. The RACE analysis kit used allows for the "true" identification of the start site by use of a specific 5' adaptor primer used for subsequent PCR amplification in conjunction with a reverse gene specific primer. In this analysis, total RNA was purified from HLEC expressing endogenous

GJA3 under 21% and 1% oxygen for 24 hours. This catches the hypoxia-adapted transcript of Cx46 in lens cells under the native growth conditions. HLEC thrive during hypoxic growth but grow at a slower rate. The goal was to use various Cx46 exon 1 specific reverse primers and the forward 5'RACE kit supplied primer to generate various RACE products of different sizes. These products have been analyzed by DNA sequence analysis to try to identify the transcriptional start sites in the *GJA3* promoter under both normoxic and hypoxic growth conditions. This method neither provides any evidence of IRES usage of which can be important in hypoxic regulation of gene translation nor does it test cell type specific effects.

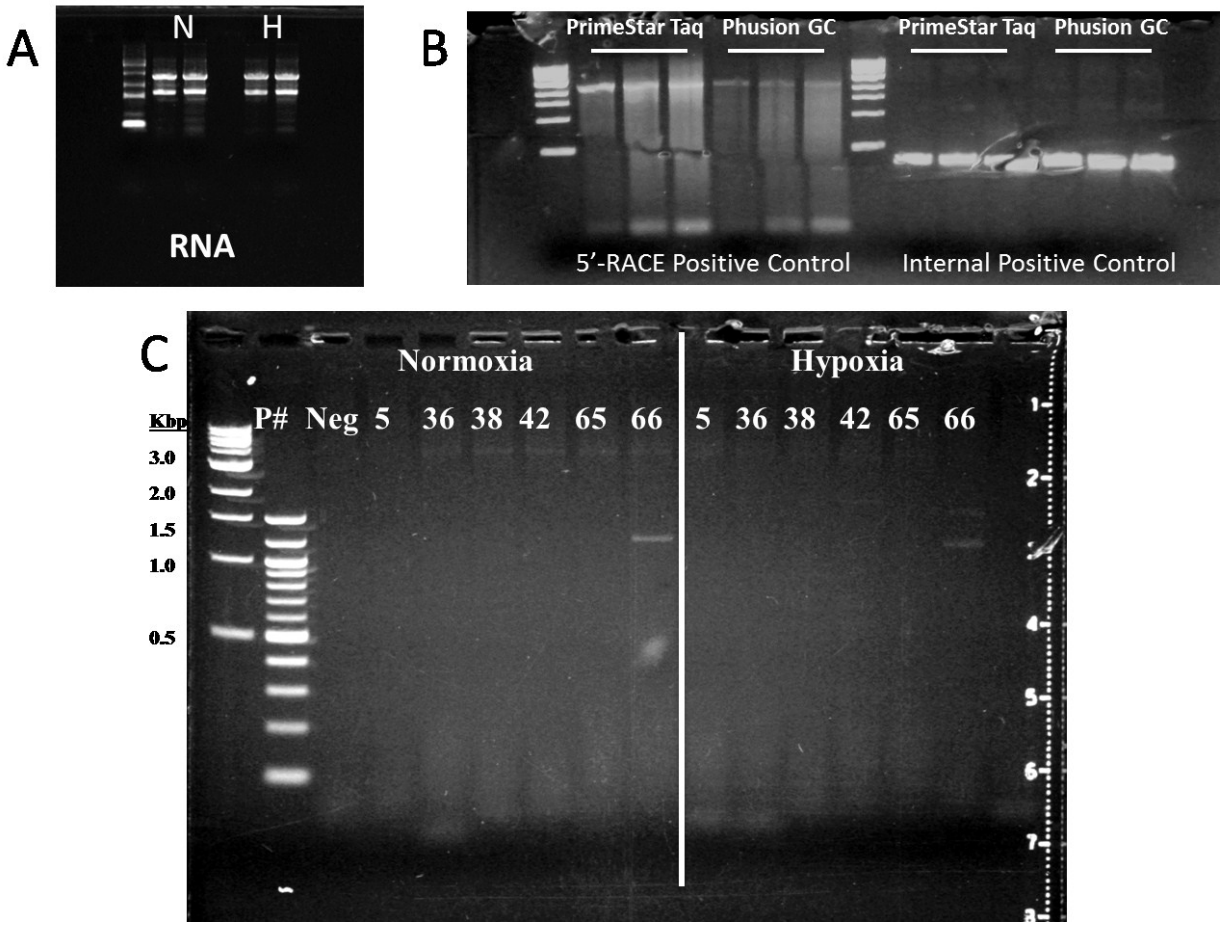

Figure 3.7. 5'RACE RNA Quality and PCR Control Reactions
A) RNAs harvested from HLEC grown in 21% oxygen (N) or 1% oxygen (H) were separated by gel electrophoresis and analyzed for r18S and r28S RNA quality (1 µg and 2.5 µg total RNA per set). B) Two different enzyme systems were used to generate the 5'RACE positive control (mouse transferrin receptor, 2.1 Kbp) and gene specific 5'RACE internal positive control (mouse transferrin receptor nested primers, 0.38 Kbp) for the gene specific RACE amplification procedure. Positive control is from mouse heart total RNA provided by the kit manufacturer. Each lane contains 1, 2 or 3 µL cDNA for each control. C) RACE negative control performed with Phusion GC polymerase master mix and a single gene specific reverse primer; the gene specific primers are noted along with the

template cDNA used. Very low non-specific products were detected above the predicted size range of each primer. Kbp: number of kilobase pairs; P#: gene specific primer number.

The cDNA library construction step of the RACE experiment relies on the ability of the 5'-adapted primer supplied in the kit to bind to all 5'UTRs present in the isolated mRNA. The RACE experiment also depends on the correct PCR cycling parameters as well as usable gene specific reverse primers to ensure efficient binding and amplification of true RACE products. For these reasons, Figure 3.7 shows that the controls for the RACE procedure worked well with the gene specific primers and cycling conditions. These conditions were used for the gene specific amplification step of the RACE experiment. Figure 3.8 shows the results of the HLEC Cx46-specific amplification of 5' cDNA ends from both 21% and 1% oxygen growth conditions over 24 hours.

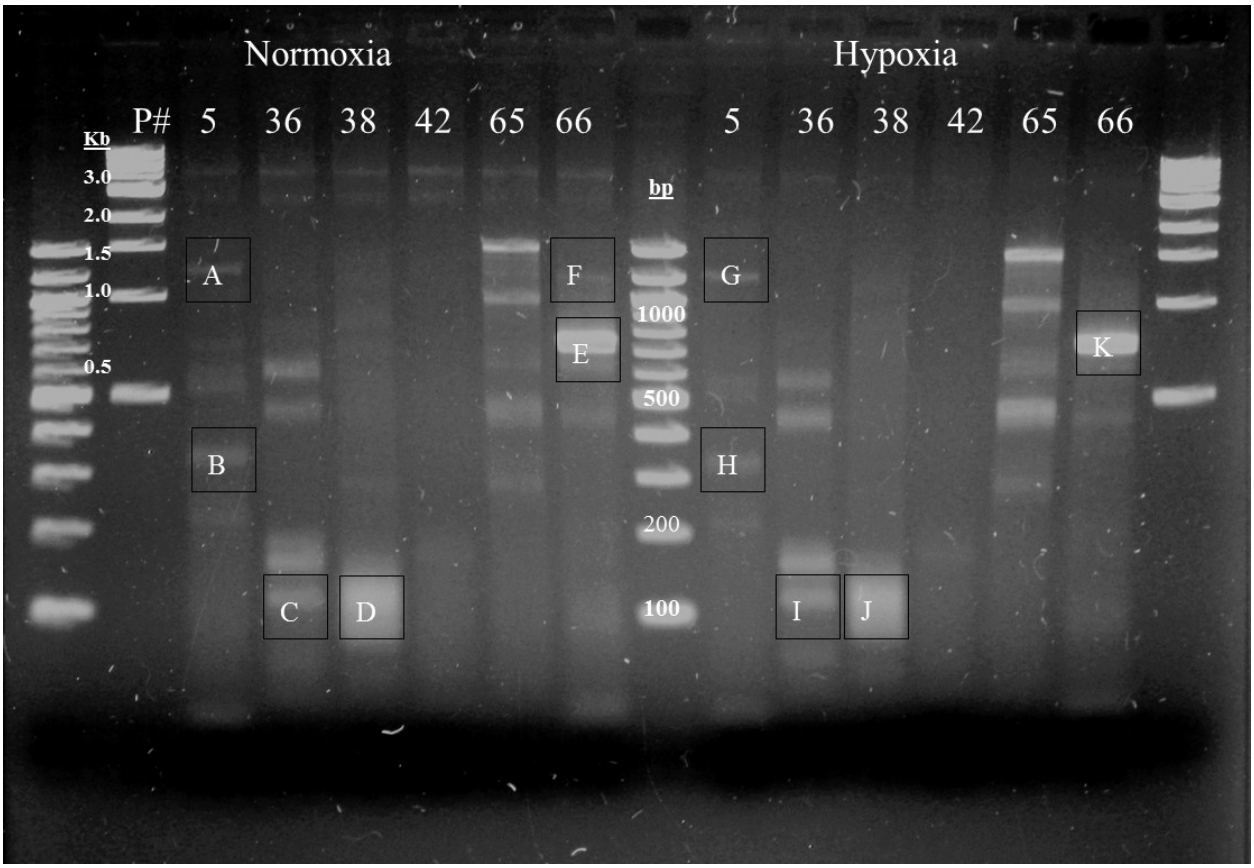

Figure 3.8. Electrophoretic Analysis of 5'-RACE Products Produced by Using HLEC Total RNA and Cx46-Specific Primers
Two normoxic and five hypoxic 5'RACE products were isolated and sent for DNA sequencing with T7 sequencing primer. Gene specific primers were generated using whole genome sequence data available at NCBI as a template.

Primers were individually BLAST searched to ensure *GJA3*-specific priming. P#: Primer number; Kb: kilobase; bp: base pairs; black box: area cut for DNA extraction.

Figure 3.8 also demonstrates the heterogeneity of 5'RACE products that were produced with Cx46-exon and 5'UTR specific primers. Primer #5 primes to the end of the Cx46 coding sequence which lies in exon 2 and the expected product length is 1477 bp referenced to NM_021954.3 (NCBI *GJA3* mRNA reference transcript). Primers #36, #38 and #42 prime in the 5'UTR and the expected product lengths are 88 bp, 21 bp, 110 bp, respectively. Primers #65-forward and #66-reverse prime the beginning and end of the Cx46 coding sequence, respectively. The forward primer is expected to not give any products, while the reverse primer is expected to give a 1480 bp product. Actual product sizes vary due to a variety of reasons like primer sequence and PCR condition mispriming, unknown and alternative Cx46 mRNA isoforms, or an incomplete or misprimed initial reverse transcription reaction.

DNA Sequence Analysis

DNA sequence analysis of the final purified PCR products gave mixed results. The results of multiple PCR products that were co-purified gave traces that resemebled multiple products in the sequencing reaction. This is indicative of 5'-RACE in which there is a wobbly transcription start site where transcription does not occur at a single site but rather a general area in the DNA sequence.

Discussion

The promoter analysis used in this chapter indicates that Cx46 is expressed at low levels in the cellular environment and is upregulated for a short amount of time (<6 hours) by hypoxia. The core promoter is contained roughly within the immediate ~300 bp upstream of the predicted TSS. Elevated transcription levels (>3-fold) were seen with the 287 and the 166 bp at all hours tested indicating an unregulated level of transcription when compared to the longer promoter fragments. Further analysis is needed to confirm that the core promoter can be turned on in other epithelial cell lines.

Of the many tissue types present in the human body, only the lens must express Cx46 to remain fully functional and disease free. Cx46 KO mice have only a noticeable cataract phenotype without any other notable differences compared to wild type littermates (36, 37). Cx46 expression has been identified in ROS 17/2.8 osteosarcoma cells, UMR 106-01

osteosarcoma cells, primary rat calvarial osteoblastic cells (24), and in neoplastic bone tissue (38), which are the only other tissues with Cx46. This is an interesting observation because osteosarcomas originate in hypoxic bone tissue, an environment which is similar to the hypoxic environment found in the lens. Koval et al. (1997) and Sanches et al. (2009) observed intracellular, perinuclear localization of Cx46 and gap junction plaques localized expression of Cx43, which we also observed in our lens cell culture system (27). Thus, a critical question is whether the Cx46 promoter is regulated by hypoxia.

When the human promoter of Cx46 was challenged with 1% atmospheric oxygen in human lens cells and normalized to a HIF1α-responsive control promoter, we observed a transient ~17-fold increase in activity, indicating that hypoxia-responsive elements are most likely located within the proximal promoter region (Figure 3.6). The Cx46 promoter also showed a sustained response with a shorter promoter fragment, suggesting that transcriptional regulation of Cx46 gene expression is tightly controlled by one or more positive and/or negative regulatory response elements present in the proximal promoter region. Of course, the possibility of long-range transcriptional regulatory effects remains open.

Analysis of the transcription start site by 5'-RACE gave mixed results. There are multiple bands in most lanes of the 5'-RACE agarose gel and when individual bands were purified and sent out for direct sequencing, each single band gave data traces indicating varied transcription start sites. Further analysis by cloning and clone isolation is warranted to sequence individual PCR products for the transcription start site or area.

Hypoxia may play an active role in the Cx43/Cx46 regulation that occurs naturally in the lens or unnaturally in solid tumors and other ischemic tissues. This possibility is being tested in lens cell cultures in addition to other tumor-derived cell lines and in other ischemic tissues. Tight regulation of Cx46 expression is not surprising since Cx46 is expressed primarily in the lens, but other lens crystallin proteins have been found in solid tumors as well, indicating that the conditions for lens protein expression exist in other tissues (39, 40). The possibility that there is overlap in the available growth factors or other receptor ligands are open; however, given the uncommon expression of Cx46 in other tissues beyond the lens, the agonist and antagonist interactions that lead to Cx46 expression may have minimal overlap in both lens and solid tumor tissues. One overlap may exist in prenatal developmental. Here there are unique global and local hypoxic environments that are present and influence cellular differentiation (41-48),

however, Cx46 expression has not been studied throughout prenatal development. This hypothesis still remains to be proven but offers interesting speculation into the unique role that Cx46 may play in physiology, independent of gap junction channel activity, which is associated with Cx46.

Acknowledgements

This work was supported in part by funds provided by grants NIH EY13421 to D.J.T., Terry C. Johnson Cancer Center Innovative Research Grant to D.J.T., and the Kansas State University Research Foundation for the Doctoral Scholarship provided to S.A.M.

References

1. Ahmad S, Martin PE, Evans WH. Assembly of gap junction channels: Mechanism, effects of calmodulin antagonists and identification of connexin oligomerization determinants. Eur J Biochem. 2001 Aug;268(16):4544-52.

2. Harris AL. Connexin channel permeability to cytoplasmic molecules. Prog Biophys Mol Biol. 2007 May-Jun;94(1-2):120-43.

