INVESTIGATION OF NOVEL FUNCTIONS OF A GAP JUNCTION PROTEIN, CONNEXIN46

by

DEBARSHI BANERJEE

M.Sc., University of Calcutta, West Bengal, India, 2005

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Biochemistry
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2010

Abstract

Connexin proteins are the principle structural components of gap junction channels that connect the cytoplasm of two cells and maintain direct intercellular communication through the exchange of ions, small molecules and cellular metabolites. Colocalization and tissue-specific expression of diverse connexin molecules are reported to occur in a variety of organs. Impairment of gap junctional intercellular communication, caused by mutations, gain of function or loss of function of connexins, is involved in a number of diseases including the development of cancer. Here the functions of a gap junction protein, connexin46 (Cx46), have been investigated in two hypoxic tissues, lens and breast tumor. We show that human breast cancer cells, MCF-7 and breast tumor tissues express connexin46 (Cx46) and it plays a critical role in protecting cells against hypoxia-induced death. Interestingly, I find that Cx46 is upregulated in MCF-7 breast cancer cells and human breast cancer tumors. Downregulation of Cx46 by siRNA promotes cell death of human lens epithelial cells (HLEC) and MCF-7 cells under hypoxic conditions. Furthermore, direct injection of anti-Cx46 siRNA into xenograft tumors prevents tumor growth in nude mice. Our result suggests that both normal hypoxic tissue (lens) and adaptive hypoxic tissue (breast tumor) utilize the same protein, Cx46, as a protective strategy against hypoxia.

In the last part of the dissertation, we show that overexpression of Cx46 induces the degradation of another connexin, connexin43, in rabbit lens epithelial NN1003A cells. Overexpression of Cx46 increases ubiquitination of Cx43. Moreover, the Cx46-induced Cx43 degradation is counteracted by inhibitors of proteasome. Taken together, these data indicate that the degradation of Cx43, upon Cx46 overexpression, is mediated by the ubiquitin-proteasome pathway. I also provide evidence that that C-terminal tail of Cx46 is essential to induce degradation of Cx43. Therefore, our study shows that Cx46 has a novel function in the regulation of Cx43 turnover in addition to its conventional role as a gap junction protein. This may contribute to protection from hypoxia in both the lens and tumors.

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In the last part of the dissertation, I show that overexpression of Cx46 induces the degradation of another connexin, connexin43, in rabbit lens epithelial NN1003A cells. Overexpression of Cx46 increases ubiquitination of Cx43. Moreover, the Cx46-induced Cx43 degradation is counteracted by inhibitors of proteasome. Taken together, these data indicate that the degradation of Cx43, upon Cx46 overexpression, is mediated by the ubiquitin-proteasome pathway. I also provide evidence that that C-terminal tail of Cx46 is essential to induce degradation of Cx43. Therefore, our study shows that Cx46 has a novel function in the regulation of Cx43 turnover in addition to its conventional role as a gap junction protein. This may contribute to protection from hypoxia in both the lens and tumors.

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Dedication

To my wonderful parents Prashanta Kumar Banerjee and Supriya Banerjee and other family members, Debajyoti, Rituparna, Debaditya, Debadrita and Debjani.

CHAPTER 1 - Introduction and Literature Review

1.1. Introduction

The animal body is a dynamic organism comprised of cells, tissues and organs that act in a highly coordinated fashion to maintain cellular homeostasis. In a multicellular organism, homeostasis is regulated by extracellular and intracellular communication processes involving hormones, growth factors, cytokines, and direct cell-to-cell contacts or cell junctions (Trosko and Ruch 1998). Cell junctions can be those that link cells together or those that link cells to the extracellular matrix. Cell junctions, connecting two adjacent cells, are tight junctions, adherent junctions and gap junctions. While tight junction connects cells at the apical surface, gap junctions are present at the basolateral surface (Shin, Fogg et al. 2006). Gap junctions can pass helpful or harmful signals to the neighboring cells and gap junction mediated communications play critical roles in normal physiological processes such as cell growth, differentiation and proliferation and in diseased processes including carcinogenesis, lens cataract formation, heart arrhythmia and many more (Loewenstein 1979; Trosko and Ruch 1998; Yamasaki, Krutovskikh et al. 1999; Laird 2006). In the dissertation, I document my research that explores the novel functions of a gap junction protein, connexin 46, in two hypoxic tissues, vertebrate lens and breast tumor. Since connexin46 belongs to the family of gap junction connexin proteins, I will review the connexins including their expression, structure, synthesis and degradation, in Chapter 1 of the dissertation, in detail. I will also review the connexins, including connexin46, which are expressed in lens and breast tumor. Hypoxic properties of lens and breast tumor will be reviewed in this chapter. In Chapter 2, a role of connexin46 protecting lens

and breast tumor from hypoxia is presented and in Chapter 3, the function of connexin46 in the degradation of another gap junction connexin, connexin 43, is presented.

1.2. Gap Junctions

Gap junctions are intercellular membrane channels that maintain direct intercellular communication through the exchange of ions, small molecules and cellular metabolites between neighboring cells (Goodenough, Goliger et al. 1996; Harris 2001). Gap junction channels are formed at the basolateral surfaces of two cells and connect directly to their cytoplasms. The cell-to-cell communication through gap junctions is known as gap junctional intercellular communication (GJIC), proper functioning of which is very important to maintain tissue homeostasis. For example, vertebrate lens is an avascular tissue and therefore GJIC is the major process, along with diffusion, by which lens epithelial and fiber cells maintain intercellular homeostasis (Mathias, White et al. 2010).

1.2.1. Gap Junctions: Structure of Connexin

One gap junction channel is composed of two hemichannels or a connexon (Goodenough 1976; Kumar and Gilula 1992). Each connexon, in turn, is formed through the hexameric oligomerization of proteins called connexins (Figure 1.1). Connexins are the multigene family of transmembrane proteins and they are the structural unit of gap junctions. So far, 21 connexin isoforms have been identified in humans (Sohl and Willecke 2004). Each of these connexin isoforms constitutes four hydrophobic

transmembrane helices, two extracellular loops (EL-1 and EL-2), a cytoplasmic loop (CL) and a carboxyl terminal (CT) and a amino terminal (AT) end, both termini are located at the cytoplasmic side (Milks, Kumar et al. 1988; Laird and Revel 1990; Zhang and Nicholson 1994; Sosinsky and Nicholson 2005) (Figure 1.1). All of the connexin isoforms show highly conserved sequence similarities within the four transmembrane domains, two extracellular loops and amino- terminal end (AT). Therefore, a highest degree of sequence diversity is seen mainly in the sequence and length of C-terminal ends (CT) and cytoplasmic loops (CL). The extracellular EL-1 and EL-2 are the most conserved residues and they are required for proper docking interaction of the hemichannels from two adjacent cells for the channel (gap junction) to form (Zhang and Nicholson 1994).

Connexons (hemichannels), from adjacent cells, can interact with each other via several ways (Sosinsky 1995; Jiang and Goodenough 1996; Desplantez, Halliday et al. 2004). If the hemichannel is composed of a single connexin isoform it is called homomeric. Heteromeric hemichannles are formed through the association of at least two different connexin isoforms. Homotypic channels are formed by the interaction of two identical homomeric hemichannels; in turn, when two different homomeric hemichannels interact to form a gap junction channel, it is known as a heterotypic channel.

Gap junction channels are aqueous channels that are permeable to several small ions including Ca^{2+}, small metabolites such as ATP, ADP, IP_3, sugars and small proteins with molecular weight less than 1KDa (Bennett and Verselis 1992; Wang, Lee et al. 2001; Bukauskas and Verselis 2004). The permeability properties shown by all

connexin channels differ and depend on the connexin isoforms that are expressed in the cell (Elfgang, Eckert et al. 1995; Goldberg, Valiunas et al. 2004; Kanaporis, Mese et al. 2008). The permeability of channels also alters in response to ions, voltage, pH, phosphorylation and other post-translational modifications (Mathias, Rae et al. 1997; Eckert 2002). For example, phosphorylation of connexin43 by the protein kinase C enzyme leads to channel closure and a decrease in GJIC in lens epithelial cells (Saleh and Takemoto 2000; Nguyen, Boyle et al. 2003).

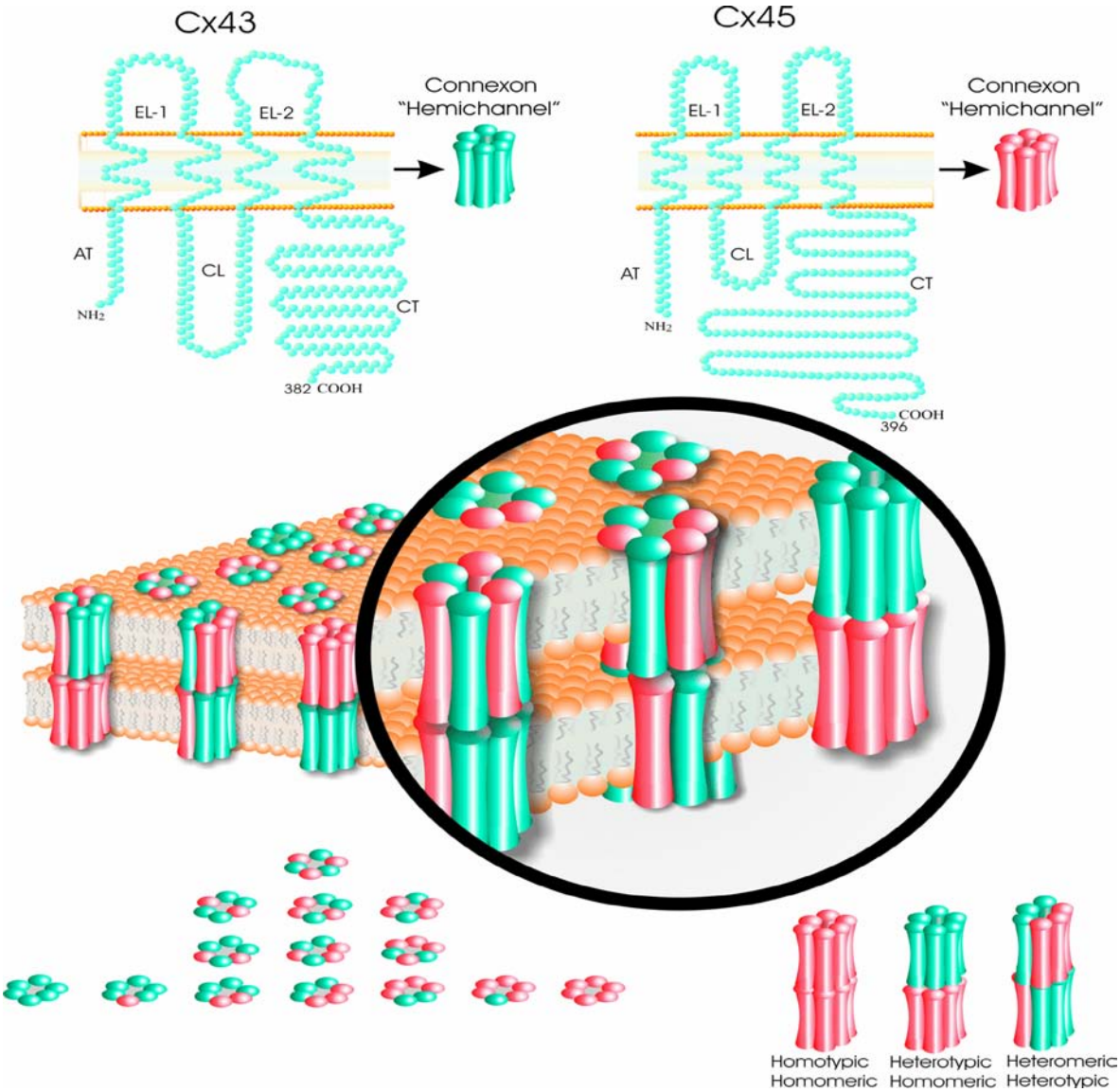

Figure 1.1 Structural organization of connexin and gap junction.