3. Griffith TM. Which connexins connect? Circ Res. 2007 Dec 7;101(12):1219-21.

4. Kanaporis G, Mese G, Valiuniene L, White TW, Brink PR, Valiunas V. Gap junction channels exhibit connexin-specific permeability to cyclic nucleotides. J Gen Physiol. 2008 Apr;131(4):293-305.

5. Pfenniger A, Wohlwend A, Kwak BR. Mutations in connexin genes and disease. Eur J Clin Invest. 2011 Jan;41(1):103-16.

6. Laird DW. The gap junction proteome and its relationship to disease. Trends Cell Biol. 2010 Feb;20(2):92-101.

7. Lai-Cheong JE, Arita K, McGrath JA. Genetic diseases of junctions. J Invest Dermatol. 2007 Dec;127(12):2713-25.

8. Mathias RT, White TW, Gong X. Lens gap junctions in growth, differentiation, and homeostasis Physiol Rev. 2010 Jan;90(1):179-206.

9. Winterhager E. Gap junctions in development and disease. Berlin ; New York: Springer; 2005.

10. Xia CH, Liu H, Cheung D, Cheng C, Wang E, Du X, et al. Diverse gap junctions modulate distinct mechanisms for fiber cell formation during lens development and cataractogenesis. Development. 2006 May;133(10):2033-40.

11. Ebihara L, Tong JJ, Vertel B, White TW, Chen TL. Properties of connexin 46 hemichannels in dissociated lens fiber cells Invest Ophthalmol Vis Sci. 2011 Feb 22;52(2):882-9.

12. Minogue PJ, Liu X, Ebihara L, Beyer EC, Berthoud VM. An aberrant sequence in a connexin46 mutant underlies congenital cataracts. J Biol Chem. 2005 Dec 9;280(49):40788-95.

13. Pal JD, Liu X, Mackay D, Shiels A, Berthoud VM, Beyer EC, et al. Connexin46 mutations linked to congenital cataract show loss of gap junction channel function Am J Physiol Cell Physiol. 2000 Sep;279(3):C596-602.

14. Dunia I, Cibert C, Gong X, Xia CH, Recouvreur M, Levy E, et al. Structural and immunocytochemical alterations in eye lens fiber cells from Cx46 and Cx50 knockout mice. Eur J Cell Biol. 2006 Aug;85(8):729-52.

15. Xia CH, Cheng C, Huang Q, Cheung D, Li L, Dunia I, et al. Absence of alpha3 (Cx46) and alpha8 (Cx50) connexins leads to cataracts by affecting lens inner fiber cells. Exp Eye Res. 2006 Sep;83(3):688-96.

16. Gong X, Cheng C, Xia CH. Connexins in lens development and cataractogenesis. J Membr Biol. 2007 Aug;218(1-3):9-12.

17. White TW. Unique and redundant connexin contributions to lens development. Science. 2002 Jan 11;295(5553):319-20.

18. Banerjee D, Gakhar G, Madgwick D, Hurt A, Takemoto D, Nguyen TA. A novel role of gap junction connexin46 protein to protect breast tumors from hypoxia. Int J Cancer. 2010 Aug 15;127(4):839-48.

19. Burr DB, Molina SA, Banerjee D, Low DM, Takemoto DJ. Treatment with connexin 46 siRNA suppresses the growth of human Y79 retinoblastoma cell xenografts *in vivo*. Exp Eye Res. 2011 Apr;92(4):251-9.

20. Vinken M, Vanhaecke T, Papeleu P, Snykers S, Henkens T, Rogiers V. Connexins and their channels in cell growth and cell death Cell Signal. 2006 May;18(5):592-600.

21. Yamasaki H, Krutovskikh V, Mesnil M, Tanaka T, Zaidan-Dagli ML, Omori Y. Role of connexin (gap junction) genes in cell growth control and carcinogenesis. C R Acad Sci III. 1999 Feb-Mar;322(2-3):151-9.

22. Rodriguez-Sinovas A, Cabestrero A, Lopez D, Torre I, Morente M, Abellan A, et al. The modulatory effects of connexin 43 on cell death/survival beyond cell coupling. Prog Biophys Mol Biol. 2007 May-Jun;94(1-2):219-32.

23. Mesnil M. Connexins and cancer Biology of the Cell. 2002;94(7-8):493-500.

24. Koval M, Harley JE, Hick E, Steinberg TH. Connexin46 is retained as monomers in a trans-golgi compartment of osteoblastic cells. J Cell Biol. 1997 May 19;137(4):847-57.

25. Anderson CL, Zundel MA, Werner R. Variable promoter usage and alternative splicing in five mouse connexin genes. Genomics. 2005 Feb;85(2):238-44.

26. Baird SD, Turcotte M, Korneluk RG, Holcik M. Searching for IRES. RNA. 2006 Oct;12(10):1755-85.

27. Banerjee D, Das S, Molina SA, Madgwick D, Katz MR, Jena S, et al. Investigation of the reciprocal relationship between the expression of two gap junction connexin proteins, connexin46 and connexin43. J Biol Chem. 2011 Jul 8;286(27):24519-33.

28. Cruciani V, Heintz KM, Husoy T, Hovig E, Warren DJ, Mikalsen SO. The detection of hamster connexins: A comparison of expression profiles with wild-type mouse and the cancer-prone min mouse. Cell Commun Adhes. 2004 Sep-Dec;11(5-6):155-71.

29. Vorderwulbecke BJ, Maroski J, Fiedorowicz K, Da Silva-Azevedo L, Marki A, Pries AR, et al. Regulation of endothelial connexin40 expression by shear stress. Am J Physiol Heart Circ Physiol. 2012 Jan;302(1):H143-52.

30. Bhattacharyya S, Wu M, Fang F, Tourtellotte W, Feghali-Bostwick C, Varga J. Early growth response transcription factors: Key mediators of fibrosis and novel targets for anti-fibrotic therapy. Matrix Biol. 2011 May;30(4):235-42.

31. Potthoff MJ, Olson EN. MEF2: A central regulator of diverse developmental programs. Development. 2007 Dec;134(23):4131-40.

32. Sykiotis GP, Bohmann D. Stress-activated cap'n'collar transcription factors in aging and human disease. Sci Signal. 2010 March 9;3(112):re3.

33. Oesch-Bartlomowicz B, Oesch F. Role of cAMP in mediating AHR signaling. Biochem Pharmacol. 2009 Feb 15;77(4):627-41.

34. Grellier M, Bareille R, Bourget C, Amedee J. Responsiveness of human bone marrow stromal cells to shear stress. J Tissue Eng Regen Med. 2009 Jun;3(4):302-9.

35. Ale-Agha N, Galban S, Sobieroy C, Abdelmohsen K, Gorospe M, Sies H, et al. HuR regulates gap junctional intercellular communication by controlling beta-catenin levels and adherens junction integrity. Hepatology. 2009 Nov;50(5):1567-76.

36. Rong P, Wang X, Niesman I, Wu Y, Benedetti LE, Dunia I, et al. Disruption of Gja8 (alpha8 connexin) in mice leads to microphthalmia associated with retardation of lens growth and lens fiber maturation. Development. 2002 Jan;129(1):167-74.

37. Tang Y, Crowley TE, Kumar NM. Global gene expression analysis of lenses from different mouse strains and in the alpha3Cx46 knockout mouse Mol Vis. 2010 Jan 27;16:113-21.

38. Sanches DS, Pires CG, Fukumasu H, Cogliati B, Matsuzaki P, Chaible LM, et al. Expression of connexins in normal and neoplastic canine bone tissue. Veterinary Pathology Online. 2009 September 01;46(5):846-59.

39. Nakatsugawa M, Hirohashi Y, Torigoe T, Asanuma H, Takahashi A, Inoda S, et al. Novel spliced form of a lens protein as a novel lung cancer antigen, lengsin splicing variant 4. Cancer Science. 2009;100(8):1485-93.

40. Deng M, Chen P, Xie S, Zhao J, Gong L, Liu J, et al. The small heat shock protein αA-crystallin is expressed in pancreas and acts as a negative regulator of carcinogenesis. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 2010 8;1802(7-8):621-31.

41. Shimoda LA, Semenza GL. HIF and the lung: Role of hypoxia-inducible factors in pulmonary development and disease Am J Respir Crit Care Med. 2011 Jan 15;183(2):152-6.

42. Patterson AJ, Zhang L. Hypoxia and fetal heart development Curr Mol Med. 2010 Oct;10(7):653-66.

43. Wan C, Shao J, Gilbert SR, Riddle RC, Long F, Johnson RS, et al. Role of HIF-1alpha in skeletal development Ann N Y Acad Sci. 2010 Mar;1192:322-6.

44. Lendahl U, Lee KL, Yang H, Poellinger L. Generating specificity and diversity in the transcriptional response to hypoxia Nat Rev Genet. 2009 Dec;10(12):821-32.

45. Shui YB, Arbeit JM, Johnson RS, Beebe DC. HIF-1: An age-dependent regulator of lens cell proliferation. Invest Ophthalmol Vis Sci. 2008 Nov;49(11):4961-70.

46. Edsjo A, Holmquist L, Pahlman S. Neuroblastoma as an experimental model for neuronal differentiation and hypoxia-induced tumor cell dedifferentiation Semin Cancer Biol. 2007 Jun;17(3):248-56.

47. Provot S, Schipani E. Fetal growth plate: A developmental model of cellular adaptation to hypoxia Ann N Y Acad Sci. 2007 Nov;1117:26-39.

48. Xie XJ, Wang JA, Cao J, Zhang X. Differentiation of bone marrow mesenchymal stem cells induced by myocardial medium under hypoxic conditions. Acta Pharmacol Sin. 2006 Sep;27(9):1153-8.

Chapter 4 - The Carboxy Terminal Tail Domain is Essential for Cx46 Function in Lens Cells

Parts of this chapter were previously published as:

Debarshi Banerjee*, Satyabrata Das*, Samuel A Molina, Dan Madgwick, Snehalata Jena, Leonie K Bossmann, Debjani Pal, Dolores J Takemoto. "Investigation of the reciprocal relationship between the expression of two gap junction connexin proteins, connexin46 and connexin43" J Biol Chem. 2011 Jul 8; 286(27):24519-33. *Co-first Author

Abstract

Connexins are the transmembrane proteins that form gap junctions between adjacent cells. The function of the diverse connexin molecules is related to their tissue specific expression and highly dynamic turnover. Although multiple connexins have been previously reported to compensate for each other's functions little is known about how connexins influence their own expression or intracellular regulation. Of the three vertebrate lens connexins, two connexins, connexin43 (Cx43) and connexin46 (Cx46), show reciprocal expression and subsequent function in the lens and in lens cell culture. In this study, we investigate the reciprocal relationship between the expression of Cx43 and Cx46. Forced depletion of Cx43, by tumor-promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), is associated with an upregulation of Cx46 at both the protein and message level in human lens epithelial cells. A siRNA-mediated downregulation of Cx43 results in an increase in the level of Cx46 protein suggesting that endogenous Cx43 is involved in the regulation of endogenous Cx46 turnover. Overexpression of Cx46, in turn, induces the depletion of Cx43 in rabbit lens epithelial cells. Cx46 induced Cx43 degradation is likely mediated by the ubiquitin-proteasome pathway as (i) treatment with proteasome inhibitors restores the Cx43 protein level and (ii) there is an increase in Cx43 ubiquitin conjugation in Cx46 overexpressing cells. We also present data which shows that the carboxy-terminal intracellular tail domain of Cx46 is essential to induce degradation of Cx43. Therefore, our study shows that Cx43 and Cx46 have novel functions in regulating each other's expression and turnover in a reciprocal manner in addition to their conventional roles as gap junction proteins in lens cells.

Introduction

Gap junctions are intercellular aqueous channels composed of trans-membrane proteins called connexins (1). The gap junction-dependent or independent functions of connexins are important in the regulation of several cellular processes including growth, proliferation, differentiation, protection and cell death (2, 3). Distinct expression patterns and highly dynamic turnover rates are the key components that regulate tissue specific activity of different connexin molecules. The expression and turnover of connexins are fine-tuned balances of several processes such as gene expression, mRNA stability, protein synthesis and transport, and degradation (4,5). Connexin turnover and function is also modulated by several intrinsic and extrinsic factors including intra- and extracellular pH, various phosphorylation events, cellular status, and chemical reagents such as the tumor-promoting phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA) (6-10).

One tissue that relies on gap junction mediated communication for normal function and growth is the vertebrate lens. The lens is naturally avascular and, therefore, gap junction mediated functions play a major role in maintaining proper homeostasis and transparency of the lens. The vertebrate lens endogenously expresses three connexin proteins required for proper lens development and function, connexin43 (Cx43), connexin46 (Cx46), and connexin50 (Cx50) (11-17). These connexins show differential spatial distributions that are related to their specific functions at different regions of the lens. Cx43 and Cx50 are mainly expressed at the lens epithelium and are required to maintain gap junction activity at the surface of the lens (17, 18). However, Cx46 is preferentially expressed in the differentiating and mature fiber cells and therefore is the major functioning connexin in the lens nuclear region (15, 19). During lens epithelial-to-fiber cell differentiation Cx43 is substantially down-regulated and Cx46 is significantly up-regulated. This reciprocal expression between Cx43 and Cx46 is critical for maintaining connexin-mediated communication in lens tissue; however, the underlying molecular mechanism of this reciprocal relationship remains to be clarified.

Previously, it was observed that Cx43 and Cx46 are reciprocally expressed in normal breast tissue and tumor xenografts (24), as well as in Y79 retinoblastoma xenografts (25). Cx43 is well known for tumor suppressor functions and has been previously reported to be downregulated in breast tumor when compared to normal breast tissue (20-23). Two prior studies demonstrated that Cx46 is significantly upregulated in tumor xenografts and functions as a proto-

oncogene favoring tumor growth while at an early hypoxic stage (24, 25). Therefore, the reciprocal expression between these two connexins plays a crucial role in the regulation of cell proliferation in tumorigenesis as well as in lens development. Multiple connexins expressed in the same tissue have been previously reported to compensate for each other's function (4, 26, 27), however, little is known about how one connexin can control the expression and turnover of another connexin.

In the present study (previously published as Banerjee D, Das S, Molina SA, Madgwick D, Katz MR, Jena S, et al. Investigation of the reciprocal relationship between the expression of two gap junction connexin proteins, connexin46 and connexin43. J Biol Chem. 2011 Jul 8; 286(27):24519-33; see original journal article for full details), we investigated the reciprocal relationship between the expression of these two connexin proteins, Cx43 and Cx46. We showed that TPA forced depletion of Cx43 protein and this is associated with an upregulation of Cx46. The upregulation of Cx46 is both at the mRNA and protein level and occurs via a protein kinase C (PKC) dependent pathway in human lens epithelial cells (HLEC). Additionally, siRNA mediated downregulation of Cx43 resulted in an increase of Cx46 indicating that endogenous Cx43 is involved in the control of Cx46 protein levels. Further and in this chapter, we showed that overexpression of Cx46 causes a reduction of Cx43 protein. Overexpression of Cx46 increased Cx43 ubiquitination and therefore mediated its degradation by a proteasome dependent pathway. In this chapter, I provide evidence that Cx46 membrane localization is not necessary for induction of degradation and that only the cytoplasmic carboxy-terminal tail domain of Cx46 is required to induce degradation of Cx43, although these two proteins failed to co-localize. Our results provide mechanistic insight into the regulation of Cx43 and Cx46 in lens cells and offers explanation of the reciprocal relationship observed unnaturally in tumor xenografts and naturally in the vertebrate lens.