Schematic diagram of connexin43 (Cx43) and connexin 45 (Cx45) protein (top). Each connexin polypeptide spans the membrane four time with amino terminal (AT) end, carboxyl terminal (CT) end and carboxyl loop (CL) in the cytoplasm. Six connexin proteins oligomerize to form one connexon or hemichannel. Connexons from two apposing cells interact and form one gap junction channel (middle). Depending on the stoichiometry of the connexin subunits, gap junctions can be homotypic homomeric (identical connexin isoform sububit), heterotypic homomeric (each connexon has identical connexin isoforms that are different in two connexons) or heteromeric heterotypic (mixed connexin isoforms) (bottom). [Figure reprinted from Laird 2006 with kind permission from Biochemical Journal]

1.2.2. Nomenclature of connexin

Connexins are named according to their molecular weight (MW). They are abbreviated as “Cx” followed by suffix that indicates the approximate molecular weight of the protein in kilo Daltons (kDa). For example, the protein Cx43 is a connexin protein that has the molecular weight of 43 kDa. Similarly, Cx46 is connexin protein with predicted molecular weight of 46 kDa.

Another nomenclature, used to identify the connexins, is developed on the based on the overall sequence similarities and length of cytoplasmic loops of connexins. This nomenclature separates connexins in two subgroups: α and β. The α-group includes Cx33, Cx37, Cx38, Cx40, Cx43, Cx45, Cx46, Cx50, and Cx56; while Cx26, Cx30, Cx30.3, Cx31.1, and Cx32 fall in to β-subgroup. This nomenclature is currently used for the official identification of different connexin genes in vertebrates. For instance, the official nomenclature of Cx43 gene in mouse is Gja1 and that in human G*Ja1*. Where the word “Gap junction” is abbreviated as “G*j*” for mouse or “G*J*” for human, “a” stands for α- subgroup connexin and “*a1”* is given to the name since Cx43

was the first identified connexin gene of the α-group in mammals. Similarly, Cx32 gene is named as G*jb1* as it is the first identified connexin gene in the β-subgroup in mouse.

So far, 21 connexin genes in the human genome and 20 connexin genes in the mouse genome have been identified (Table 1.1). Connexin 33 gene is present only in mouse whereas Cx25 and Cx59 appear only in the human genome (Laird 2006).

Mouse connexins	Human connexins
Cx23	Cx23
	Cx25
Cx26	Cx26
Cx29	Cx30.2
Cx30	Cx30
Cx30.2	Cx31.9
Cx30.3	Cx30.3
Cx31	Cx31
Cx31.1	Cx31.1
Cx32	Cx32
Cx33	
Cx36	Cx36
Cx37	Cx37
Cx39	Cx40.1
Cx40	Cx40
Cx43	Cx43
Cx45	Cx45
Cx46	Cx46
Cx47	Cx47
Cx50	Cx50
	Cx59
Cx57	Cx62

Table 1.1 Mouse and homologous human connexin family members
(Reprinted from Laird, 2006 with permission from Biochemical Journal)

1.2.3. Tissue specific expression of connexins

Connexins are expressed in almost all tissues (Table 1.2) with the exception of red blood cells, some neurons and spermatozoa (Laird 2006). In vertebrates, many tissues express two or more connexins. For example, vertebrate heart expresses Cx40, Cx43 and Cx45 (Beyer, Davis et al. 1995; Gros and Jongsma 1996; Moreno 2004) and normal breast tissue expresses Cx26, Cx32 and Cx43. When multiple connexin isoforms are expressed in one tissue type they can compensate for each other's functions particularly when one connexin is mutated. For example, Cx57-null mice shows no apparent anatomical defects though Cx57 is only expressed in horizontal cells, indicating the existence of compensation of gap junction function by another connexin when Cx57 is absent (Hombach, Janssen-Bienhold et al. 2004). However, in some other cases, the presence of two or more connexins cannot compensate for the function of the connexin that is defective (White 2003). This has been shown in the skin where a subset of loss-of-function Cx26 mutations cause skin disease though the epidermis expresses many connexin isoforms, indicating a lack of compensatory mechanism by other connexins (Richard, Rouan et al. 2002).

Some connexins are very specific and some connexins are very abundant in terms of their tissue specific expression. For example Cx43 is one of the most abundant connexins in the body as more than 35 tissues have been reported to express this protein and, over the years, it is the connexin protein that has been studied most extensively among all isoforms.

Mouse Connexin	Representative tissue/organ	Representative Cell Type
Cx23	-	-
Cx26	Liver, skin	Hepatocytes, keratinocytes
Cx29	Brain	Oligodendrocytes
Cx30	Skin	Keratinocytes
Cx30.2	Testis	Smooth-muscle cells
Cx30.3	Skin	Keratinocytes
Cx31	Skin	Keratinocytes
Cx31.1	Skin	Keratinocytes
Cx32	Liver, nervous	Hepatocytes, Schwann cells
Cx33	Testes	Sertoli cells
Cx36	Retina, nervous	Neurons
Cx37	Blood vessels	Endothelial cell
Cx39	Developing muscle	Myocytes
Cx40	Heart, skin	Cardiamyocytes, keratinocytes
Cx43	Heart, skin	Cardiomyocytes, keratinocytes
Cx45	Heart, skin	Cardiomyocytes, keratinocytes
Cx46	Lens	Lens fibre cells
Cx47	Nervous	Oligodendrocytes
Cx50	Lens	Lens fiber cells
Cx57	Retina	Horizontal cells

Table 1.2 The expression pattern of connexin family members in tissues and corresponding cell types

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1.2.4. Connexin mutations and diseases

Gap junction channels play major roles in the processes of cell growth, proliferation and development. Therefore several reports have been made on the

appearance of inherited diseases such as deafness, cataractogenesis and epidermal diseases associated with germline mutations of different connexin proteins (Liu, Walsh et al. 1997; Paznekas, Boyadjiev et al. 2003; Gerido and White 2004; van Steensel 2004) (Table 1.3). The first disease that was discovered to be associated with connexin abnormalities was chromosome-X-linked Charcot-Marie-Tooth disease (peripheral axon demyelination and limb weakness) caused by over 270 possible mutations in the *Cx32* gene (Bergoffen, Scherer et al. 1993) that resulted in the inhibition of protein transport to the cell surface and inhibition of gap junction channel formation (Krutovskikh and Yamasaki 2000; Zhou and Griffin 2003).

Mutations can happen at genes leading to impaired transcription or, at a protein coding region, interfering with the normal translation process, resulting in loss of a protein expression or appearance of an abnormal protein with defective function. A nonsense mutation in the protein coding region of the Cx26 gene results in impaired translation and formation of a mutant protein that causes deafness (Carrasquillo, Zlotogora et al. 1997). Alterations can also happen at different steps of the connexin life cycle, including synthesis at the endoplasmic reticulum (ER), assembly and transportation via the trans-Golgi pathway, disrupting proper channel formation and function. For example, mutation D66H of Cx26 leads to mutilating keratoderma with sensorineural deafness due to the inability of the mutant protein to reach the cell surface for the proper gap junction channel to form (Maestrini, Korge et al. 1999; Bakirtzis, Choudhry et al. 2003). Mutations can also affect the compatibilities of oligomerization between two different connexin proteins. Wild type Cx43 and Cx26 do

not interact to form hemichannels. However, several Cx26 mutants, that cause skin diseases, can interact and inhibit the function of Cx43 (Rouan, White et al. 2001).

Disease	Connexins
Neuropathy: Charcot-Marie-Tooth disease, X-linked type 1 (CMTX1)	Cx32
Deafness (nonsyndromic)	Cx26, Cx31,Cx30,Cx43,Cx26
Deafness (syndromic): Dominant deaf-mutism and palmoplantar keratoderma	Cx26
Deafness, peripheral neuropathy	Cx31
Skin Disease: Erythrokeratodermia variabilis	Cx31, Cx30.3
Skin Disease: Autosomal dominant hidrotic ectodermal dysplasia	Cx30
Oculodentodigital dysplasia	Cx43
Autosomal dominant zonular pulverulent catatact-3	Cx46

Table 1.3 Hereditary diseases linked with germline mutations in connexin.
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1.2.5. Connexins and cancer

Cancer was the first disease that was identified to be associated with defective intercellular gap junction communication (GJIC). For more than 40 years, the loss of gap junction communication has been shown to be an important step in the development of carcinoma. Several reports have supported that the gap junction connexins function to control cell proliferation and differentiation, however, the molecular mechanisms of which are largely unexplained. Therefore, how connexins are involved in the carcinogenesis process is yet to be known in detail and is often controversial. One important observation, that is well-established, is that tumor progression is generally associated with the loss of gap junction contacts at cell surfaces. The decreased communication of tumor cells with neighboring normal cells due to the defective intercellular gap junctions has been shown to be beneficial for tumor progression and therefore connexins are deemed tumor suppressors.

The function of connexins in cell cycle progression is not clear. The current hypothesis proposes that connexins are “gatekeepers” of cell cycle progression that allows the passage of different regulatory molecules (Vinken, Vanhaecke et al. 2006). Therefore, the downregulation of connexin is not the consequence of loss of growth control during carcinogenesis, rather, it contributes to the loss of growth control. Studies have shown that GJIC increases during G1 phase and decreases during S phase in a model of rat liver regeneration. In another rat liver model it has been found that phosphorylation of Cx43 causes disruption of GJIC and it occurs during G_0 to S phase transition (Lampe and Lau 2004). In addition, it has also been found that Cdc25A

phosphorylation can induce Cx43 phosphorylation resulting in loss of GJIC during the G1/S transition (Melchheier, von Montfort et al. 2005).

The tumor suppressive mechanism of connexins is yet to be identified; probably it is mediated through a variety of pathways. One of the pathways by which connexins act as tumor suppressors is by negatively affecting growth factors. For example, Cx32 inactivates growth factor Her-2 in metastatic renal carcinoma cell and therefore has a negative growth effect (Fujimoto, Satoh et al. 2004). Cx32 also inactivates SrC signaling pathway in the above cell line and this is associated with a reduced malignant phenotype (Fujimoto, Satoh et al. 2004; Fujimoto, Sato et al. 2005). The inactivation of Src pathways by Cx32 also contributes to the reduced production of vascular endothelial growth factor (VEGF). Another connexin, Cx43, has also been shown to regulate the growth of glioma cells by suppressing the expression of extracellular growth factor MFG-E8 (Goldberg, Bechberger et al. 2000). Cx43 also inhibits cell proliferation of osteosarcoma cells by negatively regulating S-Phase Kinase-associated Protein (Skp 2), a key cell cycle regulator (Zhang, Kaneda et al. 2003).