Materials and Methods

Cell Culture

To assure the results of this publication both human and rabbit lens epithelial cells were used. Immortalized human lens epithelial cells (HLEC) and rabbit lens epithelial N/N1003A cells (NN or N/N) were cultured as described previously (24). Briefly, cells were cultured in low glucose DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% premium fetal bovine serum

(Atlanta Biologicals, Atlanta, GA) to at least 70% confluence prior to using for experimentation. Cells were grown in a humidified atmosphere of 5% CO_2 and 37°C. All cell culture plastics were purchased from Midwest Scientific (St. Louis, MO).

Cloning and Transfection

The coding region of rat Cx43 (NM_012567.2, 382 residues), rat Cx46 (NM_024376.1, 416 residues), or rat Cx50 (NM_153465.2, 440 residues) was sub-cloned into the pEGFP-N3 vector (Clontech, Mountain View, CA) to create carboxy-terminal tagged EGFP fusion proteins. Plasmid transfection was mediated by Lipofectamine 2000 (Invitrogen) at 70-80% confluency according to the manufacturer's suggestions. The transfected cells were grown in the presence of 1 mg/mL G418 for 3-4 weeks for the selection of positive colonies. Then, the positive colonies were isolated and grown in media containing 500 µg/mL of G418, thereafter.

Wild type rat Cx46 was subcloned into pEGFP-N3 (Clontech) using the forward primer: *ratGJA3 N3 F PCR* 5'-GCAGAATTCAGGATGGGCGACTGGAGC-3' and the reverse primer: *rCx46 R* 5'-TATGGATCCGATGGCCAAGTCACCTGGTCTG-3'. Various mutants were created to test the effect of the Cx46 carboxy-terminal domain on Cx43. The Cx46Δ413 mutant is truncated before the ZO-1 interaction site (DLAI) in the carboxy-terminal tail domain. The Cx46Δ398 mutant has the majority of the PCK phosphorylation sites truncated from the wild type protein. Cx46Δ325 contains the WT protein truncated half way through the CT domain, ending at residue 325. The Cx46Δ225 mutant encodes the NT domain and the four transmembrane domains of the wild type protein and just deletes the Cx46 carboxy-terminal (CT) tail domain (also labeled as Cx46-dCT). Each mutant was cloned by PCR using the forward primer *ratGLA3 N3 F PCR* (above) and the following matched reverse primers: *Cx46Δ413*: rCx46d413-416 5'-TATGGATCCACCTGGTCTGGCTCGTCCGCT-3'; *Cx46Δ398*: rCx46d398-416 5'-TATGGATCCGTCCAGGAGGACCAACGGTGG-3'; *Cx46Δ325*: rCx46d325-416: 5'-TATGGATCCGTGGTGGCCATTGCAGTGCTTGAC-3'; *Cx46Δ225*: rCx46d225-416R 5'-TATGGATCCCATGTTGAGCACCAGTGACGCACA-3'. Amino acid residues 225-416 of wild type Cx46 were sub-cloned into pEGFP-N3 to generate an expression vector which expresses only the CT tail domain of Cx46 fused to EGFP using *46tail225-416F* 5'-ATAGAATTCATGCTAGAGATTTACCACCTGG-3' as the forward primer and *rCx46 R* as the reverse primer. Wild type rat Cx46 cDNA was also sub-cloned into the pQE-TriSystem

vector (Qiagen, Valencia, CA) to generate a C-terminal 5x-His tagged protein (courtesy of Dr. Debarshi Banerjee). The expression of Cx43-EGFP, Cx46-EGFP, Cx50-EGFP, Cx46Δ225-EGFP (Cx46-dCT-EGFP), Cx46Δ325-EGFP, Cx46Δ398-EGFP, Cx46Δ413-EGFP, Cx46Δ1-225-EGFP (Cx46Tail-EGFP), and Cx46-His were checked by western blot analyses using anti-GFP and anti-His tag antibodies, respectively.

Whole Cell Homogenate (WCH) Preparations

Cells were washed 3 times with cold phosphate-buffered saline (PBS, pH 7.2), collected by scraping from plates, and centrifuged at 4000 rpm for 5 min at 4°C. The cell pellets were lysed in ice cold 1X RIPA buffer [10 mM Tris-HCl (pH 7.5),150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 µg/mL leupeptin] containing 1 mM PMSF. Cell lysates were sonicated for 10 seconds (3 times) and protein concentration of each sample was measured using Bio-Rad Protein Assay.

Western Blotting and Antisera

Western blot analyses were performed as previously described (24, 25, 28). Mouse anti-N-terminal-Cx43 (anti-Cx43 NT) was purchased from Fred Hutchinson Cancer Center (Seattle, WA); rabbit anti-phospho-Cx43 (Ser-368) from Cell Signaling Technologies (Danvers, MA); rabbit anti-C-terminal-Cx43 (anti-Cx43 CT, 1:5000) and mouse anti-β-actin (1:10000) from Sigma-Aldrich (St. Louis, MO); rabbit anti-Cx46 (1:2000) from US Biologicals (Swampscott, MA); mouse anti-Cx50 (1:4000) from Zymed-Invitrogen (San Francisco, CA) and mouse anti-EGFP antibody (1:5000) was purchased from Clontech (Mountain View, CA). Mouse anti-pentahis tag (1:1000) was purchased from Qiagen (Valencia, CA). Mouse anti-ubiquitin antibody was purchased from Calbiochem (EMD Biosciences, OH). Rabbit anti-C-terminal-Cx43 (anti-Cx43 CT) was used to detect Cx43, if not mentioned otherwise.

Immunofluorescent Labeling and Fluorescent Microscopy

HLECs and NN1003A cells were grown on glass coverslips in six-well plates until 80% confluency. Treatments were carried out per experimental protocol. Cells were fixed with 4% formaldehyde in PBS for 20 min at room temperature, quenched with 50 mM glycine, washed with PBS, permeabilized with 0.05% Triton-X100 in PBS for 30 min, washed, and blocked with

5% BSA (blocking solution) in PBS for at least 1 h at room temperature. Cells were then treated overnight at 4°C with rabbit anti-Cx43CT antibody (1:250) or rabbit anti-Cx46 antibody (1:250) in blocking solution with constant gentle rocking. After washing three times for 5 min each in PBS, slides were labeled for 10 min in the dark at room temperature with 500 nM DAPI in PBS to stain nuclei. Finally, after three more washes for 5 min each in PBS, slides were mounted in Prolong Gold Antifade Reagent (Invitrogen-Molecular Probes, Eugene, OR) for at least 24 hours prior to visualization. The slides were viewed by a confocal microscope (LSM 510 Meta, Carl Zeiss, Göttingen, Germany) or by a fluorescence microscope (Axiovert 200M, Carl Zeiss). For fluorescence studies involving transiently transfected cells, NN1003A or HLEC cells were transfected with plasmids encoding Cx46-EGFP and mutants for no more than 48 hr prior to formaldehyde fixation. Monoclonal anti-58K/FTCD antibody (1:50, Sigma-Aldrich) was used in conjunction with donkey anti-mouse-Alexa Fluor 594 antibody (1:800, Invitrogen) to mark the Golgi apparatus in HLECs.

siRNA Transfection

HLEC were grown in 60 mm dishes. When confluency reached 70%, cells were treated with 50 nM of either anti-Cx43 siRNA (Qiagen) or All Stars Negative Control siRNA (Qiagen) and 20 µL of HiPerFect transfection reagent (Qiagen). Cells were harvested after 24 and 48 hours of transfection and WCH were prepared.

Statistical Analyses

Origin software (Microcal Software Inc., Northampton, MA) was used to generate statistical analyses and associated data graphs. The level of significance (see * in figure legends) was considered at $p < 0.05$ using paired t test analyses. All data are presented as mean ± SEM of at least 3 independent experiments.

Results

For full results of published paper, please see the original journal article. This chapter contains data generated by myself and sometimes in conjunction with fellow lab mates, as noted.

siRNA Mediated Knockdown of Endogenous Cx43 Results in Increased Cx46 Protein Levels in HLEC

As part of this study, Dr. Satyabrata Das found that pretreatment with a PKC inhibitor blocked the TPA-induced Cx43 degradation that occurred concomitantly with the inhibition of TPA-induced increase in Cx46 protein. This led us and Dr. Debarshi Banerjee to speculate whether the degradation of Cx43 is necessary for the increase of Cx46 protein. To check this, we silenced Cx43 expression by a selective anti-Cx43 siRNA and examined the effect of Cx43 downregulation on Cx46 protein levels. As shown in Figure 4.1, transfection of HLECs with anti-Cx43 siRNA decreased the Cx43 protein levels significantly after 48 hours. Interestingly, the decrease in Cx43 protein level (Figure 4.1B) was associated with a notable increase in Cx46 protein at 48 hours of siRNA transfection (Figure 4.1C). However, treatment with anti-Cx43 siRNA did not cause a change in the amount of Cx50 protein (Figure 4.1A). This data indicates that endogenous Cx43 is involved in the regulation of Cx46 protein expression in HLECs and further confirmed the endogenous reciprocal relationship of Cx46 and Cx43 expression.

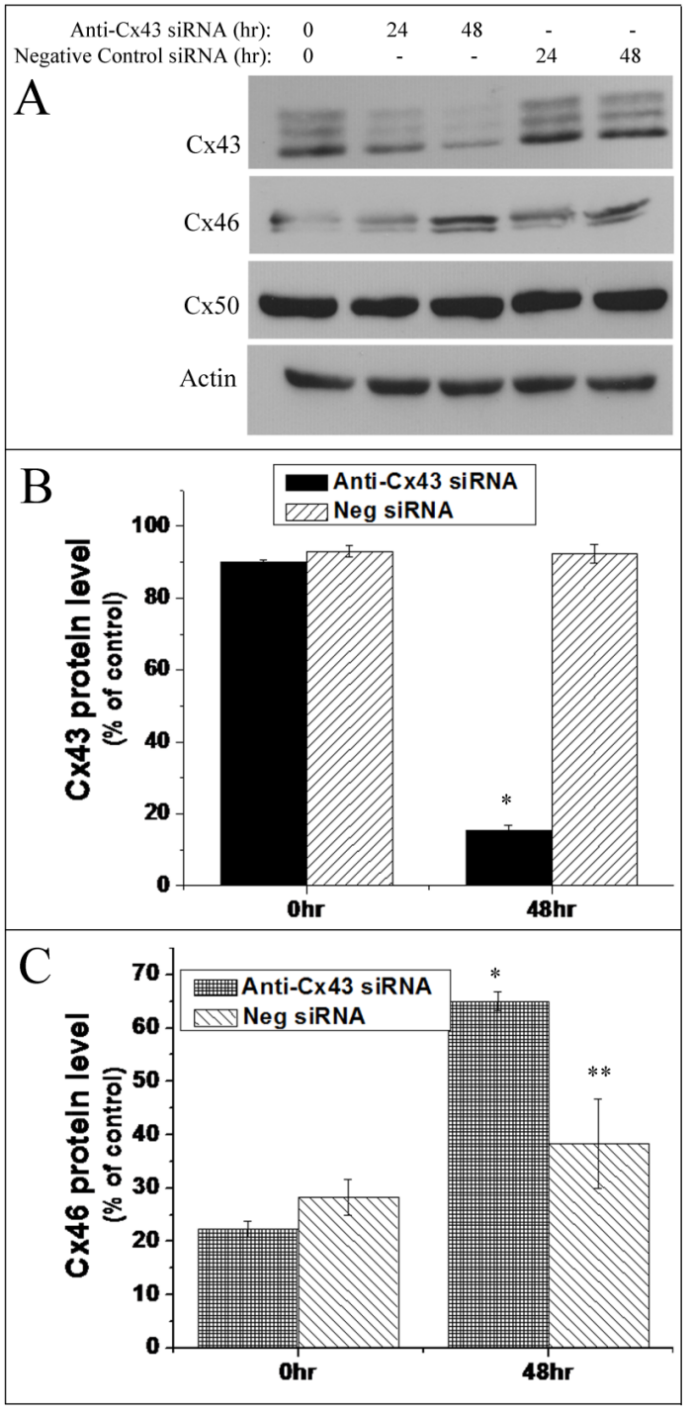

Figure 4.1 siRNA-Mediated Knockdown of Cx43 Results in Increased Cx46 Protein Levels

(A) 70% confluent HLECs were treated with 50 nM of either anti-Cx43 siRNA or 50 nM of AllStars Negative Control siRNA for the indicated time periods. Increase in Cx46 level was noticed at 48 h of anti-Cx43 siRNA treatment when Cx43 was maximally down-regulated. (B) and (C) densitometric analyses show siRNA-mediated selective down-regulation of Cx43 causes an increase in Cx46 protein levels. Data are represented as mean ± S.E. *, significant statistical difference ($p \leq 0.05$) between indicated data and control (at 0 h); **, statistical insignificance between the data and control (at 0 h).

Overexpression of Cx46 Causes a Decrease in Cx43 Protein Levels in N/N1003A Rabbit Lens Cells

Since the degradation of Cx43 was associated with an up-regulation of Cx46, we next investigated if overexpression of Cx46 had an effect on Cx43. To examine this, we stably overexpressed the Cx46-EGFP fusion protein in rabbit lens epithelial N/N1003A cells and analyzed the effect of Cx46 overexpression on the other lens connexins endogenously present in these cells as well as other membrane proteins. The Cx46-EGFP expressing cells showed the expression of fusion protein as two distinct bands around 75 kDa as determined by western blot using an anti-EGFP antibody (Figure 4.2A). The two bands in Figure 4.2A are suspected to be phospho-Cx46-EGFP isoforms. Interestingly, stable overexpression of Cx46 decreased the Cx43 protein levels of the phosphorylated isoforms P0, P1 and P2. Stable overexpression of Cx46 reduced the Cx43 protein levels to 50% when compared to untransfected or EGFP transfected cells (Figure 4.2B). However, Cx50 protein level was not changed due to the stable overexpression of Cx46 (Figure 4.2A and 4.2C). We also examined the effect of overexpression of Cx50 on Cx43 protein levels. Stable overexpression of Cx50-EGFP had no effect on Cx43 protein levels (Figure 4.2D). Collectively, these data indicate that only Cx46 influences the decrease of Cx43 and that another closely related alpha-type connexin, Cx50, does not.