The loss of intercellular gap junctional communication (GJIC) in tumor cells is believed to favor tumor progression. Aberrant localization of connexins, mainly lack of plasma membrane localization, has been observed in many tumor cell lines (Krutovskikh, Mazzoleni et al. 1994; de Feijter, Matesic et al. 1996; Kanczuga-Koda, Sulkowski et al. 2005). In several colorectal cancer specimens, Cx26 was found to be predominantly present in the cytoplasm. Similarly, the expression of Cx26 is only seen in the

cytoplasm of breast tumor tissues (Kanczuga-Koda, Sulkowski et al. 2005). The cytoplasmic localization of connexin could be due to the impaired localization via a trans-Golgi network pathway to the cell surface or due to post transcriptional or post translational defects of those proteins in neoplastic cells. The cytoplasmic localization of connexin may also function to promote tumorigenesis by pathways independent of GJIC. Qin *et al*. (2002) found that restoration of Cx43 in human breast cancer cells suppressed tumor growth via mechanisms that were distinct and independent of GJIC (Qin, Shao et al. 2002). Forced expression of Cx43 inhibits proliferation of osteosarcoma cells without forming gap junction channels (Zhang, Kaneda et al. 2003).

Several reports have been made suggesting that connexins can directly affect the gene expression of different proteins involved in the process of cell growth and proliferation. Cx43 expression increases the expression of *cyr61,* a gene that encodes for a cysteine-rich heparin-binding protein involved in several cellular pathways (O'Brien and Lau 1992). In HEK293 cells, co-expression of Cx43 increases the expression of cyclin-dependent kinase inhibitor p21 which is a key regulator of the cell cycle at the G1 phase (Kamei, Toyofuku et al. 2003). All these results point out the definite role of gap junctions in carcinogenesis but the exact mechanism of action is largely unknown.

1.3. Connexin turnover

Connexins have a very short half-life of 1.5 – 4 hr, depending on the cell type. The short half-lives of connexins have been well studied mainly in cell culture and in

tissue environments (Table 1.4) (Fallon and Goodenough 1981; Laird, Puranam et al. 1991; Beardslee, Laing et al. 1998; Hertlein, Butterweck et al. 1998; Diestel, Eckert et al. 2004; Herve, Derangeon et al. 2007). This short half-life of a connexin is very unusual for an integral membrane protein whose half-life is expected to be long (>20 hr)(Lukacs, Chang et al. 1993; Huh and Wenthold 1999; Kleyman, Zuckerman et al. 2001). The reason for the rapid turnover rate of connexins is not well documented; however, it is assumed that connexins are continuously degraded and synthesized to increase or decrease the GJIC as per a cell's physiological requirement. For instance, in the myometrium tissue of uterus, it has been found that steroid hormones increase total gap junctions to more than 5-fold just before the labour onset; however, the gap junctions declined significantly after the labour, indicating a requirement or lack of requirement of GJIC before and after the process, respectively (Risek, Guthrie et al. 1990; Hendrix, Mao et al. 1992; Risek, Klier et al. 1995; Risek and Gilula 1996).

Connexin	Half life (h)	Cell or tissue
Cx26	1.5-2	Cultured mouse hepatocytes (Fallon and Goodenough 1981)
Cx31	4	Hela cells (Diestel, Eckert et al. 2004)
	2.5-3	Mouse embryo hepatocytes (Traub, Look et al. 1987)
Cx43	1.5	Chicken lens epithelial cells (Musil, Cunningham et al. 1990)
	2.3	Bovine retinal endothelial cells (Fernandes, Girao et al. 2004)
	2.5	E36 Chinese hamster ovary cells (Laing and Beyer 1995)
	2	SK-HEP-1 cells
Cx45	4.2	HeLa cells (Hertlein, Butterweck et al. 1998)
Cx56	2-3	Chicken lens cultured cells (Berthoud, Bassnett et al. 1999)

Table 1.4 The half-lives of few connexin isoforms in several cells or tissues.

1.3.1. Connexin biosynthesis

Connexins are synthesized within the endoplasmic reticulum (ER) and co-translationally inserted into ER membranes (Figure 1.2) (Zhang, Chen et al. 1996; Segretain and Falk 2004). Connexins are then transported to the plasma membrane via Golgi-apparatus (Figure 1.2) and trans-Golgi network (VanSlyke and Musil 2000). In the ER, connexins are synthesized with four transmembrane segments and are not glycosylated. Connexin oligomerization to connexon can take place in the ER or in the trans-Golgi network depending on the connexin isoform (Falk, Kumar et al. 1994; Falk and Gilula 1998; Ahmad, Diez et al. 1999; Maza, Das Sarma et al. 2005). Cx32 oligomerizes in the ER (Kumar, Friend et al. 1995; Das Sarma, Wang et al. 2002). However, Cx43 and Cx46 have been found to be present in monomeric forms in the ER and Golgi apparatus and oligomerize in the trans-Golgi compartment (Musil and Goodenough 1993; Koval, Harley et al. 1997).

Upon exit from the ER, connexins pass through the Golgi apparatus to reach the trans-Golgi network. Several studies have documented that the role of the Golgi apparatus is to aid connexin transport (Laird, Castillo et al. 1995; Lauf, Giepmans et al. 2002; Thomas, Telford et al. 2004; Thomas, Jordan et al. 2005). Few studies also show that connexins, mainly Cx26, can reach the plasma membrane via a Golgi-independent pathway (George, Kendall et al. 1999; Martin, Errington et al. 2001). Upon exiting the trans-Golgi network (TGN), connexins are delivered to the cell surface with the help of exocytic vesicles (Figure 1.2) (Jordan, Solan et al. 1999; Thomas, Jordan et al. 2005). The delivery process of connexins from TGN to cell membrane is aided by microtubules

that increase the efficiency of the delivery process (Feldman, Kim et al. 1997; Johnson, Meyer et al. 2002). When a connexon (hemichannel) arrives at the plasma membrane, it docks, head-to-head, with a hemichannel from adjacent cell to form the complete gap junction channel (Unger, Kumar et al. 1999; Lauf, Giepmans et al. 2002; Segretain and Falk 2004). Gap junction channels cluster to form gap junctional plaques that may contain as many as 10,000 channels per plaque. In lens fiber cells, these gap junction plaques cover more than 50% of the cell surface (Musil 2009).

1.3.2. Connexin degradation

There is much evidence that suggests that connexins are degraded by lysosomal as well as proteasomal degradation pathways (Vaughan and Lasater 1992; Laing, Tadros et al. 1997). Degradation of connexins was found to be delayed in the presence of both lysosomal and proteasomal pathway in cell culture systems. During synthesis in the ER, misfolded or unfolded connexins are tagged with poly-ubiquitins and degraded via the endoplasmic reticulum associated degradation (ERAD) pathway, as a part of the process known as “quality control” (VanSlyke and Musil 2002). The pathway by which degradation of connexins, present at the cell surface, occurs has been under intense investigation in recent years and shows that mainly lysosome is involved (Figure 1.2). In this pathway, connexins at the surface are internalized into one of two adjacent cells as double-membrane structures, commonly known as annular junctions or connexosomes (Figure 1.2) (Jordan, Chodock et al. 2001; Leithe, Brech et al. 2006; Piehl, Lehmann et al. 2007; Nickel, DeFranco et al. 2008). Connexins are then trafficked to early endosomes, by a process which is not clearly understood but may

involve fusion of connexosomes to endosomes (Leithe, Kjenseth et al. 2009). Connexins are then delivered to late endosomes and ultimately to lysosomes where they are degraded (Figure 1.2) (VanSlyke, Deschenes et al. 2000; Qin, Shao et al. 2003; Leithe, Sirnes et al. 2006). Various external factors such as changes in voltage, calcium concentration, stress and the phosphorylation of connexins by mitogen activated protein kinase (MAPK) or protein kinase C (PKC) have been implicated in the regulation of the internalization and degradation process in many cells (Leithe and Rivedal 2004; Solan and Lampe 2009).

Ubiquitination of connexins also acts as a major signal to mediate the Cx43 degradation. Ubiquitin is a 76 amino acid protein that, upon multiple conjugation, (polyubiquitination) targets proteins to proteasomal pathway for degradation (Hershko and Ciechanover 1998). Laing et al (1995) first showed that ubiquitin was involved in the degradation of connexins when they found Chinese hamster ovary CHO-ts20 cells had increased levels of Cx43 when ubiquitin-activating enzymes were defective. Subsequent studies conducted in the last few years have established that Cx43 is ubiquitinated at the plasma membrane by E3 ubiquitin ligase Nedd4 (Figure 1.2) (Leykauf, Salek et al. 2006). Cx43 has a Nedd4 binding site (PY motif) at its C-terminal region (S282PPGY286) that binds to Nedd4 which is recruited by MAPK dependent pathway. The Nedd4 mediated ubiquitination leads to Cx43 degradation via the proteasome.

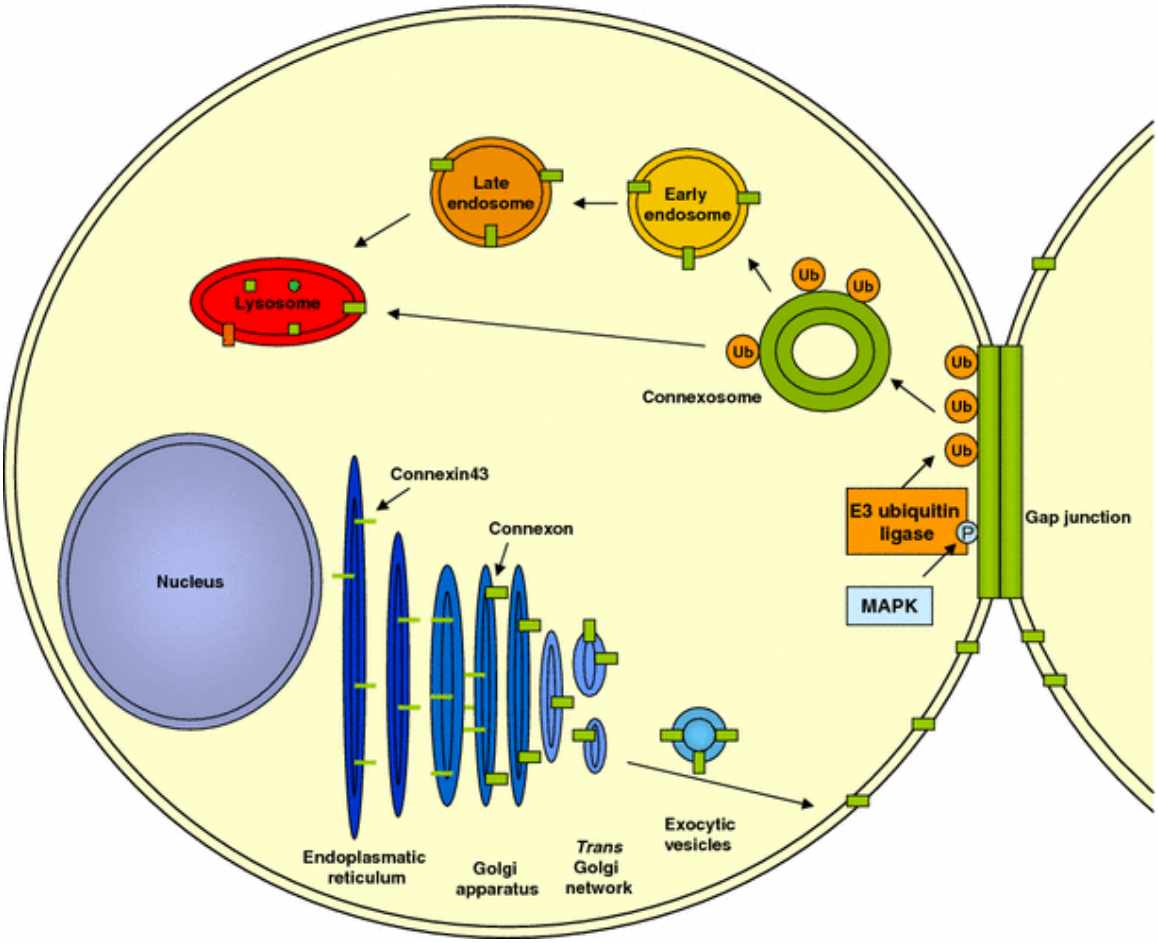

Figure 1.2 Intercellular trafficking of connexin.

Connexins are synthesized within the ER and transported to the plasma membrane via Golgi-apparatus and trans-Golgi network. Connexins, at the cell surface, are internalized and degraded via lysosome. A E3 ubiquitin ligase Nedd4 ubiquitinates Cx43 by the MAPK dependent pathway [Taken from Leithe 2007 with kind permission from Journal of Membrane Biology].

Studies in chapter 2 and 3 of this dissertation investigated the function of Cx46 protein in lens and breast tumor tissues. Therefore next section will address the cellular organization and gap junctions of lens.

1.4. Lens

Vertebrate lens is a biconvex transparent organ whose main function is to focus the refracted light onto the retina. Lens is located at the anterior portion of the eye. At the anterior side of the lens is the aqueous humor and at the posterior side is the vitreous humor. The anterior surface of the lens is less curved than the posterior surface.

1.4.1. Cellular organization in lens

Vertebrate lens is composed of two types of cells, epithelial and fiber cells. Lens is surrounded by a basement membrane (capsule) that is elastic in nature and provides protective and contractive function to the lens. The lens can be broadly divided into three distinct regions based on cellular organization and presence of organelles: anterior epithelium, cortex and nucleus (Figure 1.3). The anterior surface of the lens is covered with a single layer of epithelial cells (Zampighi, Eskandari et al. 2000). These cells are hexagonal in morphology and their basal surface is in contact with the lens capsule that completely surrounds the lens. The lens epithelium contains most of the organelles and expresses most of the active transport proteins such as the Na-K ATPase pump. The epithelial cells at the anterior pole of the lens differ from the epithelial cells that reside in the equator in shape and property. The epithelial cells at the anterior pole are flat and do not divide (Gao, Sun et al. 2000). The epithelial cells,

located halfway between the anterior pole and equator, are cubic in shape and undergo mitosis. Cells at the equator are elongated with low mitotic activity and they eventually are differentiated to form differentiating fiber cells (DF) at the cortex region.

The cortex region consists of two types of fiber cells, differentiating fiber cells at the outer side and the inner mature fiber (MF) cells (Zampighi, Eskandari et al. 2000). The central part of the lens is known as the nucleus. This region contains mainly mature fiber cells (Kuszak 1995).

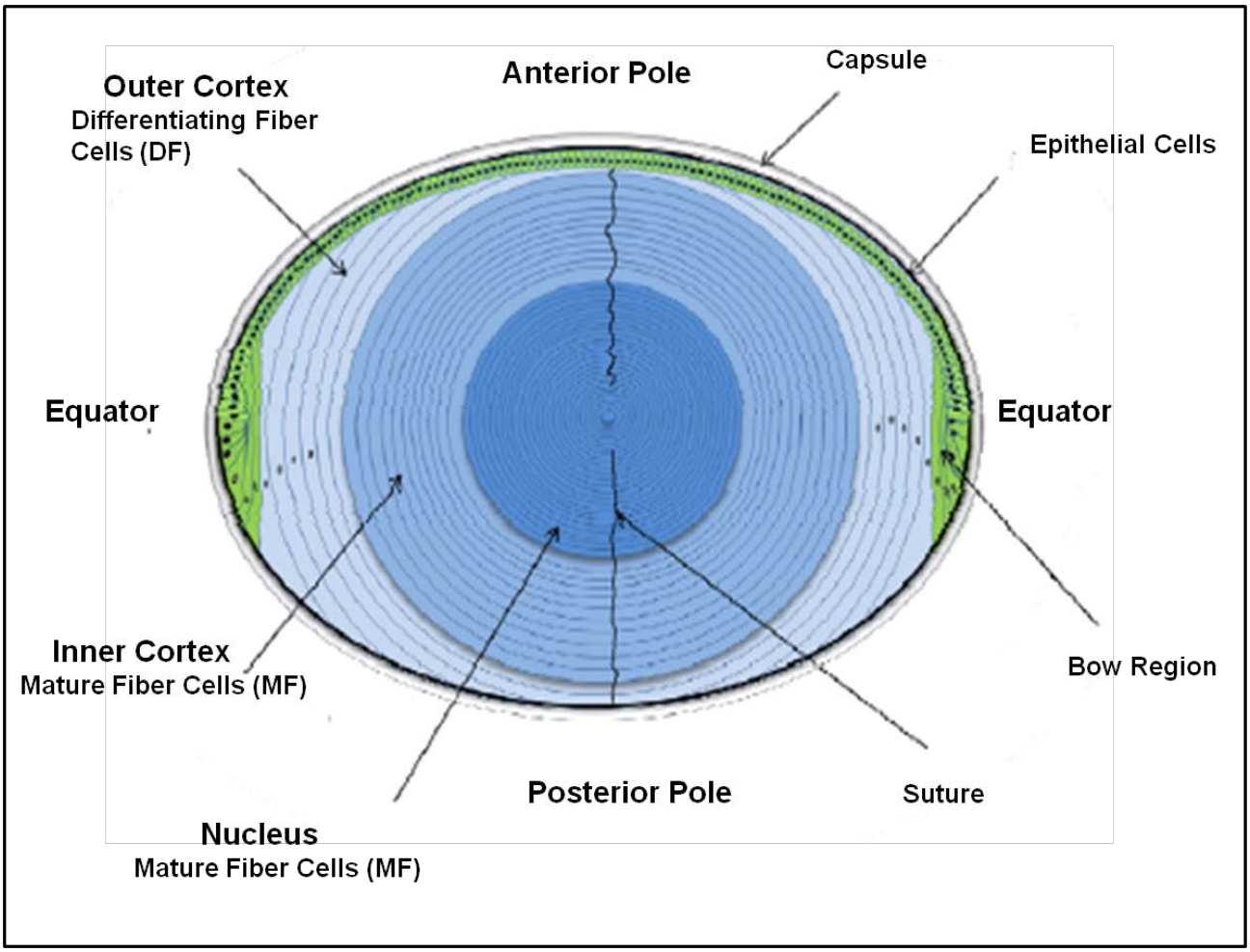

Figure 1.3 Cellular organization of the lens showing capsule, epithelial monolayer at the anterior surface, cortical differentiating fiber cell (DF) and mature fiber cells (MF) and nuclear mature fiber (MF) cells.
[Taken from Satyabrata Das PhD dissertation (2009) with kind permission].

1.4.2. Lens differentiation

As stated earlier, surface epithelial cells are differentiated into differentiating fiber cells (DF) at the equator region of the lens. This differentiation process is accompanied with changes in the expression of membrane proteins and upregulation of crystallins in the cytoplasm of DF. The transition from DF to mature MF occurs at the inner side of the cortex region. The mature fiber cell cytoplasm does not contain any organelles such as mitochondria, nuclei, Golgi bodies, endoplasmic reticulum and is rich in protein crystallins (Bassnett and Beebe 1992; Bassnett 1995). The crystallin proteins account for more than 85% of total proteins present in the lens. The differentiation of epithelial cells to DF is a continuous process and the lens grows throughout life. The newly synthesized fiber cells are deposited over the existing fiber cells at the surface pushing the old or existing fiber cells towards the center of the lens. The old fiber cells at the nucleus are as old as the organism itself. Several growth factors such as fibroblast growth factors (FGF) (Robinson, Overbeek et al. 1995), Wingless (Wnt) (Lyu and Joo 2004), transforming growth factor-B (TGF-β) proteins (de Iongh, Lovicu et al. 2001), as well as several cell signaling pathways, including mitogen activated protein kinase (MAPK) signaling pathway (Wang, Stump et al. 2009), have been implicated to be involved in lens cell proliferation and differentiation.

1.4.3. Lens gap junction

Vertebrate lens has two unique properties. First, the lens is hypoxic. The hypoxic nature of the lens will be discussed later. The second important characteristic of the lens is its avascular nature or absence of blood vessels. Therefore, lens homeostasis and cell-to-cell communication are mediated by a network of gap junctional intercellular communication (GJIC). Human lens endogenously expresses three connexin isoforms, connexin43 (Cx43), connexin46 (Cx46) and connexin50 (Cx50) (Goodenough 1992; Goodenough, Goliger et al. 1996; Donaldson, Kistler et al. 2001; Berthoud and Beyer 2009). The orthologs of these three connexins have been identified in human, mouse, rat, rabbit and chicken. These three connexin isoforms show distinct expression and spatial distribution inside the lens. Lens epithelium mainly contains Cx43 and Cx50. During epithelial-to-fiber cell differentiation, the Cx43 is significantly downregulated and Cx46 is highly upregulated in the fiber cells (Musil, Beyer et al. 1990; Paul, Ebihara et al. 1991; Dahm, van Marle et al. 1999). Lens differentiation does not change Cx50 expression as high levels of Cx50 protein persist in both epithelial layers and DF and MF layers (White, Bruzzone et al. 1992).

1.4.3.1. Function of gap Junctions in lens

The gap junction channels formed by each of the three connexins show unique gating and permeability properties suggesting their important physiological roles in regulating the cell-to-cell communication at different regions of lens (White 2002;

White, Gao et al. 2007). Lens epithelial gap junctions are mainly composed of Cx43 and Cx50 whereas Cx46 and Cx50 gap junctional channels are present in DF and in MF (Beyer, Kistler et al. 1989; Paul, Ebihara et al. 1991; Jiang and Goodenough 1996). Cx46 and Cx50 form heteromeric connexons in the fiber cells. The fiber-epithelial junctions may be heterotypic channels composed of Cx43 and Cx46 (Mathias, Rae et al. 1997; Mathias, White et al. 2010). These three connexins have been shown to play significant roles in the proper lens development and differentiation. Among all the studies, mutational studies, in particular, have been able to shed more light on the functions of these connexins in lens.

The study of Cx43 function is greatly impeded by the fact that Cx43 knockout is lethal in mice (Reaume, de Sousa et al. 1995). However, lenses from Cx43KO pups have been isolated and studied (Gao and Spray 1998). Cx43 knockout, apparently, did not interfere with the normal lens development in the pups but intracellular large vacuoles appeared at the fiber cells. Lack of Cx43 expression also resulted in separation of epithelial cells and between epithelial cells and fiber cells suggesting a possible function of Cx43 in epithelial to fiber cell communication.

Cx46 plays a major role in maintaining fiber cell homeostasis. Cx46 channels are the major functional channels inside the center of the lens and are required in maintaining low intercellular Ca^{2+} concentration. It is speculated that the Cx46 channel cycles out Ca^{2+} from fiber cells to surface epithelial cells where the Ca^{2+} pumps are located. The support for this speculation came from the observations that Cx46

knockout mice developed severe cataracts at the nucleus of the lens with increased intercellular Ca^{2+} concentration (Gong, Li et al. 1997; Baruch, Greenbaum et al. 2001; Gao, Sun et al. 2004). The cataract is developed at the nuclear region of the lens with some opacity found at the cortical region (Figure 1.4C). The deletion of the Cx46 gene, however, does not inhibit normal lens development and growth. The increased intercellular concentration of Ca^{2+} in the fiber cells of Cx46 knockout mice activates Ca^{2+}-activated calpain protease Lp82 (calpain 3) that cleaves and precipitates gamma-crystallin protein resulting in cataract formation (Baruch, Greenbaum et al. 2001). Further support came from the work with Cx46/calpain3 double knockout mice where formation of cataract was found to be delayed significantly due to the absence of crystallin cleavage size (Shakespeare 2009).