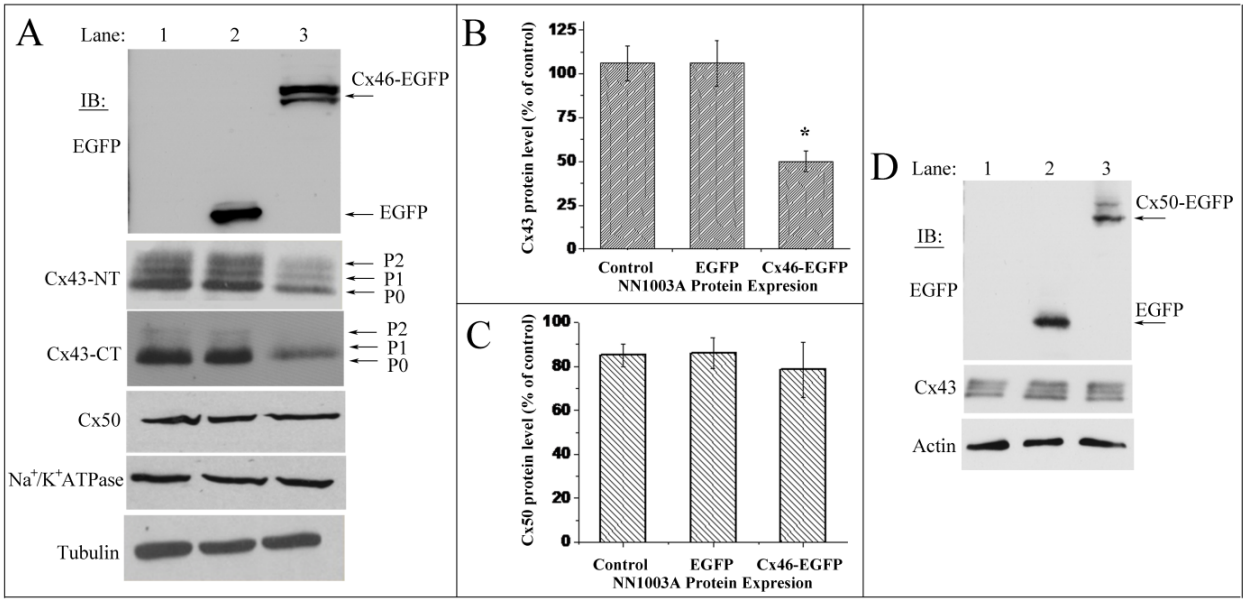

Figure 4.2 Overexpression of Cx46 Causes a Reduction in Cx43 Protein Levels in Rabbit Lens Epithelial N/N1003A Cells

(A) Western blot analyses of Cx46-EGFP, Cx43, Cx50, tubulin, and Na^+/K^+ATPase in untransfected control cells and in cells stably overexpressing EGFP or Cx46-EGFP. Whole cell lysates were made, and equal amounts of total protein were run in SDS-PAGE followed by immunoblot. The blot was probed with anti-EGFP antibody to detect Cx46-EGFP expression. The blots were also probed with Cx43CT (against C-terminus) and Cx43NT (against N terminus) antibodies to detect Cx43 to rule out any C-terminal truncation of Cx43. (B) Densitometric analyses showed significant reduction of Cx43 protein levels in Cx46-EGFP stably transfected N/N1003A cells, whereas (C) The level of Cx50 protein showed no change. The bands representing Cx43, Cx50, and tubulin were digitized by UN-SCAN-It gel software. The average pixel values were calculated for Cx43 bands, normalized against average pixel values for loading control (tubulin), and then plotted in percent of control. Data are represented as mean ± S.E. of three independent experiments. *, Significant statistical differences ($p \leq 0.05$) between indicated data and control cells. (D) Overexpression of Cx50 does not cause a decrease in Cx43 protein levels. N/N1003A cells were stably transfected with plasmid encoding Cx50-EGFP. Cell lysates were prepared, and equal amounts of total protein were run on SDS-PAGE followed by Western blot. The Cx50-EGFP and Cx43 proteins were detected by probing the blots with EGFP and Cx43 antibodies, respectively.

Cx46 is Predominantly Localized to the Intracellular Compartments in N/N1003A and HLEC Lens Cells

We determined the intracellular localization of Cx46 in the stably transfected cells by intrinsic EGFP fluorescence (Figures 4.3 and 4.4) and immunofluorescence microscopy (Figures 4.5 and 4.6). Green fluorescence of Cx46-EGFP expression was predominantly detected to an intracellular perinuclear compartment (Figure 4.3, 4.4, and 4.6A). Fluorescence signal of Cx46-EGFP was not observed at the plasma membrane region. In Figure 4.3, it can be noted that wild type Cx46 does not traffic well to the plasma membrane of mouse N2A cells when the cells are either opposed or free and that acute 1% oxygen does not affect this trait. However, the carboxy-terminal tail deletion mutant shows diffuse localization different than that of EGFP and wild type Cx46. This is most likely due to the loss of sorting and/or packaging transport ability because of the truncation. The CT domain of any connexin contains many cryptic signals that are yet to be understood. However, hypoxia seems to not affect localization of the wild type Cx46 protein. Overall Cx46-EGFP proteins fluorescence increased when subjected to hypoxia but the differences were not quantitated for this study. Figure 4.4 highlights the trafficking and truncation correlation further by displaying each of the mutant subcellular localizations. All mutants except the Cx46Δ1-225 (Cx46-Tail) and Cx46Δ225 (Cx46-dCT) domains localized in punctate perinuclear structures. The two exceptions displayed diffuse expression patterns suggesting that all domains are necessary for correct protein trafficking with the signals for oligomerization/stabilization being contained in amino acid residues 226-325. However, for complete and correct construction of connexons, residues 226-325 must be connected to the first 225 residues of Cx46. Cx46 localization does not seem to be largely affected by the PKC activator TPA (Figure 4.5B) whereas Cx43 is negatively affected (Figure 4.5A) by TPA treatment (Dr. Satyabrata Das). Again, wild type Cx46 is punctate and perinuclear (Figure 4.5C).

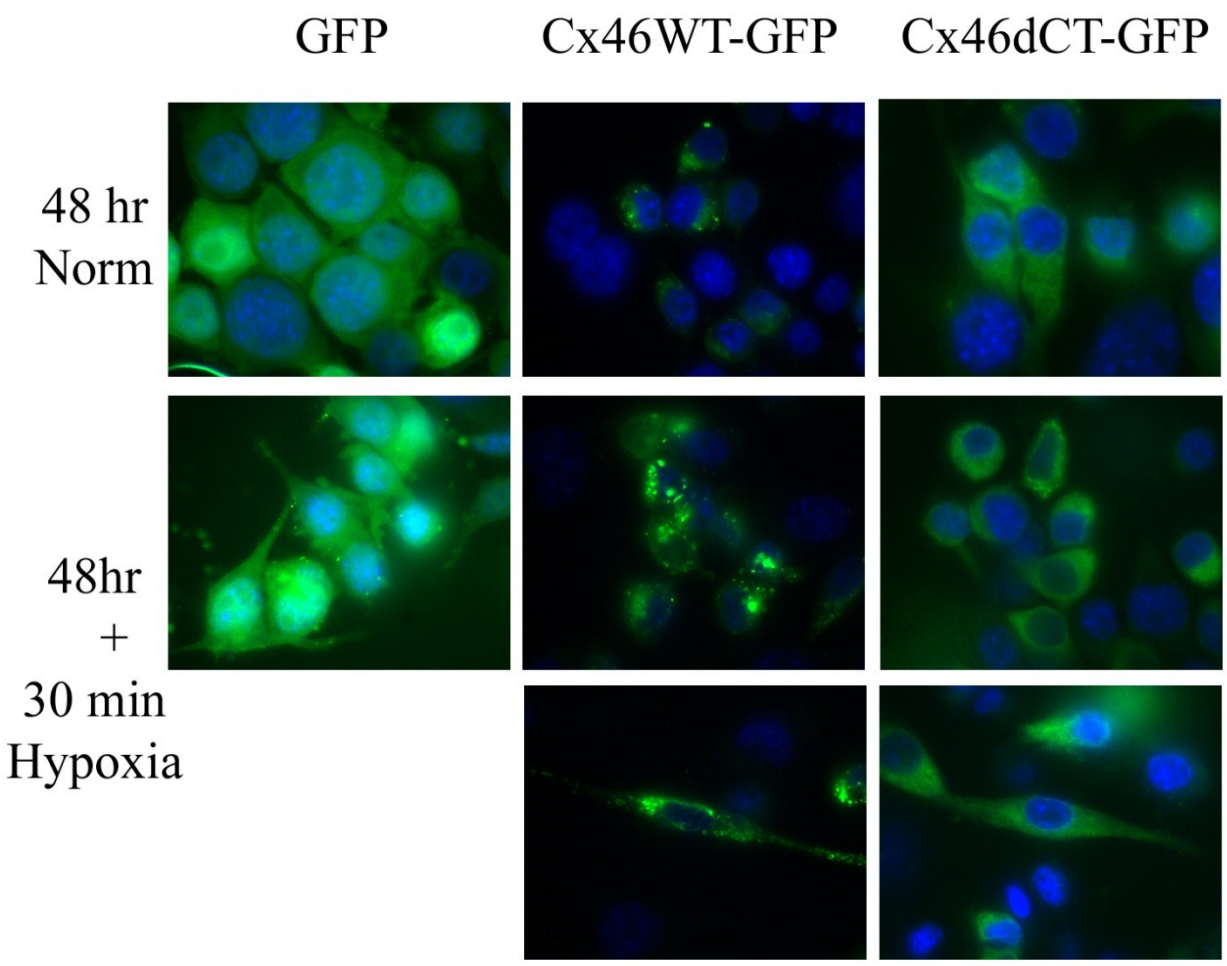

Figure 4.3 Intracellular Localization of Rat Cx46-EGFP in Mouse N2A Cells
Mouse N2A cells were transiently transfected with either EGFP, wild type Cx46 (Cx46WT-GFP), or a Cx46 deletion mutant where the entire carboxy-terminal tail domain is truncated at residue 225 (Cx46dCT-GFP). Wild type Cx46 (middle) shows perinuclear (nuclei: blue) localization under both normoxic and acute 1% oxygen conditions, whereas the truncated Cx46 mutant (right) is perinuclear and diffuse, similar to EGFP only (left). The difference in localization and packaging can be attributed to the loss of cryptic molecular signals contained in the CT domain of all connexins.

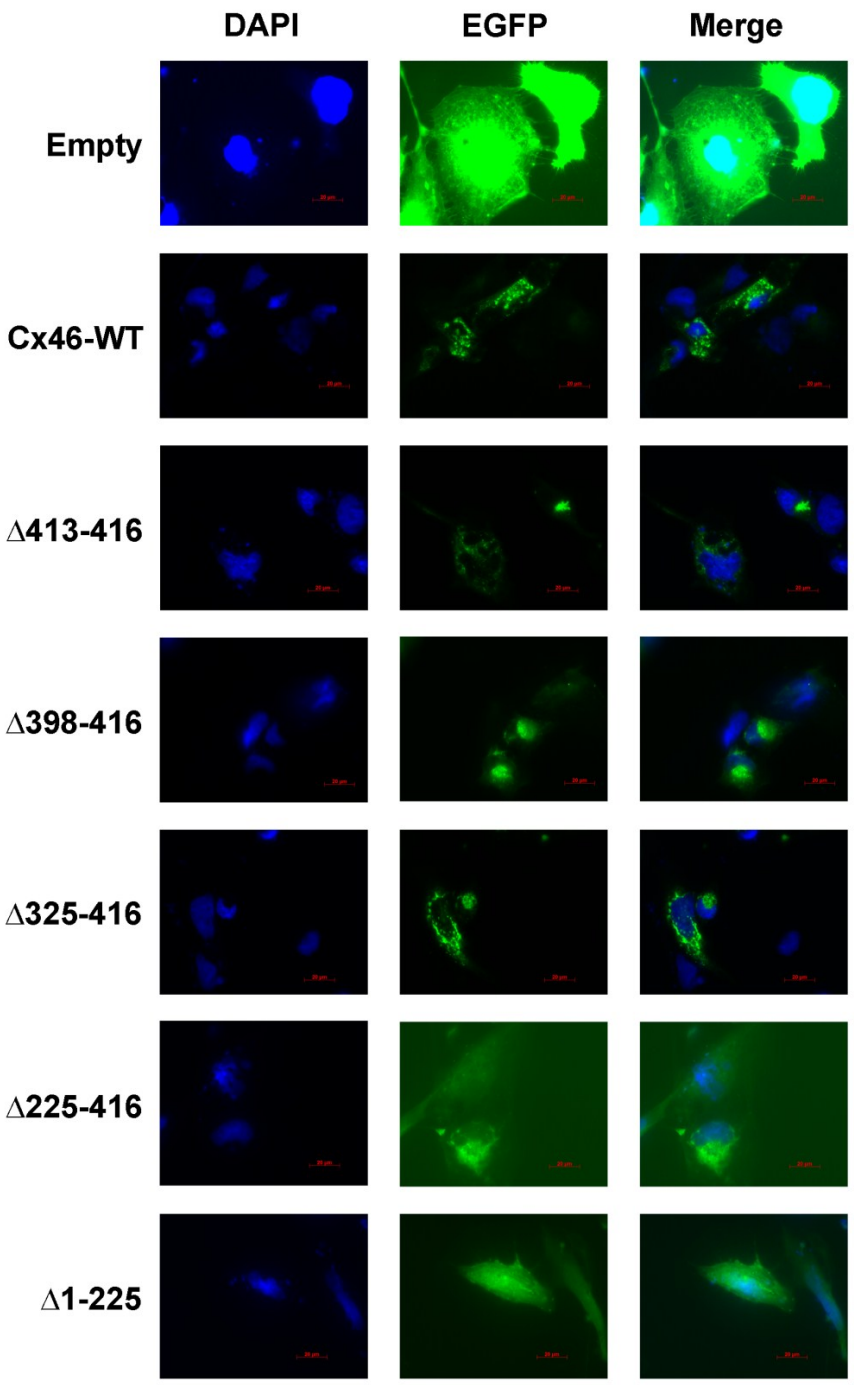

Figure 4.4 Intracellular Localization of Rat Cx46 Protein Mutants Labeled with EGFP in Human Lens Epithelial Cells

Punctate Cx46-EGFP proteins were localized to the perinuclear region for all mutants except for the Cx46Δ225 (Cx46-dCT) CT domain deletion and the Cx46Δ1-225 NT (Cx46-Tail), and transmembrane domain deletion mutants.