Cx50 is also instrumental in lens development. Cx50 is the only connexin that is present in both epithelial and fiber cells. Deletion of Cx50 causes mild cataract (Rong, Wang et al. 2002; White 2002) (Figure 1.4 B) and significantly reduces lens growth that resulted from fewer, rather than smaller, lens fiber cells. Cx50 is required for lens cell proliferation at post natal stages. Cx50 knockout mice or Cx50KI46 (where one allele of *Cx50* gene is replaced with *Cx46*) mice show reduced numbers of dividing cells during the first week of postnatal growth, indicating that Cx50 mediated communication is involved in the regulation of mitosis (Sellitto, Li et al. 2004). Cx46/Cx50 double knockout mice show severe cataract and significant reduction in lens size (Xia, Cheng et al. 2006) (Figure 1.4 D). Cx50/Calpain3 double knockout mice show absence of cataract and similar reduction in lens size (Shakespeare 2009). All these results suggest that Cx50 is

influential for maintaining proper lens growth whereas Cx46 acts to keep lens transparency.

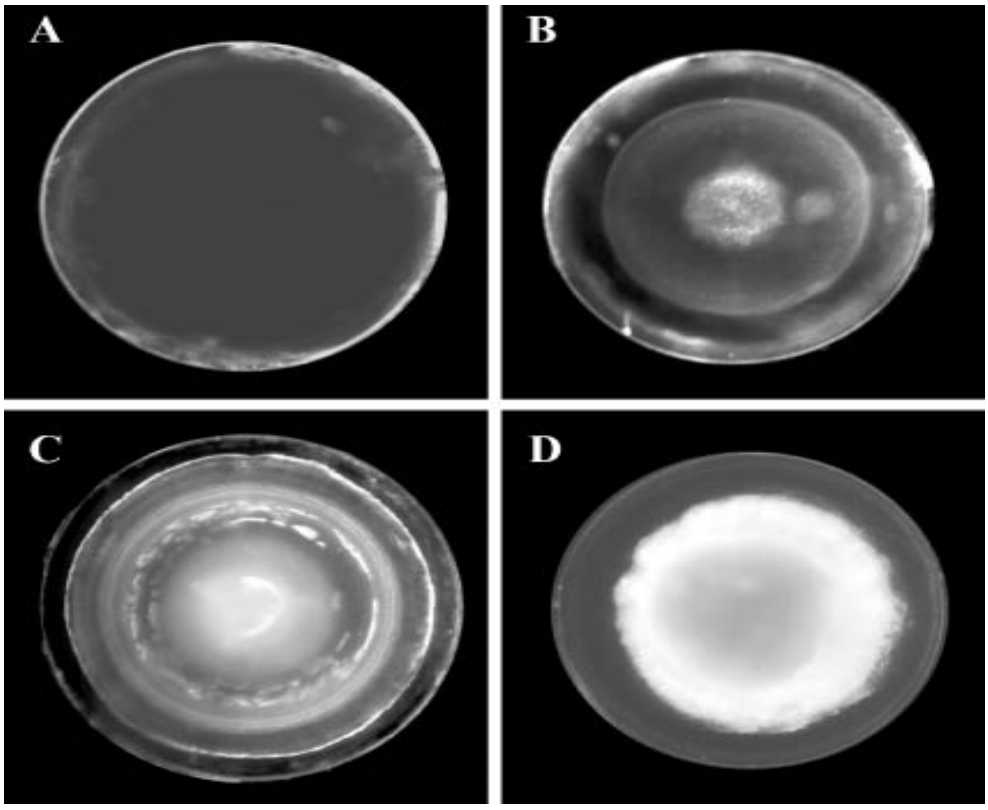

Figure 1.4 Images of lenses from normal wild-type, Cx50KO, Cx46KO, and Cx46/Cx50 double knockout mice emphasizing the importance of connexin genes for lens homeostasis.

(A). Wild-type lens which has normal size and clarity. (B). Cx50KO lens with mild nuclear cataract. (C). Cx46KO lens with normal size but severe dense nuclear cataract. (D). Cx46/ Cx50 double knockout lens with both a severe cataract and reduced lens size. [Taken from Shakespeare 2009 with kind permission from Springer eBook].

This dissertation delves into the novel functions of Cx46 in breast tumor. Therefore the breast carcinogenesis and the roles that other connexins play in growth

and development of normal mammary gland and breast tumors will be reviewed in the next section.

1.5. Breast Cancer

Breast cancer is the most common form of cancer and the second leading cause of mortality in women around the world. Over 182,500 (female) and 1,990 (male) estimated new cases were reported in United States in 2008 (Jemal, Siegel et al. 2008). Due to the high mortality rate, breast cancer has been a subject of intense research for the last 10 years.

Two most significant risk factors for breast cancer are hormonal imbalance and hereditary susceptibility (Seidman, Stellman et al. 1982). Hormonal factors, mainly estrogen and progesterone, are the key regulators of breast cancer progression. Estrogen and progesterone are the two main hormones in the female body that are required for proper development. Estrogen causes breast cancer by inducing proliferation of existing mutant cells. These mutations are acquired through heredity or caused by environmental factors such as chemicals, radiation, cigarette smoking etc. Estrogen action inside the cells is mediated mainly by its cognate receptor, estrogen receptor α (ERα) (Katzenellenbogen, Montano et al. 2000; Sommer and Fuqua 2001). The interaction of estrogen with its receptor results in the dimerization of estrogen receptor and the formation of the ER-complex. The ER-complex exerts its effect by binding to the estrogen response element of several genes that collectively control the growth and proliferation of mammary epithelium (Levin 2005). About 70% of breast cancer cases are associated with overexpression of ER receptor (ER positive) (Fabris,

Marchetti et al. 1987). Aberrant expression of ER receptor causes estrogen mediated uncontrolled cell proliferation and DNA replication leading to mutations that promote breast tumorigenesis.

Hereditary factors include mutations in the two tumor suppressor genes, breast cancer susceptibility gene 1 (BRCA1) and breast cancer susceptibility gene 2 (BRCA2) (Eisinger, Stoppa-Lyonnet et al. 1996; Robson, Gilewski et al. 1998; Choi, Lee et al. 2004). Mutations in this two genes account for less than 10% of the breast cancer cases and confer a lifetime risk of breast cancer of between 60-80% (King, Marks et al. 2003). Current treatment of breast cancer includes surgery, radiation therapy and hormone blocking therapy with a drug tamoxifen.

1.5.1. Connexins in mammary gland development

Human mammary gland is an intricate organ that is composed of glandular, fatty and fibrous tissues. Mature mammary gland consists of a series of alveoli that are organized into milk producing glands called lobules (McLachlan, Shao et al. 2007). Each lobule is connected towards the nipple via ducts that transport milk from the lobules to the nipple. A single layer of luminal epithelial cells surrounds the ducts and alveoli and a basal myoepithelial cell layer surrounds the epithelium at the surface (Figure 1.5A). A layer of fatty tissue surrounds the breast glands and extends throughout the breast. The major development and differentiation of the mammary glands occurs post puberty. During involution and pregnancy, the mammary gland also undergoes extensive differentiation and remodeling to attain a lactating structure. From birth to post pregnancy, the development of mammary glands is regulated by the signal

pathways that include hormones, local growth factors and interactions between epithelial cells with surrounding stroma (Robinson, Karpf et al. 1999; Lamote, Meyer et al. 2004).

The gap junctional intercellular communication (GJIC) also plays a major role in the proper development, differentiation and functioning of vertebrate mammary glands at different stages of growth, from post-puberty to post-pregnancy. Human mammary glands have been shown to express two connexin isoforms Cx43 and Cx26 (Lee, Tomasetto et al. 1991; Wilgenbus, Kirkpatrick et al. 1992). In addition to these connexins, mouse mammary glands have been found to express two more connexin isoforms, Cx30 and Cx32 (Pozzi, Risek et al. 1995; Locke, Perusinghe et al. 2000; Locke, Jamieson et al. 2007). The expression, localization and channel formation of all these connexins are distinct and controlled in a precise manner throughout the mammary gland development.

Cx26 is the first connexin identified in human mammary glands. Several studies have shown that Cx26 channels are predominantly located in between the luminal cells (Figure 1.5B) indicating its selective function in luminal cell proliferation (Talhouk, Elble et al. 2005). Another connexin, Cx43, is found to form gap junction channels only between myoepithelial cells (Figure 1.5B) and is speculated to maintain myoepithelial differentiation in resting human mammary gland (Talhouk, Elble et al. 2005). In mouse, two other connexins Cx30 and Cx32 are also expressed in the luminal cells (Figure 1.5B) during lactation (Monaghan, Perusinghe et al. 1994; Pozzi, Risek et al. 1995). The expression of Cx30 and Cx32 are not detected

in human mammary glands which indicates that these connexins may have a distinct function in mouse mammary glands that is either compensated or not required in human counterparts.

1.5.2. Functions of connexins in mammary gland

The mammary glands gap junction channels formed by different connexins have distinct functions. Northern blot and microarray studies have shown that Cx26 mRNA is detected in the mouse mammary gland during lactation (Locke, Stein et al. 2004), pregnancy as well as in all stages of development. The expression of Cx26 protein goes up during lactation and declines in involution (Talhouk, Elble et al. 2005). At early stages of mammary gland development, Cx26 functions in luminal epithelial cell proliferation and, in later stages of development, along with Cx32, Cx26 is required for the proper production of milk by secretory cells (McLachlan, Shao et al. 2007). Inhibition of Cx26 expression in the mammary epithelium, before puberty, results in impaired lactation and development (Bry, Maass et al. 2004). Like Cx26, Cx30 expression also is increased at the onset of lactation and decreased in involution in mouse. Immunohistochemical studies have detected Cx32 at all stages of development indicating its importance in the regulation of mammary gland growth in mouse (Talhouk, Elble et al. 2005).

Mammary gland connexins, Cx26 and Cx32, can compensate for each other's function (Locke, Stein et al. 2004). Conditional knockout of Cx26, during pregnancy, does not affect the normal mammary gland function. Similarly, Cx32 null mice are

associated with proper mammary gland development and functioning. A study, done by Locke et al (2004), shows that gap junction channels can be formed by hetero-hexamer containing Cx26 and Cx32, in the luminal epithelial cells, during the later stage of pregnancy with stoichiometric ratio greater for Cx26. During lactation the ratio of Cx32 increases within the hexamer and eventually homomeric Cx32 is formed. The change of Cx26-Cx32 connexon to Cx32 connexon is speculated to be in accordance with the cell's biological need. Channels formed by Cx32 alone are much wider than the heteromeric Cx32-Cx26 channels and allow the free passage of larger molecules such as cAMP and cGMP (Bevans, Kordel et al. 1998; Weber, Chang et al. 2004), the metabolites that act in the several pathways involved in the regulation of mammary gland growth and differentiation.

The function of Cx30 has not been investigated to date and elucidation of the function of Cx43 is impaired due to the fact that Cx43 knockout is lethal in mouse embryos (Reaume, de Sousa et al. 1995). However some studies with knock-in technology shed light on the role of Cx43 in breast development. Heterozygous Cx43KICx32 mice (where an allele of *Cx43 gene* is replaced with *Cx32* allele) show normal milk production but impaired milk ejection indicating a possible role of Cx43 in the functioning of the mammary gland (Plum, Hallas et al. 2000).