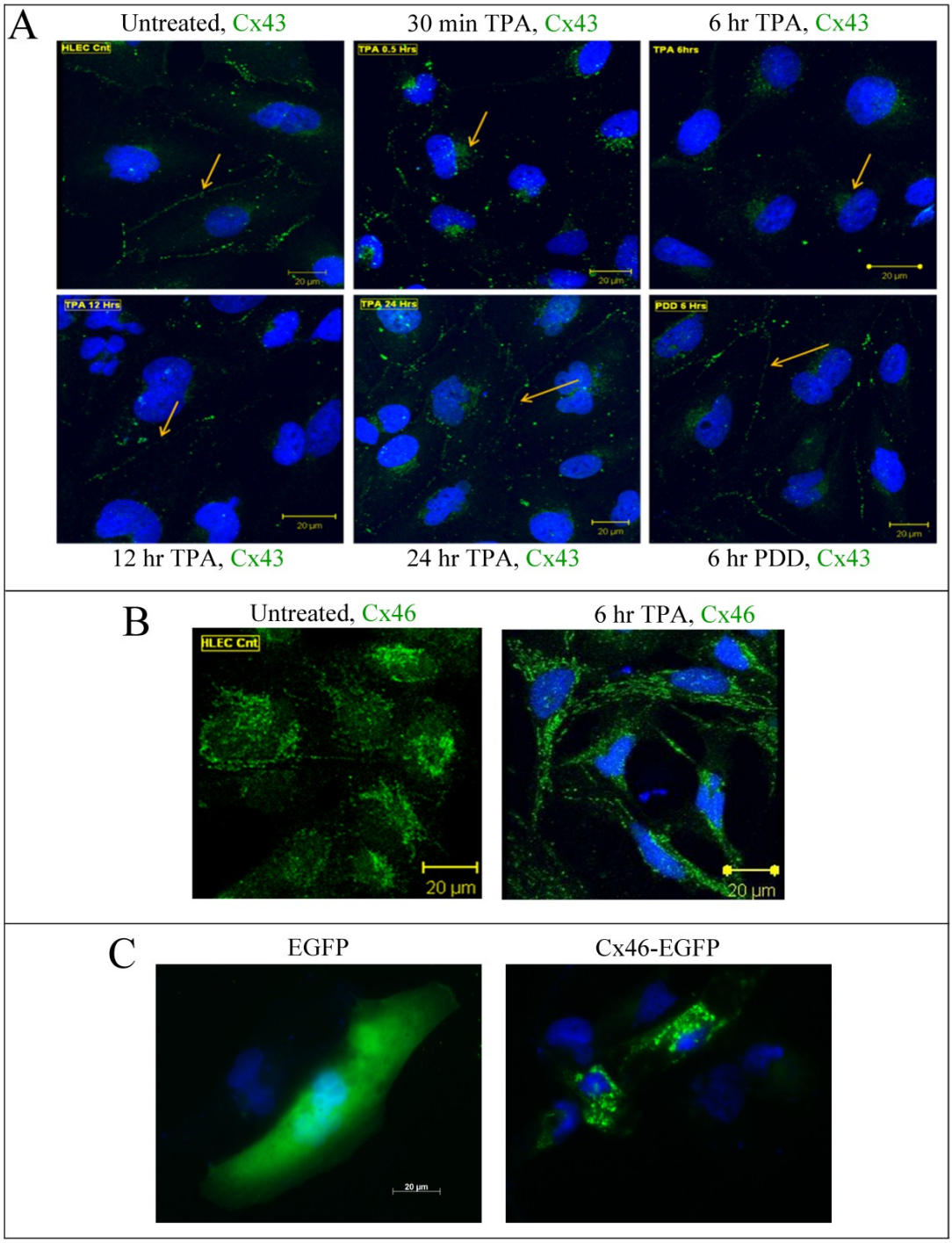

Figure 4.5 TPA-Induced Changes in Localization of Cx43 and Cx46 in HLECs
(A, credit to Dr. Satyabrata Das) HLECs were treated with or without 300 nM TPA for different time periods as indicated in the figure and labeled with anti-Cx43 antibody (green). DAPI (blue) was used to stain nuclei. Arrows point to the gap junction plaques at the membrane or in the cytosol. (B, credit to Dr. Debarshi Banerjee) Control HLECs or TPA-treated (300 nM, 6 h) HLECs were labeled with anti-Cx46 antibody (green) and DAPI (blue). Cx46 staining was found predominantly in the intercellular compartments of HLEC cells. (C) Fluorescence microscopy of HLEC cells transiently expressing either EGFP (green) or Cx46-EGFP (green) with DAPI staining of the nuclei (blue). Again, Cx46-EGFP localized to the perinuclear region.

The EGFP protein is a rather large fusion protein tag and has a molecular weight of 27 kDa. Therefore, we speculated whether EGFP tagging at the C-terminus of Cx46 prevented it from reaching the cell surface. To investigate this, we transfected cells with a plasmid encoding Cx46-His, which encodes six histidine residues fused to the carboxy-terminus of Cx46. We then examined the localization of Cx46-His fusion protein via immunofluorescence in order to assess differences in the localization of Cx46. Immunofluorescence using the anti-His antibody also demonstrated that Cx46 localized predominantly to the intracellular perinuclear compartments and not to the plasma membrane (Figure 4.6B, red channel).

To confirm the localization of Cx46, untransfected or stably transfected cells were fractionated to isolate plasma membrane proteins. Western blot analyses with antibodies against EGFP and Cx46 detected no immunoreactive bands that corresponded to endogenous or Cx46-EGFP or Cx46 protein in the plasma membrane fraction of untransfected or transfected cells (Figure 4.6C). In all the preparations of plasma membrane fractions, a marker for plasma membrane (Na^+/K^+ATPase) was enriched; meanwhile, the cytosolic marker GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was not, confirming the purity of the membrane fractions.

To further demonstrate that EGFP-tagging does not affect the localization of connexin proteins in N/N1003A cells, cells were transiently transfected to express Cx43-EGFP. Using western blot analysis, Cx43-EGFP fusion protein (around 70 kDa) was detected in plasma membrane fractions isolated from Cx43-GFP overexpressing N/N1003A cells (Figure 4.6D). These data clearly suggested that EGFP tagging had no influence on the Cx43 or Cx46 transport and localization within the lens cell. Our data are consistent with other literature confirming that C-terminal tagging of connexins does not inhibit their intracellular transport (41, 42).

Since Cx46 is localized to the perinuclear region, we further characterized which compartment Cx46 is localizing to by using three-color fluorescence microscopy. Figure 4.6E shows that Cx46-EGFP localizes to the perinuclear region in HLECs but does not co-localize well with the Golgi marker 58K/FTCD protein. This suggests that Cx46-EGFP could reside in the endoplasmic reticulum compartment of HLECs.

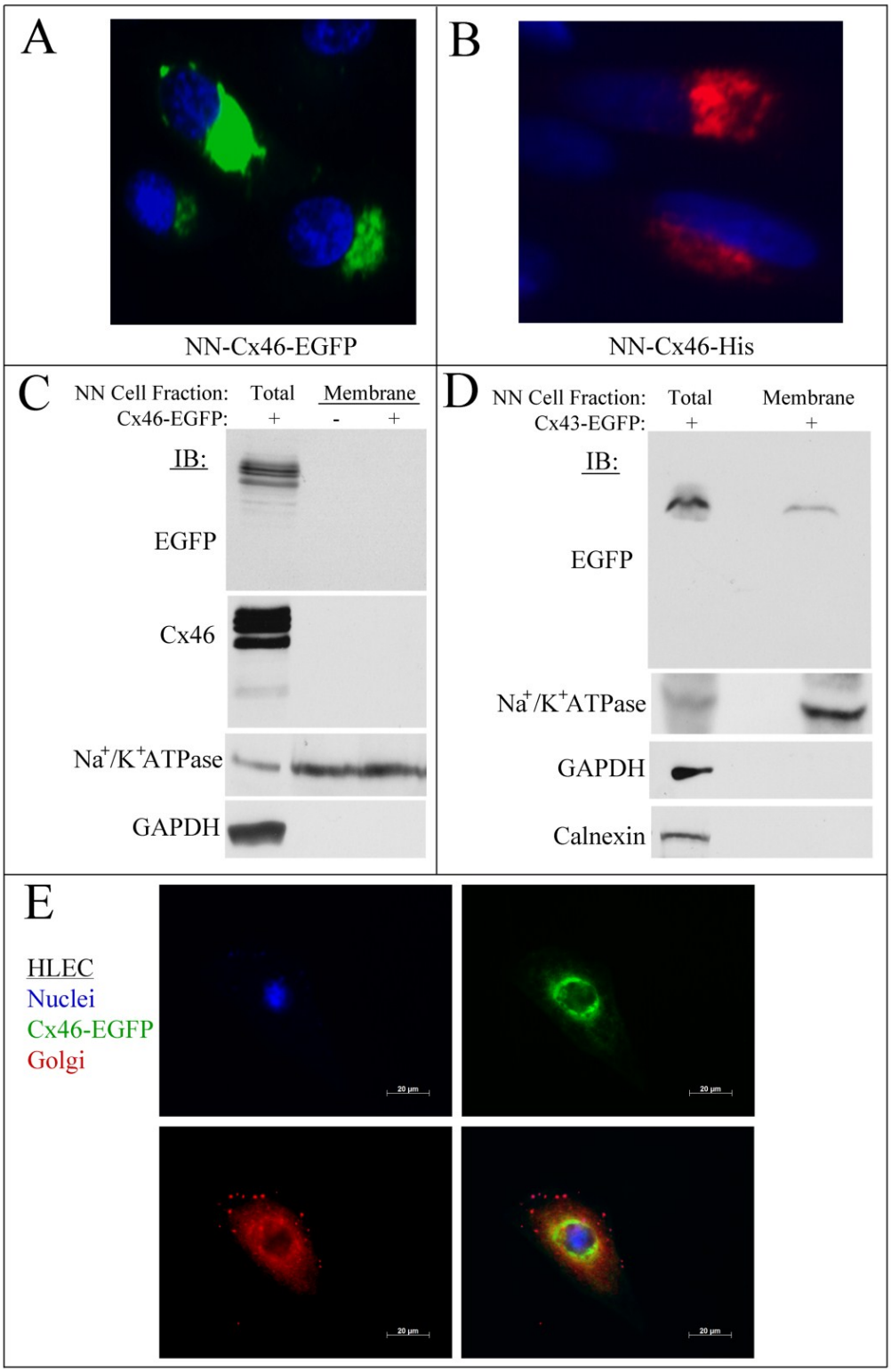

Figure 4.6 Cx46 Protein is Predominantly Localized to the Intercellular Compartments in Lens N/N1003A Cells

(A) Fluorescence microscopy images of NN1003A cells stably overexpressing Cx46-EGFP. All cells were fixed and stained with DAPI to visualize nuclei. (B) The localization of Cx46-His protein (penta-histidine-tagged) in

N/N1003A cells. Cells were transfected with plasmid encoding Cx46-His and, after 24 hr of transfection, cells were fixed, permeabilized, and labeled with penta-histidine antibody (red) and DAPI (blue). (C) Western blot analyses were performed for Cx46 (with anti-EGFP or anti-Cx46) on equal amounts of total protein from lysates of Cx46-EGFP-expressing cells (lane 1) and plasma membrane protein fractions isolated from untransfected control cells (lane 2) or Cx46-EGFP stable (lane 3) cells. The blots were also probed with antibodies against a plasma membrane marker Na^+/K^+ATPase, and a cytosolic marker (GAPDH) to demonstrate the purity of plasma membrane protein extracts. (D) Western blot analyses show the localization of Cx43-EGFP in the plasma membrane of Cx43-EGFP-expressing N/N1003A cells. Cells were transiently transfected with plasmid encoding Cx43-EGFP for 24 hr. Then, the cells were fractionated into plasma membrane protein fractions. Equal amounts of total protein of lysate and plasma membrane fractions of Cx43-EGFP expressing cells were subjected to Western blot analyses with antibodies against EGFP, Na^+/K^+ATPase, GAPDH, and calnexin (an ER marker). (E) Fluorescence microscopy images of HLEC cells expressing Cx46-EGFP (green) that were fixed, permeabilized, and labeled with 58K/FTCD antibody (red, a Golgi marker) and DAPI (blue). Cx46-EGFP did not co-localize well with the Golgi marker. NN: N/N1003A cells. Data in panels A-D were collected with Dr. Debarshi Banerjee.

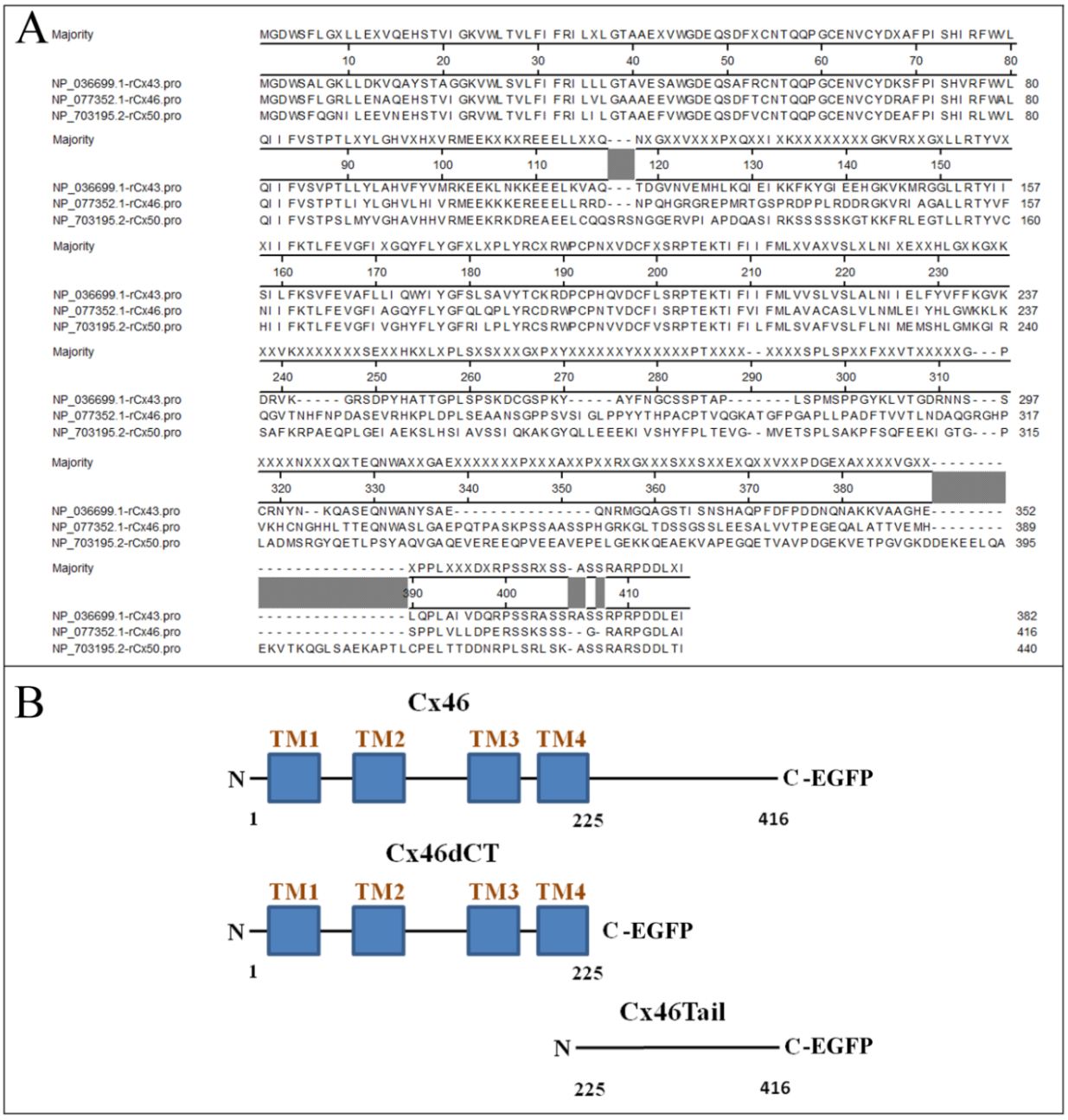

Figure 4.7 Protein Sequence Alignment of Rat Cx43, Cx46, and Cx50 and Schematic of Cx46 Mutants
(A) The amino acid residues of Cx43, Cx46, and Cx50 are given as a ClustalW alignment produced with Lasergene 9 MegAlign software (DNAStar, Inc., Madison, WI). Sequences are numbered according to the Cx46 sequence. The NCBI reference sequences used are NP_036699.1 for Cx43, NP_077352.1 for Cx46, and NP_703195.2 for Cx50. (B) Schematic diagram of wild-type Cx46 and Cx46 C-terminal deletion mutant (Cx46dCT, deletion of amino acids 225–416), as well as a schematic of the Cx46 tail domain mutant (Cx46Tail, deletion of amino acids 1–224).

The Carboxy-Terminal Tail Domain of Cx46 is Necessary and Sufficient for Induction of Cx43 Degradation

Primary amino acid sequence alignments of rat Cx43, Cx46, and Cx50 show significant sequence similarity at their N-terminal and transmembrane domains similar to that of all alpha-type connexins (48). The major variability in the amino acid sequences of Cx43, Cx46, and Cx50 are found at the cytoplasmic carboxy-terminal tail domain (Figure 4.7A). Since Cx50 overexpression was not found to mediate Cx43 degradation, we speculated whether the C-terminal tail domain (amino acids 225-416) is the active domain of Cx46 that induces Cx43 degradation. To test this hypothesis, we created two Cx46 mutants. One mutant contains the N-terminus and all four transmembrane domains (amino acids 1-225, named Cx46dCT). The other Cx46 mutant contains amino acids 225-416, which encode only the C-terminal tail domain (named Cx46Tail). Each mutant is expressed as an EGFP fusion protein, similar to Cx46-EGFP (Figure 4.7B).

Next, we transfected N/N1003A cells with either the plasmid encoding wild type Cx46-EGFP, Cx46dCT-EGFP, or Cx46Tail-EGFP, and then analyzed the effect of mutant Cx46 protein on the Cx43, Cx50, Na^+/K^+ATPase, and ZO-1 protein levels (Figure 4.8A-D). Cx46dCT-EGFP was expressed at the predicted molecular weight of ~50 kDa as determined by western blot using anti-EGFP antibody (Figure 4.8A). Notably, overexpression of Cx46dCT-EGFP did not induce Cx43 degradation at 24-48 hr after transfection (Figure 4.8A). The role of the C-terminal tail domain of Cx46 in inducing Cx43 degradation was confirmed by overexpressing Cx46Tail-EGFP. Overexpression of Cx46Tail-EGFP (Figure 4.8C), like wild type Cx46-EGFP (Figure 4.8B), was able to cause a reduction of Cx43 protein at 24 hr and 36 hr of transient transfection (Figure 4.8D). Additionally, Cx46Tail-EGFP exhibited no specific localization within HLECs as shown by fluorescence microscopy (Figure 4.8E). These data demonstrate that the carboxy-terminal cytoplasmic tail domain of Cx46 is responsible for inducing the degradation of Cx43 in lens cells. Similarly, Figure 4.9 shows that the degradation feature of Cx46 depends on its carboxy-terminal tail and that the effect is unique to only the Cx46 effect on Cx43 stability. Cx50 does not have this same effect. Figure 4.10 shows that each mutant expressed the desired product in the cells tested in this study.

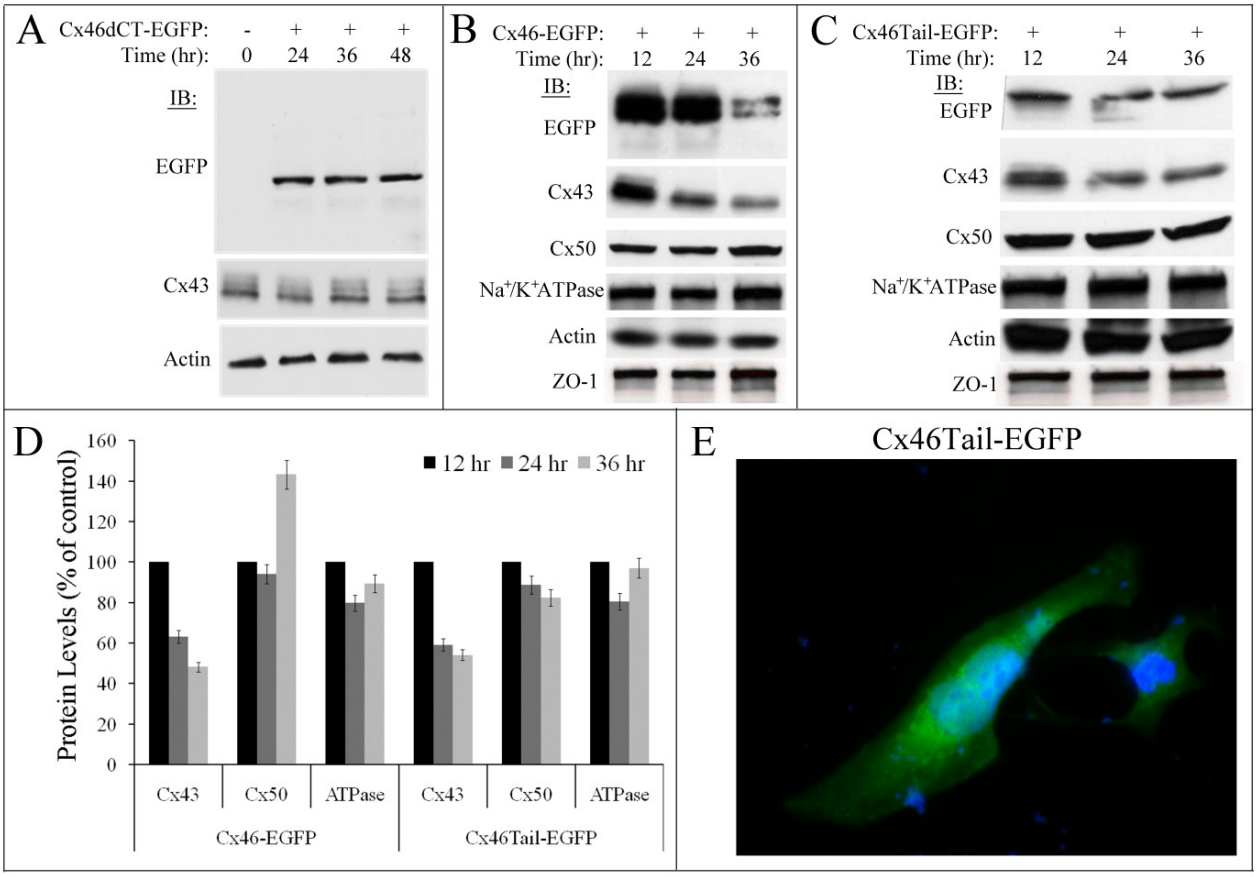

Figure 4.8 The C-Terminal Tail Domain of Cx46 is Required for Cx43 Degradation
(A) Over-expression of a Cx46 deletion mutant (Cx46dCT-EGFP) does not induce Cx43 degradation. Western blot analyses of Cx46dCT-EGFP and Cx43 in untransfected cells (0 hr) or in cells transiently transfected with plasmid encoding the Cx46 deletion mutant (Cx46dCT-EGFP). The expression of Cx46dCT-EGFP was confirmed by probing the blot with the EGFP antibody. The blot was also probed with Cx43CT and β-actin antibodies. (B) NN1003A cells were transiently transfected with full-length Cx46-EGFP, and whole cell lysates were prepared after 12, 24, and 36 hr post-transfection. Equal amounts of total protein were analyzed by 10% SDS-PAGE followed by immunoblot. The blots were probed with antibodies to detect EGFP, Cx43, Cx50, Na^+/K^+ATPase, and β-actin. There is no change in any of the protein levels of membrane-associated proteins Cx50 or Na^+/K^+ATPase, indicating that overexpression of Cx46-EGFP only affects Cx43 protein levels. (C) Over-expression of the Cx46 C-terminal tail domain (Cx46Tail-EGFP, residues 225–416 of wild type) in NN1003A cells caused reduction in Cx43 protein levels after 24 and 36 hr of transient transfection. This provides evidence that the CT tail domain of Cx46 induces the degradation of Cx43 through either direct or indirect interaction. (D) Densitometric analysis of Western blots used in *B* and *C*. Blots were digitized by UN-SCAN-It gel software. The average pixel value was calculated for all protein bands, normalized, and plotted in percent of control (β-actin). Data are represented as the mean of two independent experiments with S.E.M. shown. (E) Fluorescence microscopy of HLECs expressing Cx46Tail-EGFP. The tail domain did not localize to any specific intracellular compartment, confirming cytoplasmic expression and not membrane association.

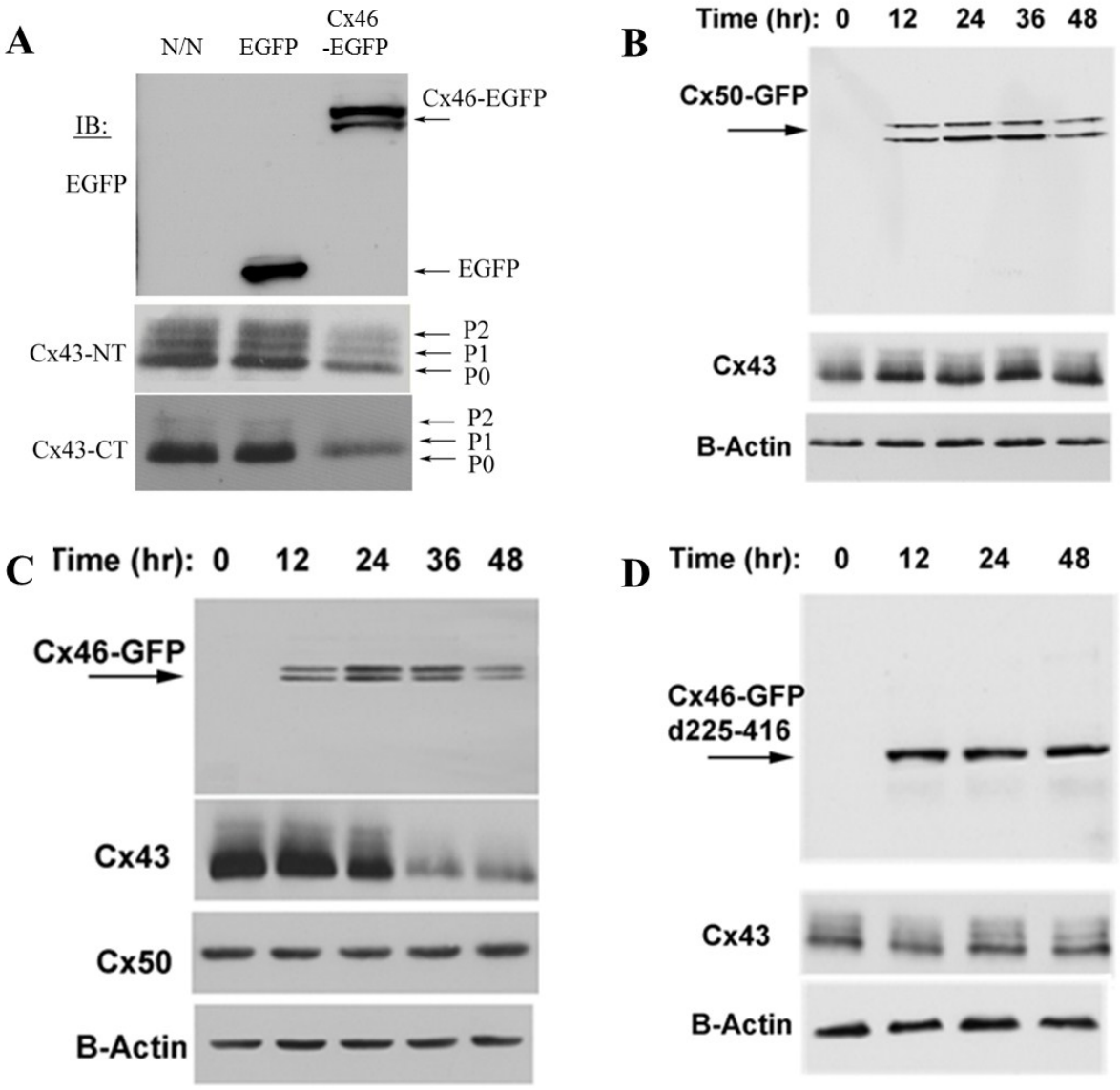

Figure 4.9 Effect of Cx46, Cx50 and the Cx46-Tail Deletion Mutant on Cx43 Protein Levels in N/N1003A Cells

(A) Immunoblot showing the Cx46 specific effect on reducing Cx43 protein levels with two different primary antibodies against Cx43. (B) Cx50 expression does not affect Cx43 protein expression over 48 hours. (C) Cx46 expression results in a reduction in the Cx43 protein levels while there was no change in Cx50 protein expression during the same experiment. (D) When the CT domain of Cx46 is deleted (Cx46d225-416), the reduction in Cx43 protein levels is abolished. This indicates that the minimum necessary domain for Cx43 interaction is the CT tail domain, which is predicted to be unstructured (an intrinsically disordered region).