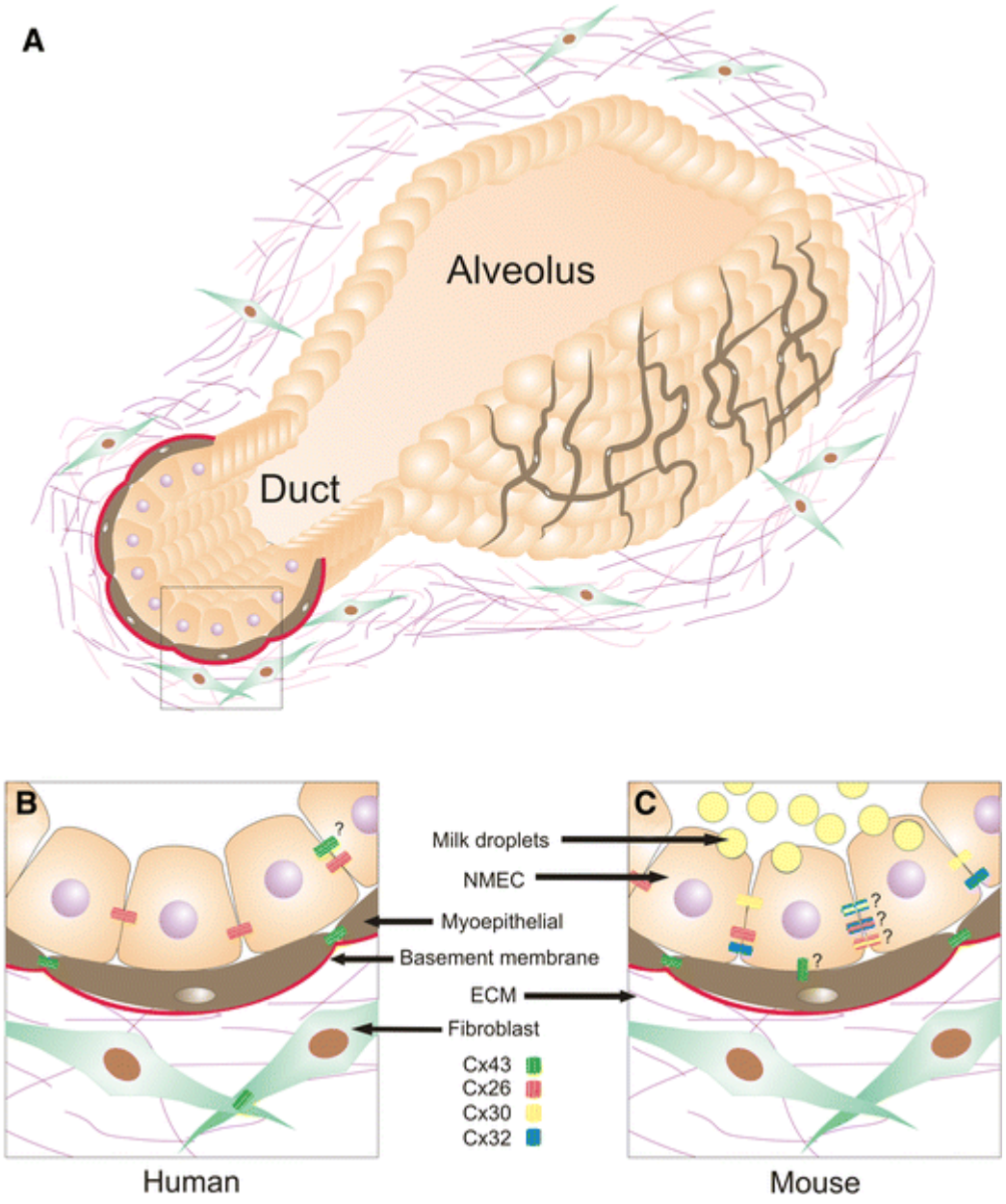

Figure 1.5 The microanatomy of mammary gland with expression and localization of different connexins.

(A). Structural organization of mammary gland with duct, alveolus, myoepithelial cell layer and luminal cells. (B). The localization of connexins in human and mouse mammary gland. [Figure reprinted from McLachlan 2007 with kind permission from the Journal of Membrane Biology].

1.5.3. Connexin and breast tumor

Cx26 and Cx43 are well-documented for their tumor-suppressive roles in several carcinomas, including breast tumors. Cx26 and Cx43 are deemed breast tumor suppressors since 1991 when Lee et al first identified them as the candidates for tumor suppressor genes by subtractive hybridization techniques. However, till today, the correlation of expression of connexins with the function as a tumor suppressor at different stages of breast carcinogenesis is far from clearly understood and is often contradictory. Breast tumor progression is generally accompanied with a loss of gap junctional intercellular communication when compared to the matched normal tissues (Wilgenbus, Kirkpatrick et al. 1992; Laird, Fistouris et al. 1999; Kanczuga-Koda, Sulkowski et al. 2006). The loss of communication of tumor cells with the neighboring normal cells, due to defective or lack of GJIC, promotes tumor progression. Studies in the early 90s detected low levels of Cx26 and Cx43 mRNA in the primary cells derived from human breast tumors and several breast cancer cell lines (Laird, Fistouris et al. 1999; Singal, Tu et al. 2000). The reason for the repression of Cx26 gene in breast tumors is clearly not known, though occurrence of methylation at the promoter region could contribute to the gene silencing (Singal, Tu et al. 2000; Tan, Bianco et al. 2002). Support from this notion came from the work of Singal *et.al*. (2000) where they found that Cx26 is hypermethylated in MCF-7 breast cancer cells leading to gene silencing and reduced expression. Another study with tumor tissues from breast cancer patients reported that the Cx26 promoter was methylated in more than 50% of the tissues irrespective of the stage of cancer (Tan, Bianco et al. 2002).

Cx43 protein expression was also found to be downregulated in human tumor tissues as well as in several breast cancer cells when compared to their normal counterparts. Laird *et al.*, (1999) showed that reduced Cx43 expression can be used as an independent marker for the detection of breast tumors. Lack of Cx43 gap junctions was observed in ductal carcinomas *in situ*, infiltrating ductal carcinomas, and infiltrating lobular carcinomas with no correlation with the level of estrogen and progesterone, the hormones that regulate Cx43 expression. However, there are also few reports made on the occurrence of connexin upregulation in breast tumor. One study reports that 50% of invasive carcinomas express Cx43 (Jamieson, Going et al. 1998). In these samples Cx43 were only detected in the cytoplasm and the presence of gap junctions were not observed. Jamieson et al (1998) also showed that Cx26 expression was detected in 75% of breast ductal carcinoma *in situ*, a value which is much higher relative to normal breast tissues. Another study reported increased expression of Cx26 and Cx43 in lymph node metastases (Naoi, Miyoshi et al. 2007). One common observation, in all the above studies, was that connexins were typically retained within the cytoplasm and not localized at cell surfaces to mediate their gap junctional function.

1.5.3.1. Effect of re-expression of connexins in cancer (breast)

Several reports have been made on the inhibition of tumor growth upon reconstitution or re-expression of Cx26 or Cx43 in breast cancer cells (McLachlan, Shao

et al. 2007). Re-expression of Cx43 or Cx26 resulted in growth suppression and reversed the malignancy of human mammary carcinoma cell, MDA-MB-435 (Hirschi, Xu et al. 1996). Re-expression of Cx26 reduced the levels of total ß1 integrin, and the matrix metalloproteinase-9 (MMP-9) activity. Similarly, overexpression of Cx43 in MDA-MB-231 cells though did not effect *in vitro* cell proliferation but inhibited the tumor growth in mice (Qin, Shao et al. 2002). Transfection of C6 glioma cells with Cx43 (Zhu, Caveney et al. 1991; Bond, Bechberger et al. 1994) and the transfection of human hepatoma cell, SKHep1, with Cx32 (Eghbali, Kessler et al. 1991) suppressed the growth of both transfected cells and xenograft-tumors in nude mice. Transfection of liver carcinoma HepG2 cells with Cx26 changed the morphology of the cells and decreased xenograft tumor growth (Yano, Hernandez-Blazquez et al. 2001).

In most of the studies, mentioned above, the restoration of connexin expression in the tumor cells did not result in gap junction formation (Kalra, Shao et al. 2006) and therefore the suppression of tumor growth is speculated to be mediated by mechanisms independent of GJIC. The GJIC independent roles of connexins have also been found in the regulation of apoptosis. Kanczuga-Koda *et al* (2005) found that Cx26 and Cx43 expression correlated with proapoptotic factor Bak but not with Bcl-2. Cx26 and Cx43 are also considered to be involved in the inhibition of angiogenesis in breast cancer. Cx26 was found to inhibit angiogenesis via mechanisms that are both GJIC dependent and independent. Overexpression of a functional or a non-functional Cx26 variant upregulated the expression of an anti-angiogenic molecule, thrombospondin-1 (TSP-1), in MDA-MB-435 cells (Qin, Shao et al. 2003). Similarly, downregulation of Cx43, by a siRNA, reduced the expression of angiogenesis inhibitor TSP- 1 and

resulted in an aggressive cell phenotype of breast cancer Hs578t cells (Shao, Wang et al. 2005). A similar result was found by another group (McLachlan, Shao et al. 2006) when they observed that MDA-MB-231 cells, overexpressing Cx26 or Cx43, showed increases in the expression of anti-angiogenic factors over pro-angiogenic molecules.

The mechanisms by which connexins act as tumor suppressors are yet to be identified at the molecular level. The majority of the data, to date, indicates that connexins suppress tumor growth by GJIC- independent mechanisms. Most of the studies are done using *in vitro* cell culture systems, where the environments are not the actual representation of *in vivo* conditions. Another important question that is yet to be fully answered; does the loss of GJIC promotes tumor progression or is it a consequence of it? Therefore more elaborate investigations are required to ascertain the role of connexins in tumorigenesis.

This dissertation investigates hypoxia-specific function of connexin46 and therefore the roles that hypoxia or hypoxia regulated pathways play in lens and breast carcinogenesis will be reviewed below.

1.6. Hypoxia

Hypoxia refers to the condition of reduced availability oxygen. In tissue, hypoxia is a pathological condition, known as ischemia, which arises due to the lack of proper blood flow that results in reduced oxygen and nutrients supply to the cells.

Hypoxia is involved in the development of several life-threatening diseases such as tumorigenesis, kidney disease, pulmonary hypertension, inflammation and Alzheimer disease and therefore, it has been a subject of intense scientific research for centuries.

1.6.1. Hypoxia and lens

A unique property of lens is its hypoxic nature. Lens is one of the natural hypoxic tissues in the human body. Several studies have reported that the oxygen concentration in the cortical region of lens is below 5% and around (or below) 1% in the nucleus (Helbig, Hinz et al. 1993; McNulty, Wang et al. 2004; Shui, Fu et al. 2006; Beebe 2008; Shui and Beebe 2008). Lens is also located at the low oxygen environment of the anterior segment of the eye. The average P_{O2} in the aqueous humor, which is located at the anterior side of the lens, of rabbit eye is 38 mm Hg (Fitch, Swedberg et al. 2000) and in the vitreous humor (posterior side of the lens) of human eye is 16 mm Hg (Sakaue, Negi et al. 1989; Maeda and Tano 1996). These P_{O2} values are much smaller than, for example, the P_{O2} in arteries which is 100 mm Hg. The P_{O2} value at the anterior region, which is immediately adjacent to the lens, was found to be 2-3 mm Hg in cat, chicken and rabbit eyes (Jacobi and Driest 1966; Ormerod, Edelstein et al. 1987; Bassnett and McNulty 2003).