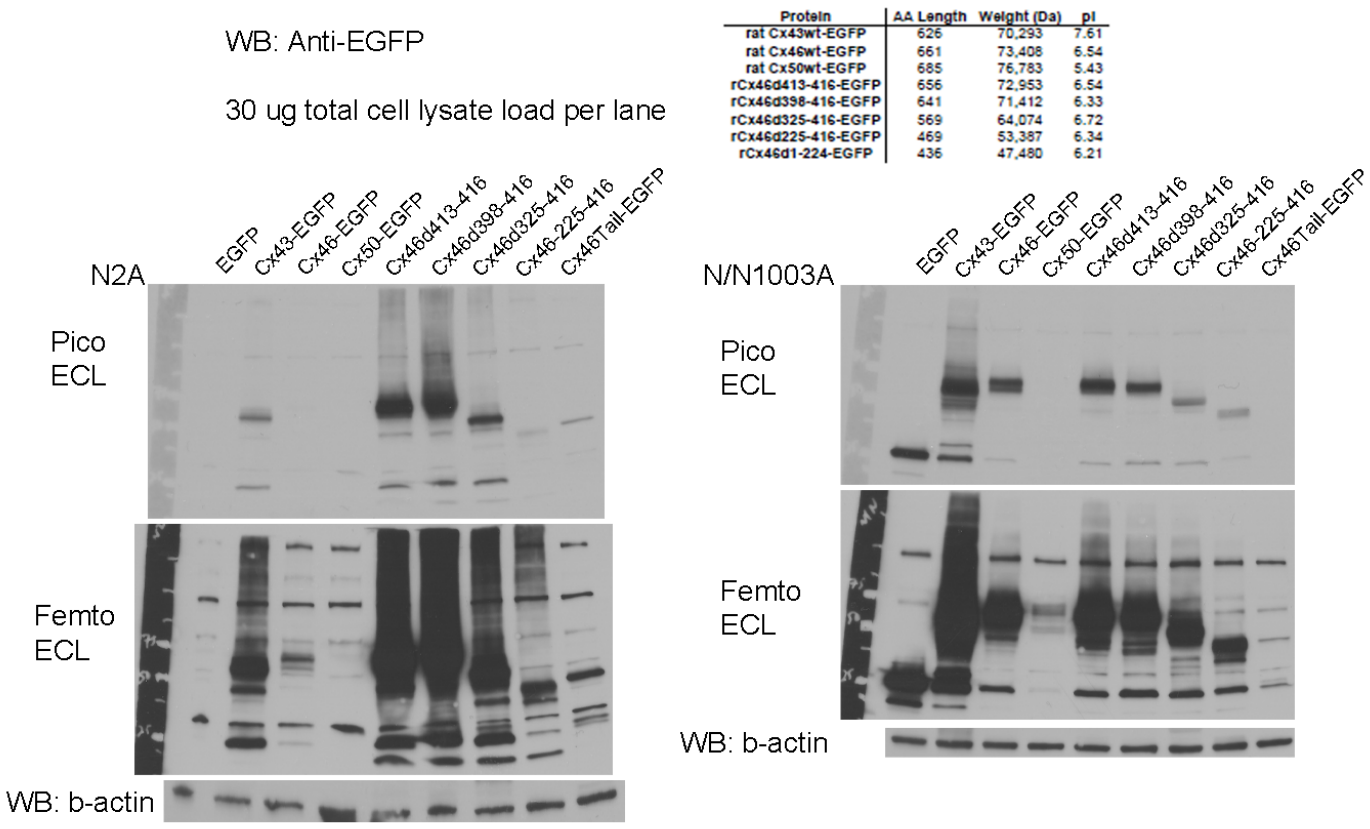

Figure 4.10 Western Blot of the Expression Pattern of Various Cx46 Tail Deletion Mutants
Cx46 mutants were expressed in mouse N2A and rabbit N/N1003A cells and western blotted for EGFP. This ensures each mutant is expressed although at various levels within each cell type.

Discussion

In the present study we investigated the reciprocal relationship between expression and turnover of two gap junction connexin proteins, Cx43 and Cx46, in lens epithelial cells. The use of vertebrate lens as a system to study gap junction communication and connexins function has been increasing in recent years. The developing lens also serves as a highly accessible paradigm for the universal study of cell proliferation, epithelial function, and cell differentiation, as well as studies of hypoxia. Here we show that TPA induces internalization and degradation of Cx43 in human lens epithelial cells. Previously, we have reported the association of PKCγ with Cx43 in response to TPA-treatment in HLECs (28, 37). TPA induced Cx43 hyper-phosphorylation and internalization from membranes has been well established in the cultured rat liver epithelial cell line IAR20 (31). In HLEC, TPA induced degradation of Cx43 was associated with an increase in phosphorylation of S368 of Cx43 and treatment with PKCγ inhibitor prevented the

phosphorylation at this residue and Cx43 degradation. Therefore our study shows that the PKCγ mediated phosphorylation of Cx43 on S368 acts as a critical signal for internalization and degradation (credit to Dr. Satyabrata Das for PKCγ work).

The reciprocal expression of Cx43 and Cx46 was further observed in rabbit lens epithelial cells overexpressing Cx46-EGFP. These cells exhibited a reduction of endogenous Cx43 protein levels. We confirmed the reduction of all three phospho-isoforms of Cx43 protein (P0, P1, and P2) by western blotting with antibodies against C-terminal and N-terminal domains. This precaution aids in ruling out the possibility that proteolytic cleavage of Cx43 epitopes may be contributing to the reduction of Cx43 observed via western blotting.

Several studies have previously reported the involvement of both proteasomal and lysosomal pathways in the degradation of connexins (4, 43-45). The lysosomal pathway was shown not to be involved in the degradation of Cx43 when it is internalized from cell membranes. Treatment with lysosome inhibitors leupeptin and chloroquine showed no effect on the degradation of Cx43 in Cx46-EGFP expressing cells. But, the treatment with proteasome inhibitors ALLN and MG132 strongly counteracted Cx46-induced Cx43 degradation. In addition, co-immunoprecipitation analyses showed an increase in Cx43 ubiquitination associated only with Cx46-EGFP expressing cells. Together, these results suggest that Cx46-induced degradation of Cx43 is mediated by the ubiquitin-proteasome pathway while the lysosome degradation pathway does not appear to be involved (credit to Dr. Debarshi Banerjee for degradation pathway work).

In this study, we observed that Cx46 is mainly localized to the intracellular compartments of lens epithelial cells. Even upon overexpression of Cx46 or after TPA treatment, the localization of Cx46 was not detected in the plasma membrane, as determined by confocal immunofluorescence and EGFP-intrinsic fluorescence microscopy. This was further confirmed by western blot analyses of cellular fractions. The intracellular localization of Cx46 was also shown in bone osteoblast (39) and lung alveolar cells (40). Conventional techniques such as fluorescence microscopy and western blots do not completely rule out the possibility of membrane localization of Cx46 in our lens cultures because Cx46 has a major membrane-localized function *in vivo* in the lens. Connexins have rapid turnover rates with half-lives of a few hours (50-52). Therefore, if Cx46 is rapidly internalized after being delivered to the plasma membrane, it would not be detectable at the cell surface by conventional techniques.

Nonetheless, since membrane localization was not observed, it is unlikely that overexpression of Cx46 induced Cx43 degradation by facilitating loss from the plasma membrane.

Due to its observed intracellular localization, Cx46 is likely to induce Cx43 degradation by a gap junction independent mechanism. This leads us to speculate two possibilities for the degradation of Cx43. One, that endoplasmic reticulum associated degradation (ERAD) mediates the Cx46-induced degradation of ubiquitinated Cx43 species observed in our studies (either folded or unfolded Cx43). Or two, Cx46 associates with Cx43 either directly or indirectly in the ER prior to transit to the cellular membrane and induces the degradation of Cx43 outside of the ER. Nonetheless, these hypotheses need to be thoroughly tested.

Additionally, the Cx46dCT-EGFP mutant failed to induce Cx43 degradation. However overexpression of the Cx46Tail-EGFP mutant, which localizes to the cytoplasm, caused a reduction of Cx43 protein levels. This is interesting because the Cx46Tail mutant does not contain the membrane anchoring signals found in the amino-terminus of Cx46, yet it can still trigger degradation of endogenous Cx43. It is possible that Cx46Tail stimulates ERAD of Cx43 during translation but this does not rule out potential Cx46Tail interactions with Cx43 at the plasma membrane. Therefore, it is likely that Cx46 mediates its action through putative binding partners at the C-terminal tail domain. The cytoplasmic tail domain of Cx46 has predicted binding sites for various kinases, such as PKCγ, CK-1, Akt/PKB, and MAPK, among others. It is also predicted to be rather unstructured, so it could take on altered functional conformations depending upon its binding partner. Further studies with mutant Cx46 proteins that are defective in phosphorylation sites or kinase binding sites are required to unravel the mechanism of Cx43 degradation upon Cx46 overexpression. Studies elucidating the roles of various cellular compartments aiding in the interaction between Cx43 and Cx46 would also provide insight into which kinases or other binding proteins are involved in the reciprocal relationship.

Our results can be extended to understand the molecular mechanism of connexin-influenced regulation of oncogenesis. Cx43 has anti-tumor properties and expression of Cx43 can be down-regulated in tumors (22-25). Moreover, in previous studies, we have shown that Cx46 is up-regulated in breast tumor samples, MCF-7 and Y79 retinoblastoma xenografts, and acts as an oncogene to favor early tumor growth (24, 25). Therefore, we speculate that up-regulation of Cx46 promotes tumor growth by inducing the degradation of the tumor suppressor protein Cx43. Indeed, in a recent study we have found that the downregulation of Cx46 by

siRNA inhibited Y79 retinoblastoma xenograft tumor growth which was associated, interestingly, with an increase in Cx43 protein levels *in vivo* (25).

In conclusion, our data indicate that Cx43 and Cx46 are expressed and regulated in a reciprocal manner in lens epithelial cells. Depletion of Cx43 is associated with an upregulation of Cx46 and both processes are mediated by PKC. We also provide evidence that endogenous Cx43 regulates endogenous Cx46 at the protein level. Exogenous expression of Cx46, on the other hand, induces degradation of Cx43. The C-terminal tail domain of Cx46, which has less sequence similarity with other connexins, is required to induce degradation of Cx43. The ubiquitination of Cx43 is strongly increased in Cx46 over-expressing cells and proteasome inhibitors counteract Cx43 degradation. Therefore, Cx46-induced Cx43 degradation is likely to be mediated via the ubiquitin-proteasome pathway in the perinuclear region of lens cells, although the exact cellular mechanism warrants further characterization.

Acknowledgements

This work was supported in part by funds provided by grants NIH EY13421 to D.J.T., Terry C. Johnson Cancer Center Innovative Research Grant to D.J.T., and Terry C. Johnson Cancer Center Summer Stipend to D.B. and S.D., and NIH K-INBRE grant P20RR016475 to D.M. Thank you to the Kansas State University Research Foundation for the Doctoral Scholarship provided to S.A.M.

References

1. Paul DL, Ebihara L, Takemoto LJ, Swenson KI, Goodenough DA. Connexin46, a novel lens gap junction protein, induces voltage-gated currents in nonjunctional plasma membrane of xenopus oocytes. J Cell Biol. 1991 Nov; 115(4):1077-89.

2. Goodenough DA, Goliger JA, Paul DL. Connexins, connexons, and intercellular communication. Annu Rev Biochem. 1996; 65:475-502.

3. Loewenstein WR. Junctional cell-to-cell communication and growth control. Ann N Y Acad Sci. 1980; 339:39-45.

4. Yamasaki H, Naus CC. Role of connexin genes in growth control. Carcinogenesis. 1996 Jun; 17(6):1199-213.

5. Laird DW. Life cycle of connexins in health and disease. Biochem J. 2006 Mar 15; 394(Pt 3):527-43.

6. Salameh A. Life cycle of connexins: Regulation of connexin synthesis and degradation. Adv Cardiol. 2006; 42:57-70.

7. Brissette JL, Kumar NM, Gilula NB, Dotto GP. The tumor promoter 12-O-tetradecanoylphorbol-13-acetate and the ras oncogene modulate expression and phosphorylation of gap junction proteins. Mol Cell Biol. 1991 Oct; 11(10):5364-71.

8. Lampe PD. Analyzing phorbol ester effects on gap junctional communication: A dramatic inhibition of assembly. J Cell Biol. 1994 Dec; 127(6 Pt 2):1895-905.

9. Bevans CG, Harris AL. Regulation of connexin channels by pH. direct action of the protonated form of taurine and other aminosulfonates. J Biol Chem. 1999 Feb 5; 274(6):3711-9.

10. Trexler EB, Bukauskas FF, Bennett MV, Bargiello TA, Verselis VK. Rapid and direct effects of pH on connexins revealed by the connexin46 hemichannel preparation. J Gen Physiol. 1999 May; 113(5):721-42.

11. Lampe PD, Lau AF. The effects of connexin phosphorylation on gap junctional communication. Int J Biochem Cell Biol. 2004 Jul; 36(7):1171-86.

12. Musil LS, Beyer EC, Goodenough DA. Expression of the gap junction protein connexin43 in embryonic chick lens: Molecular cloning, ultrastructural localization, and post-translational phosphorylation. J Membr Biol. 1990 Jun; 116(2):163-75.

13. Dahm R, van Marle J, Prescott AR, Quinlan RA. Gap junctions containing alpha8-connexin (MP70) in the adult mammalian lens epithelium suggests a re-evaluation of its role in the lens. Exp Eye Res. 1999 Jul; 69(1):45-56.

14. Rong P, Wang X, Niesman I, Wu Y, Benedetti LE, Dunia I, et al. Disruption of Gja8 (alpha8 connexin) in mice leads to microphthalmia associated with retardation of lens growth and lens fiber maturation. Development. 2002 Jan; 129(1):167-74.

15. TenBroek EM, Johnson R, Louis CF. Cell-to-cell communication in a differentiating ovine lens culture system. Invest Ophthalmol Vis Sci. 1994 Jan; 35(1):215-28.

16. White TW, Bruzzone R, Goodenough DA, Paul DL. Mouse Cx50, a functional member of the connexin family of gap junction proteins, is the lens fiber protein MP70. Mol Biol Cell. 1992 Jul; 3(7):711-20.

17. Mathias RT, White TW, Gong X. Lens gap junctions in growth, differentiation, and homeostasis Physiol Rev. 2010 Jan; 90(1):179-206.

18. Beyer EC, Kistler J, Paul DL, Goodenough DA. Antisera directed against connexin43 peptides react with a 43-kD protein localized to gap junctions in myocardium and other tissues. J Cell Biol. 1989 Feb; 108(2):595-605.

19. Gong X, Li E, Klier G, Huang Q, Wu Y, Lei H, et al. Disruption of alpha3 connexin gene leads to proteolysis and cataractogenesis in mice. Cell. 1997 Dec 12; 91(6):833-43.

20. Lee SW, Tomasetto C, Paul D, Keyomarsi K, Sager R. Transcriptional downregulation of gap-junction proteins blocks junctional communication in human mammary tumor cell lines. J Cell Biol. 1992 Sep; 118(5):1213-21.

21. Hirschi KK, Xu CE, Tsukamoto T, Sager R. Gap junction genes Cx26 and Cx43 individually suppress the cancer phenotype of human mammary carcinoma cells and restore differentiation potential. Cell Growth Differ. 1996 Jul; 7(7):861-70.

22. Kanczuga-Koda L, Sulkowska M, Koda M, Reszec J, Famulski W, Baltaziak M, et al. Expression of connexin 43 in breast cancer in comparison with mammary dysplasia and the normal mammary gland. Folia Morphol (Warsz). 2003 Nov; 62(4):439-42.

23. Shao Q, Wang H, McLachlan E, Veitch GI, Laird DW. Down-regulation of Cx43 by retroviral delivery of small interfering RNA promotes an aggressive breast cancer cell phenotype. Cancer Res. 2005 Apr 1; 65(7):2705-11.

24. Banerjee D, Gakhar G, Madgwick D, Hurt A, Takemoto D, Nguyen TA. A novel role of gap junction connexin46 protein to protect breast tumors from hypoxia. Int J Cancer. 2010 Aug 15; 127(4):839-48.

25. Burr DB, Molina SA, Banerjee D, Low DM, Takemoto DJ. Treatment with connexin 46 siRNA suppresses the growth of human Y79 retinoblastoma cell xenografts *in vivo*. Exp Eye Res. 2011 Apr; 92(4):251-9.

26. Hombach S, Janssen-Bienhold U, Sohl G, Schubert T, Bussow H, Ott T, et al. Functional expression of connexin57 in horizontal cells of the mouse retina. Eur J Neurosci. 2004 May; 19(10):2633-40.

27. Kelsell DP, Dunlop J, Stevens HP, Lench NJ, Liang JN, Parry G, et al. Connexin 26 mutations in hereditary non-syndromic sensorineural deafness. Nature. 1997 May 1; 387(6628):80-3.

28. Akoyev V, Takemoto DJ. ZO-1 is required for protein kinase C gamma-driven disassembly of connexin 43. Cell Signal. 2007 May; 19(5):958-67.

29. Solan JL, Lampe PD. Connexin43 phosphorylation: Structural changes and biological effects. Biochem J. 2009 Apr 15; 419(2):261-72.

30. Zampighi GA, Planells AM, Lin D, Takemoto D. Regulation of lens cell-to-cell communication by activation of PKCgamma and disassembly of Cx50 channels. Invest Ophthalmol Vis Sci. 2005 Sep; 46(9):3247-55.

31. Leithe E, Rivedal E. Ubiquitination and down-regulation of gap junction protein connexin-43 in response to 12-O-tetradecanoylphorbol 13-acetate treatment. J Biol Chem. 2004 Nov 26; 279(48):50089-96.

32. Rivedal E, Yamasaki H, Sanner T. Inhibition of gap junctional intercellular communication in syrian hamster embryo cells by TPA, retinoic acid and DDT. Carcinogenesis. 1994 Apr; 15(4):689-94.

33. Matesic DF, Rupp HL, Bonney WJ, Ruch RJ, Trosko JE. Changes in gap-junction permeability, phosphorylation, and number mediated by phorbol ester and non-phorbol-ester tumor promoters in rat liver epithelial cells. Mol Carcinog. 1994 Aug; 10(4):226-36.

34. Musil LS, Cunningham BA, Edelman GM, Goodenough DA. Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and -deficient cell lines. J Cell Biol. 1990 Nov; 111(5 Pt 1):2077-88.

35. Musil LS, Goodenough DA. Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques. J Cell Biol. 1991 Dec; 115(5):1357-74.

36. Lampe PD, Lau AF. Regulation of gap junctions by phosphorylation of connexins. Arch Biochem Biophys. 2000 Dec 15; 384(2):205-15.

37. Akoyev V, Das S, Jena S, Grauer L, Takemoto DJ. Hypoxia-regulated activity of PKCepsilon in the lens. Invest Ophthalmol Vis Sci. 2009 Mar; 50(3):1271-82.

38. Das S, Wang H, Molina SA, Martinez-Wittinghan FJ, Jena S, Bossmann LK, et al. PKCgamma, role in lens differentiation and gap junction coupling. Curr Eye Res. 2011 Jul; 36(7):620-31.

39. Koval M, Harley JE, Hick E, Steinberg TH. Connexin46 is retained as monomers in a trans-golgi compartment of osteoblastic cells. J Cell Biol. 1997 May 19; 137(4):847-57.

40. Avanzo JL, Mesnil M, Hernandez-Blazquez FJ, da Silva TC, Fukumasu H, Mori CM, et al. Altered expression of connexins in urethane-induced mouse lung adenomas. Life Sci. 2006 Nov 2; 79(23):2202-8.

41. Paemeleire K, Martin PE, Coleman SL, Fogarty KE, Carrington WA, Leybaert L, et al. Intercellular calcium waves in HeLa cells expressing GFP-labeled connexin 43, 32, or 26. Mol Biol Cell. 2000 May; 11(5):1815-27.

42. Thomas T, Jordan K, Simek J, Shao Q, Jedeszko C, Walton P, et al. Mechanisms of Cx43 and Cx26 transport to the plasma membrane and gap junction regeneration. J Cell Sci. 2005 Oct 1; 118(Pt 19):4451-62.

43. Laing JG, Tadros PN, Westphale EM, Beyer EC. Degradation of connexin43 gap junctions involves both the proteasome and the lysosome. Exp Cell Res. 1997 Nov 1; 236(2):482-92.

44. Laing JG, Beyer EC. The gap junction protein connexin43 is degraded via the ubiquitin proteasome pathway. J Biol Chem. 1995 Nov 3; 270(44):26399-403.

45. Qin H, Shao Q, Igdoura SA, Alaoui-Jamali MA, Laird DW. Lysosomal and proteasomal degradation play distinct roles in the life cycle of Cx43 in gap junctional intercellular communication-deficient and -competent breast tumor cells. J Biol Chem. 2003 Aug 8; 278(32):30005-14.

46. Leithe E, Rivedal E. Ubiquitination of gap junction proteins. J Membr Biol. 2007 Jun; 217(1-3):43-51.

47. White TW, Bruzzone R, Wolfram S, Paul DL, Goodenough DA. Selective interactions among the multiple connexin proteins expressed in the vertebrate lens: The second extracellular domain is a determinant of compatibility between connexins. J Cell Biol. 1994 May; 125(4):879-92.

48. Li GY, Lin HH, Tu ZJ, Kiang DT. Gap junction Cx26 gene modulation by phorbol esters in benign and malignant human mammary cells. Gene. 1998 Mar 16; 209(1-2):139-47.

49. Fallon RF, Goodenough DA. Five-hour half-life of mouse liver gap-junction protein. J Cell Biol. 1981 Aug; 90(2):521-6.

50. Beardslee MA, Laing JG, Beyer EC, Saffitz JE. Rapid turnover of connexin43 in the adult rat heart. Circ Res. 1998 Sep 21; 83(6):629-35.

51. Herve JC, Derangeon M, Bahbouhi B, Mesnil M, Sarrouilhe D. The connexin turnover, an important modulating factor of the level of cell-to-cell junctional communication: Comparison with other integral membrane proteins. J Membr Biol. 2007 Jun; 217(1-3):21-33.

Chapter 5 - Summary and Conclusions of Work Presented

In summary, this work has provided insights into why Cx46 is unique to hypoxic environments. I have shown that Cx46 protein up-regulation is associated with hypoxic environments and that Cx43 is negatively affected by the CT domain of Cx46. I have also shown that Cx46 ablation is an effective anti-tumor growth therapy with the mechanism of action correlating with an up-regulation of anti-tumor Cx43 protein. Additionally, I have provided insight into the hypoxic regulation of Cx46 at the gene level which suggests that Cx46 is tightly controlled in a tissue and time-dependent manner.

This work has led me to uncover an additional biological role of Cx46. The protein seems to be important to mitigate the hypoxic insults encountered in a low oxygen environment. While it is nearly impossible to detect and measure the type of molecules that traverse individual pores with the current state of channel technology, one can suggest that metabolites, both basic and toxic, are passed freely between cells connected with Cx46 gap junctions. This is inferred from the lens where it is avascular and hypoxic in nature. Cx46 comprises the majority of functional gap junction channels in the mature fiber cells and loss of function results in cataract by enhanced oxidation and degradation of structural proteins such as crystallins. Cx46 channel function is critical to prevent oxidation-based cataracts. This may be true in solid tumors as well where Cx46 becomes up-regulated in the hypoxic environment of the tumor.

We propose that the function of Cx46 in hypoxic tissues is related to the hypoxic growth state of the tumor, where Cx46 functions as a molecular portal in which necessary metabolites and molecular signals are shared more efficiently than metabolite paracellular diffusion and uptake. Prevention of Cx46 mRNA translation by siRNA treatment in solid tumors results in slow growing or even halted tumor growth, suggesting that Cx46 channels are needed for expansion of the tumor mass. Once vascularization occurs, Cx46 channels no longer would be needed but may persist due to tumor transcription factor expression. This certainly can be the case since many lens crystallins are expressed in solid tumors, as discussed in the introduction of this dissertation. This hypothesis needs to be tested further to confirm.

The mechanism of action we propose in the lens and tumor follow similar patterns. Figure 5.1 demonstrates the mechanism in schematic detail. As Cx46 becomes up-regulated by the increasing hypoxic environment, Cx43 expression halts and the protein becomes

degraded by perinuclear (most likely Golgi) interactions with Cx46 protein in a ubiquitin-dependent manner. In the cytoplasm, Cx46 may interact with Cx43 at the plasma membrane, as suggested by the CT domain experiments. The Cx46 tail domain may bring ubiquitin ligases specific for Cx43 to the gap junction plaque, therefore enhancing the Cx43 ubiquitination effect and subsequent degradation.

Of note is the natural pattern of connexin expression in the lens where Cx50 is expressed in all regions whereas Cx43 is only in the oxygenated epithelia and Cx46 is the hypoxic fiber cell region. We believe Cx43 is affected by the increase in Cx46 by the transitioning transcription factor expression present in the differentiating and hypoxic fiber cells. Cx46 could be upregulated in this area of the lens where Cx43 expression could be halted and the remaining Cx43 protein being affected by the Cx46 protein. Cx46 persists into the hypoxic lens where Cx43 protein is not found.

Therefore, we present a mechanism where induction of Cx46 is concurrent with the halting of Cx43 production and where Cx46 is responsible for the enhanced destruction of Cx43 gap junction plaques in lens cells. Cx46 functions as an important channel molecule for hypoxic survival and expression of Cx46 can be turned on in hypoxic environments when it is most needed to perform similar to vasculature.

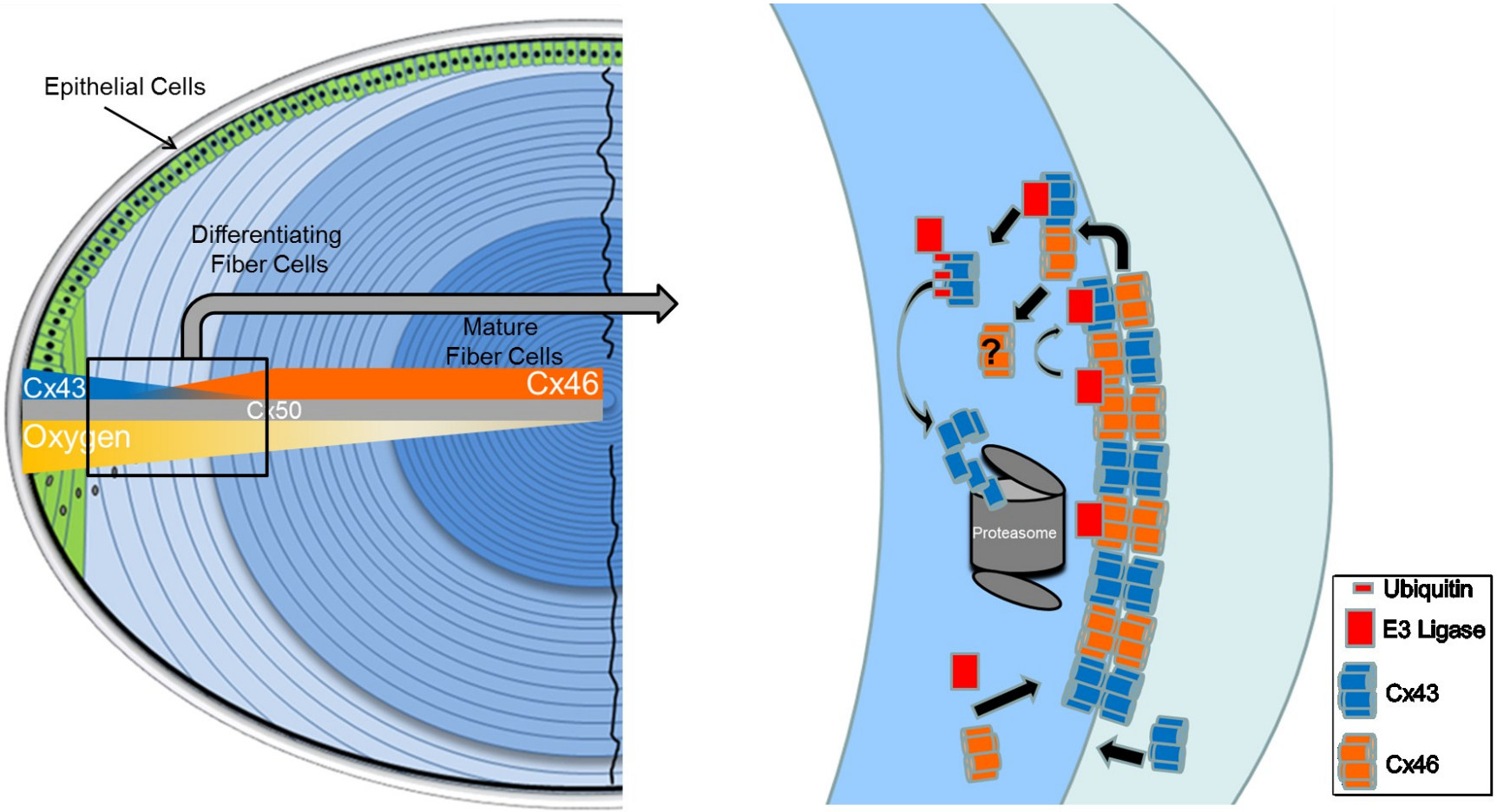

Figure 5.1 Proposed Regulatory Mechanism of Action between Cx43 and Cx46 in the Lens
Cx43 is degraded by Cx46 protein interaction at the ER/Golgi and the cellular membrane in gap junction plaques. Degradation is proposed to occur via the endoplasmic reticulum associated degradation (ERAD) scheme and by enhanced Cx43 gap junction destruction by ubiquitin signals. This is proposed to occur in solid tumors as well as in the lens where this mechanism can explain the connexin protein patterning that was observed during our studies.

Abbreviations Used

Standard biochemical and molecular biology abbreviations assumed.

	A
AT	Amino-terminal
	B
bHLH	basic helix-loop-helix protein domain
	C
CK1	Casein kinase 1
CT domain	Carboxy-terminal domain of each connexin; sequence after TM4
CMTX	Charcot-Marie-Tooth disease, X-linked
CMV	Cytomegalovirus
CNS	Central nervous system
Cx	Connexin protein
	D/E
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated degradation
EL	Extracellular loop
	F
FITC	Fluorescein isothiocyanate
	G
GJ	Gap junction
GJIC	Gap junction intracellular communication
GPCR	G-protein coupled receptor
	H
HIF	Hypoxia-inducible factor
HLEC	Human lens epithelial cell
HRE	Hypoxia response element
Hyp	Hypoxia

I

IRES	Internal ribosome entry site
IL	Intracellular loop

J/K/L/M/N

N2A	Mouse neuro2A cell
N/N1003A	Rabbit lens epithelial cell
Norm	Normoxia
NT	Amino-terminal domain

O

ODDD	Oculodentodigital dysplasia, malformation
OFZ	Organelle-free zone

P

PAS	Per-Arnt/AhR-Sim-like protein domain
PET	Positron emission tomography
PGE_2	Prostaglandin E2
PKCγ	Protein kinase C, gamma isoform
PM	Plasma membrane

Q/R

RACE	Rapid amplification of cDNA ends
ROS	Reactive oxygen species – superoxide, peroxides, free radicals

S/T

TCA	Tricarboxylic acid
TGN	Trans-golgi network
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TM	Transmembrane
TSS	Transcription start site

U

UTR	Untranslated region

V/W/X/Y/Z

WB	Western blot