The lens requires hypoxia to maintain its growth and optimum transparency. Exposure of lens to higher concentrations of oxygen leads to oxidation and precipitation of crystallin proteins that results in increased light scattering and ultimately in the

development of nuclear cataract (Dische and Zil 1951; Palmquist, Philipson et al. 1984; Giblin, Padgaonkar et al. 1995; Holekamp, Shui et al. 2006). Low oxygen suppresses the growth of the epithelial cells which is essential to maintain the proper growth and size of the lens in vertebrates. A study by Shui *et al* (2008) showed that the lenses of one-month old rats contained low levels of oxygen. The lens cell proliferation and lens growth declined with low oxygen concentration after one-month of growth of those rats. If the oxygen concentration was elevated to 60% the lens growth also increased. It suggests that hypoxia is essential to maintain the proper growth and size of the lens in vertebrates. Low oxygen is speculated to control lens growth by inhibiting synthesis or secretion of growth factors in the intraocular fluids and by suppressing epithelial cell response to the growth factors.

1.6.2. Hypoxia and tumor (breast)

Hypoxia is an important feature of solid tumor growth. Hypoxia arises due to the lack of blood vessel formation around the developing mass in a solid tumor. Impaired blood supply, due to the inadequate vascularization, results in very low oxygen concentration inside the tumor (Brizel, Rosner et al. 1995). Studies, where the oxygen measurement was done by needle electrode, showed that the oxygen concentration can go down as low as 1% inside the solid breast tumor (Vaupel, Kallinowski et al. 1989; Vaupel, Schlenger et al. 1991). Breast tumors use hypoxia as an adaptation to grow and survive. The role of hypoxia in the progression of breast carcinogenesis has been studied extensively. Hypoxia brings about changes in the morphological and physiological characteristics of the cells to shift the balance towards tumor progression

over tumor regression during tumorigenesis. Hypoxia alters the gene expression of several signaling pathways including angiogenesis, apoptotic and glycolytic, to promote breast tumor progression and malignancy (Knowles and Harris 2001).

1.6.1.1. Hypoxia and tumor angiogenesis

Hypoxia favors primary tumor growth by promoting angiogenesis to increase the blood vessel formation around the developing tumor. Hypoxia significantly upregulates the pro-angiogenic molecule vascular endothelial growth factor (VEGF) and its receptor flk-1 (Shweiki, Itin et al. 1992; Carmeliet 2000). Hypoxia exerts its effect at the gene level by inducing transcription factor hypoxia inducible factor-alpha 1 (HIF-1α). HIF-1α is a part of a heterodimer HIF-1. In normoxic condition, an enzyme prolyl hydroxylase converts HIF-1α to a hydorxylated form that interacts with the tumour suppressor Von Hippel–Lindau (VHL) protein (Figure 1.6). VHL is part of a larger complex that contain ubiquitin-conjugating enzyme (E2). This complex, with the help of a ubiquitin-activating enzyme (E1), mediates the ubiquitylation (Ub) of HIF-1α and subsequent proteasomal degradation. During hypoxia, prolyl hydroxylase cannot modify HIF-1α allowing it to remain stable. Stabilized HIF-1α s then translocates to the nucleus and interacts with cofactors such as aryl hydrocarbon receptor nuclear translocator (ARNT), CBP/p300 and the DNA polymerase II (Pol II) complex (Figure 1.6). This large complex then binds to hypoxia-responsive element (HREs) and activates transcription of the target genes including VEGF. HIF-1α has been found to be upregulated in a broad range of human malignancies including primary breast cancers (Bos, van der Groep et al. 2003; Semenza 2003). Several studies have also shown that HIF-1α can

induce other angiogenetic factors interleukin-1(IL-1) (Shreeniwas, Koga et al. 1992), erythropoietin (Goldberg, Dunning et al. 1988) and platelet derived growth factor (PDGF) (Deberardinis, Sayed et al. 2008).

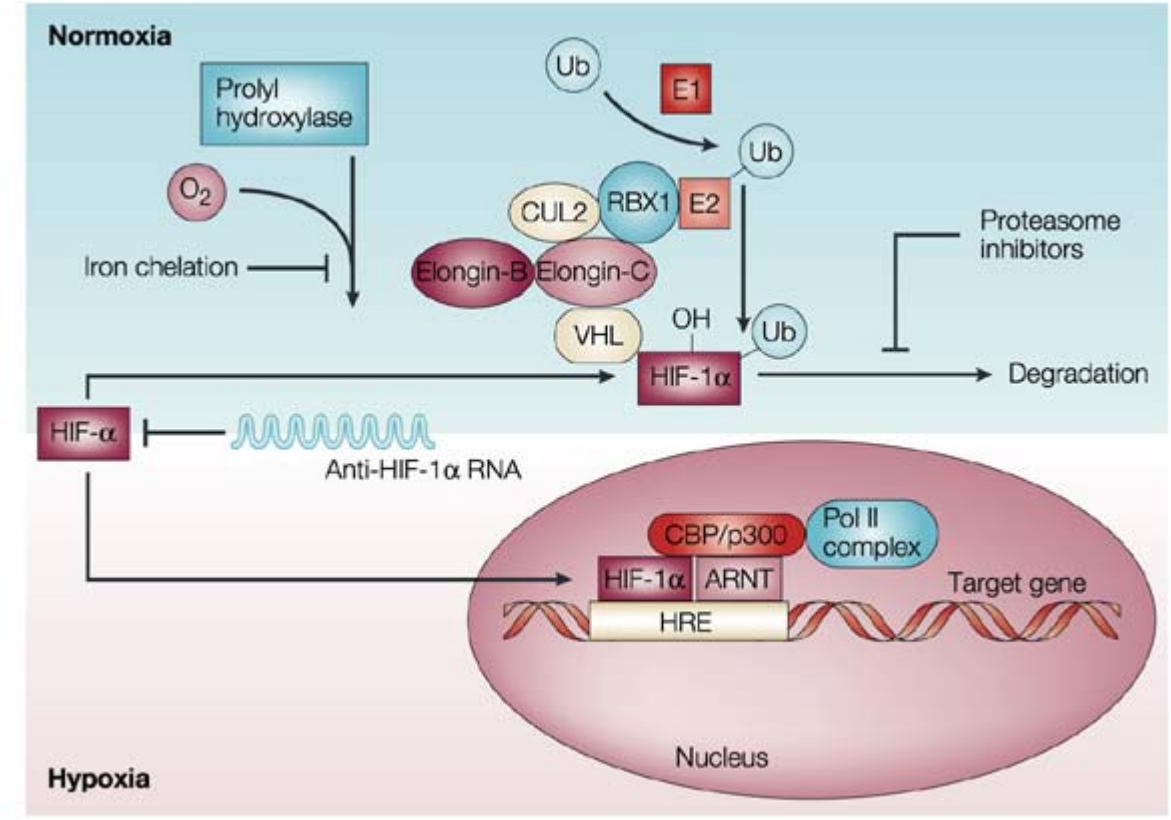

Figure 1.6 The molecular mechanism of hypoxia mediated activation of HIF-1α.
In presence of oxygen (normoxia) the enzyme prolyl hydroxylase modifies HIF-1α to interact with Von Hippel–Lindau (VHL) protein. This interaction mediates ubiquitination of HIF-1α and subsequent degradation. In presence of low oxygen (hypoxia), prolyl hydroxylase is inactivated and thus no longer modifies HIF-1α allowing it to remain stable. Stabilized HIF-1α then translocates to the nucleus and with the help of other proteins, binds to the hypoxia-responsive element (HRE) and induces the target gene transcription. [Figure reprinted from Harris, 2002 with kind permission from the journal Nature Reviews of Cancer].

1.6.1.2. Hypoxia and apoptosis

One of the hypoxia mediated signal pathways that kill normal cells is apoptosis (Malhotra, Lin et al. 2001). Howver, in tumor cells, hypoxia inhibits apoptotic pathway to prolong their survival. In liver tumor cells (HepG2), hypoxia inhibits apoptosis, reduces mitochondrial permeability and cytochrome *c* release in the cytoplasm, decreases the activity of the downstream component, caspase 3, and also reduces the pro-apoptotic bax to anti-apoptotic bcl-2 ratio (Baek, Jang et al. 2000). Another mechanism by which cells can escape hypoxia-induced apoptotic death is by the process of selection and clonal expansion of the cells with mutations in the components of the apoptotic pathway (Graeber, Osmanian et al. 1996). When oncogenically transformed mouse embryonic fibroblast cells were treated with hypoxia, cells with no pro-apoptotic protein p53 ($p53^{-/-}$) were selected over wild type cells. It was also observed that little apoptosis occurred in the hypoxic regions of p53 deficient murine tumors whereas high levels of apoptosis occurred in the highly hypoxic regions of p53 wild type tumors. $p53^{-/-}$ cells are apoptotic resistant cells due to the deletion of the p53 pro-apoptotic gene. Therefore, it is speculated that, in the primary tumor tissues, the hypoxic environment contributes to malignancy by passively selecting the cells with acquired mutation that confers resistance to the apoptotic death.

1.6.1.3. Hypoxia and the glycolytic shift

Another major change in tumor cells, in response to hypoxia, is the shift of the respiratory pathway to anaerobic glycolysis. The presence of low oxygen reduces the

ATP production via the electron transport chain and therefore, hypoxic tumors attain their metabolic need via an increased glycolytic pathway. Therefore, one of the characteristics of rapidly growing tumor is a high rate of glucose consumption (Vaupel, Kallinowski et al. 1989). Hypoxia activates the expression of several glycolytic enzymes including phosphoglycerate kinase1, lactate dehydrogenase A, pyruvate kinase M, glyceraldehyde-3-phosphate dehydrogenase, enolase1 and aldolase A (Semenza, Roth et al. 1994). Low oxygen also regulates the expression of the glucose transporter genes, *glut1* and *glut3*, in human tissues to maximize glucose uptake (Maxwell, Dachs et al. 1997). All of these adaptations enable tumor cells to maximally utilize glucose for efficient energy production that aids tumor cells to grow under the adverse conditions where normal cells cannot.

1.7. Connexin46 (Cx46)

As mentioned earlier, Cx46 is a connexin protein with predicted molecular weight of 46 kDa. The Cx46 protein is expressed in human and orthologs are found in rabbit, rat, bovine and chicken. The rat Cx46 is a 416 amino acid long protein with four transmenbrane domains (residues 20-42, 77-99, 155-177 and 207-229). It has a short N-terminal (about 20 amino acid) and a relatively long C –terminal tail (about 187 amino acid), both of which reside at the cytoplasm. Among the members of connexin family isoforms, connexin 43 (Cx43) has been studied most extensively. There are more than 2000 published article covering different aspects of Cx43 life cycle including synthesis, degradation, post-translational modification and function. Some of the reasons that

Cx43 has been well-studied are its endogenous expression in over 30 distinct animal tissues and its role as a potent tumor suppressor.

In contrast to Cx43, Cx46 has not been extensively studied. Though Cx46 primary transcripts have been found in many mice tissues (Anderson, Zundel et al. 2005) the actual protein expression have only been reported, to my knowledge, in vertebrate lens (Donaldson, Kistler et al. 2001), lung adenoma (Avanzo, Mesnil et al. 2006) and lung alveolar cells (Abraham, Chou et al. 1999), Schwann cells (Chandross 1998) and bone osteoblastic cells (Koval, Harley et al. 1997). The gap junctional function of Cx46 in lens has been investigated previously. As mentioned earlier, Cx46 gap junction channels are required to maintain Ca^{2+} homeostasis inside the lens. However the function of Cx46 in other tissues including tumor has not been investigated. Cx46 was highly expressed at urethane-induced mouse lung adenomas though the proper function was not reported (Avanzo, Mesnil et al. 2006). Cx46 proteins had been found to be localized to intercellular compartments in bone osteoblastic cells (Koval, Harley et al. 1997) and upregulated in Schwann cells after nerve injury (Chandross 1998).

The lens is a naturally hypoxic tissue in an animal body. But how the lens survives against the all adversity of low oxygen is not well-known. Initially, we have found that Cx46 protein is also highly expressed in early hypoxic breast tumor tissues. In my dissertation work, I have studied the role of Cx46 in a natural hypoxic tissue, lens and a diseased hypoxic tissue, breast tumor. I have found that Cx46 provide protection,

to both lens and breast tumor, against hypoxia-induced death and regulates the degradation of Cx43 in lens. The findings of my research have been presented in Chapter 2 and 3 of the thesis.

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CHAPTER 2 - A novel role of gap junction connexin46 protein to protect breast tumors from hypoxia

2.1. Introduction

Gap junctions are intercellular membrane channels that maintain direct intercellular communication through the exchange of ions, small molecules and cellular metabolites between neighboring cells. Gap junction channels are formed through the hexameric oligomerization of transmembrane proteins called connexins (Goodenough, Goliger et al. 1996). Over the years, several studies have shown that the gap junctions are involved in the regulation of important cellular processes including cellular homeostasis, cell growth and differentiation, morphogenesis and cell death (Loewenstein 1979).

For more than 40 years, the loss of gap junction communication has been shown to be an important step in the development of carcinoma (Trosko and Ruch 1998). There is also increasing evidence that loss of connexin protein expression or dysfunction of gap junction intercellular communication (GJIC) is associated with breast cancer malignant progression (Lee, Tomasetto et al. 1991; Laird, Fistouris et al. 1999; Kanczuga-Koda, Sulkowska et al. 2003). The connexins, well-documented for tumor-suppressive functions in breast carcinoma, are connexin43 (Cx43) and connexin26 (Cx26) (Lee, Tomasetto et al. 1992; Hirschi, Xu et al. 1996; Qin, Shao et al. 2002; Kanczuga-Koda, Sulkowski et al. 2005; Shao, Wang et al. 2005). These connexins are required for differentiation and proper development of normal mammary gland. Studies

have shown that Cx26 and Cx43 were downregulated at the mRNA and protein level in human breast tumors and several human mammary tumor cell lines (Lee, Tomasetto et al. 1992). Re-expression of Cx26 or Cx43 reverses the malignancy of human mammary carcinoma cells MDA-MB-435 (Hirschi, Xu et al. 1996). Similarly, overexpression of Cx43 in MDA-MB-231 cells inhibits the tumor growth in mice (Qin, Shao et al. 2002). Cx43 is also considered to be involved in the inhibition of angiogenesis in breast cancer. For instance, downregulation of Cx43 by siRNA in breast cancer Hs578t cells results in the reduced expression of angiogenesis inhibitor thrombospondin-1 (TSP-1) and in aggressive cell phenotype (Shao, Wang et al. 2005).

Another important feature in breast carcinoma is adaptation to hypoxia that favors tumor growth and survival. Several studies have shown that the inside oxygen levels, in a solid breast tumor, can go down as low as 1% (Vaupel, Schlenger et al. 1991; Vaupel, Briest et al. 2002). Hypoxia which arises due to lack of formation of new blood vessels in a developing tumor mass induces hypoxia-inducible factor-1α (HIF-1α) (Wang, Jiang et al. 1995). HIF-1α is a key transcription factor that activates gene expression of pro-angiogenic factor vascular endothelial growth factor (VEGF) (Maxwell, Dachs et al. 1997). In solid tumors, HIF-1α also induces expression of other growth factor such as transforming growth factor-β and platelet-derived growth factor to promote cell proliferation (Denko, Schindler et al. 2000; Koong, Denko et al. 2000; Wykoff, Pugh et al. 2000). Hypoxia also regulates the gene expression of several other signaling pathways including apoptosis and glycolysis to enhance tumor progression

and metastasis (Semenza, Roth et al. 1994; Van Meir 1996; Baek, Jang et al. 2000; Chandel and Simon 2008).

There is another tissue that is naturally hypoxic, vertebrate lens. The oxygen concentration in the cortical region of lens is below 5% and around (or below) 1% in the nucleus (McNulty, Wang et al. 2004; Holekamp, Shui et al. 2005; Holekamp, Shui et al. 2006; Shui, Fu et al. 2006). Lens requires hypoxia to prevent opacification and maintain optimum transparency (Beebe 2008; Shui and Beebe 2008; Akoyev, Das et al. 2009). However, how lens survives against the all adverse effects of hypoxia is not well known. Lens tissue endogenously express 3 connexin isoforms Cx43 (α1), Cx50 (α8) and Cx46 (α3) (Goodenough 1992; Goodenough, Goliger et al. 1996; Donaldson, Kistler et al. 2001; Rong, Wang et al. 2002). Connexin46 (Cx46, $M_w \sim 46$ kD) is preferentially expressed in lens fiber cells, inside the even more hypoxic center of the lens (Tenbroek, Arneson et al. 1992). Cx46 is believed to be essential for maintaining proper Ca^{2+} homeostasis in fiber cells to prevent cataract formation. Cx46 knockout mice show a marked increase in nuclear cataract (Gong, Li et al. 1997)with increased Ca^{2+}.

The role of Cx46 in tissues other than lens has not been extensively investigated. Though Cx46 primary transcript was detected in several mouse tissues but actual protein expression has been reported only in lung adenoma and lung alveolar cells (Abraham, Chou et al. 1999; Avanzo, Mesnil et al. 2006), Schwann cells (Chandross 1998) and bone osteoblastic cells (Koval, Harley et al. 1997). Cx46 is highly expressed at urethane-induced mouse lung adenomas though the proper function was

not reported (Avanzo, Mesnil et al. 2006). In this study, I investigated a novel role of Cx46 protein to protect cells from hypoxia-induced death. I found that Cx46 protein is highly expressed in MCF-7 breast cancer cells and human breast tumors. I hypothesized that the presence of Cx46 protein aids breast tumors' growth and also lens homeostasis under hypoxia. Downregulation of Cx46 in hypoxia-resistant human lens epithelial cells (HLEC) and MCF-7 breast cancer cells confers susceptibility to hypoxia. In contrast, overexpression of Cx46 in hypoxia-sensitive neuronal gap junction deficient N2A cells endows resistance against hypoxia. Further studies demonstrated that knockdown of Cx46 *in vivo* inhibited human breast tumor xenograft growth in nude mice. Thus Cx46, unlike other connexins, may play a significant role in the growth and development of early hypoxic breast tumors and, in the natural hypoxic lens

2.2. Methods and Materials

2.2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM; low glucose), trypsin-EDTA, gentamicin, and penicillin/streptomycin were purchased from Invitrogen Corp. (Carlsbad, CA). Bovine serum albumin (BSA) and dithiothreitol (DTT) were purchased from Fisher Scientific (Hampton, NH). Fetal bovine serum was purchased from Atlanta Biologicals (Norcross, GA, # S11050). RIPA buffer (10X) was purchased from Cell Signaling Technology (Boston, MA, #9806). Protease inhibitor cocktail (# P8340) was purchased from Sigma-Aldrich (St. Louis, MO). Phosphatase inhibitor cocktail (#

524625) was purchased from Calbiochem (La Jolla, CA). SuperSignal West Femto chemiluminescence substrate kit (#1856136) was purchased from Pierce (Rockford, IL).

2.2.2. Antibody

Rabbit polyclonal anti-Cx46 antibody was purchased from USBiological (Swampscott, MA, #C7858-07A). Mouse Anti-Cx50 (#33-4300), mouse anti-Cx26 (#13-8100) and mouse anti-α-tubulin antibodies were purchased from Zymed-Invitrogen (Carlsbad, CA). Mouse anti-β-actin antibody was purchased from Sigma (St. Louis, MO). Secondary anti-mouse IgG (#31430) and anti-rabbit IgG (#31460) conjugated with horseradish peroxidase were purchased from Pierce (Rockford, IL).

2.2.3. Cell culture and transfection

NN1003A rabbit lens epithelial cells, human lens epithelial cells (HLEC) and mouse neuronal N2A cells were cultured in Dulbecco's modified Eagle's medium (DMEM, low glucose; Invitrogen, CA) supplemented with 10% fetal bovine serum and 50 µg/mL gentamicin, 0.05 U/mL penicillin, and 50 µg/mL streptomycin , pH 7.4 at 37^{O}C in an atmosphere of 90% air and 10% CO_2. Breast cancer MCF-7 (Human breast adenocarcinoma cell) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). MCF-7 cells were grown in Minimal Essential Media (MEM, ATCC, # 30-2003) supplemented with 0.01 mg/ml bovine insulin and fetal bovine serum to a final concentration of 10%.

To generate stably transfected neuronal N2A cells overexpressing Cx46 or Cx43 the cDNA of rat Cx46 (Figure 2.1) or rat Cx43 (NCBI Reference Sequence: NM_012567.2) was sub-cloned into pEGFP-N3 vector (Clontech, Mountain View, CA) at the EcoR1 and BamH1 restriction sites in the proper reading frame with GFP coding sequences (Figure 2.2) and transfection was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The selection of transfected cells was done by growing cells in the presence of G418 antibiotic (500 µg /ml) for 6 weeks. The expression of Cx46-GFP and Cx43-GFP proteins in stably transfected cells were confirmed by western blot analyses using anti-GFP antibody.

```
   1 atgggcgact ggagcttcct ggggcggctg ctggagaatg cgcaggagca ctctacagtc
  61 atcggcaagg tgtggctgac cgtcctgttc atcttccgca ttctggtgct gggggcggca
 121 gccgaggagg tgtggggtga tgagcagtcg gacttcacct gcaacacaca gcagccaggc
 181 tgtgagaacg tctgctacga ccgtgccttc cccatctcgc acattcgctt ctgggcgctg
 241 caaatcatct tcgtgtccac gcccaccctc atctatctgg gccacgtgct gcacatcgtg
 301 cgcatggagg agaagaagaa agagcgggag gaggagctgc tgaggagaga caaccctcag
 361 cacggccgtg gtcgggaacc aatgcgtaca gggagcccgc gggacccgcc actgcgcgat
 421 gaccgtggca aggtgcgnat cgcaggcgcg ctgctgcgga cctacgtctt caacatcatc
 481 ttcaagacgc tcttcgaagt ggggttcatc gcgggccagt actttctata tggcttccag
 541 ctgcagccgc tttaccgctg cgaccgctgg ccctgcccca acaccgtgga ctgcttcatc
 601 tccaggccca cggagaagac catctttgtt atcttcatgt tggctgtggc ctgtgcgtca
 661 ctggtgctca acatgctaga gatttaccac ctgggctgga agaagctcaa gcagggagtt
 721 accaaccact tcaacccaga tgcctcagaa gtcaggcaca agcccttgga ccccctatcc
 781 gaggccgcca actctggccc tcccagcgtc tccattgggt tgccacctta ttacacacac
 841 cctgcctgtc ccacagtaca gggaaaggcc acagggtttc ctggggcccc actgctacca
 901 gcagacttca cagtggtgac cctaaacgat gcgcaaggca gaggccaccc ggtcaagcac
 961 tgcaatggcc accacctgac gacagagcag aactgggcca gcctaggggc agagccgcag
1021 actccagcca gcaagccctc ttcagcagcc tccagccctc atggccgcaa ggggctcact
1081 gacagcagtg gcagcagctt agaggagagt gccttggtgg tgacaccaga gggagagcag
1141 gctttggcga ccacagtgga gatgcactcc ccaccgttgg tcctcctgga cccagaaagg
1201 tccagcaagt ccagcagcgg acgagccaga ccaggtgact tggccatcta g
```

Figure 2.1 The cDNA sequence of rat Cx46 obtained from www.ncbi.nlm.nih.gov (NCBI Reference Sequence: NM_024376.1)

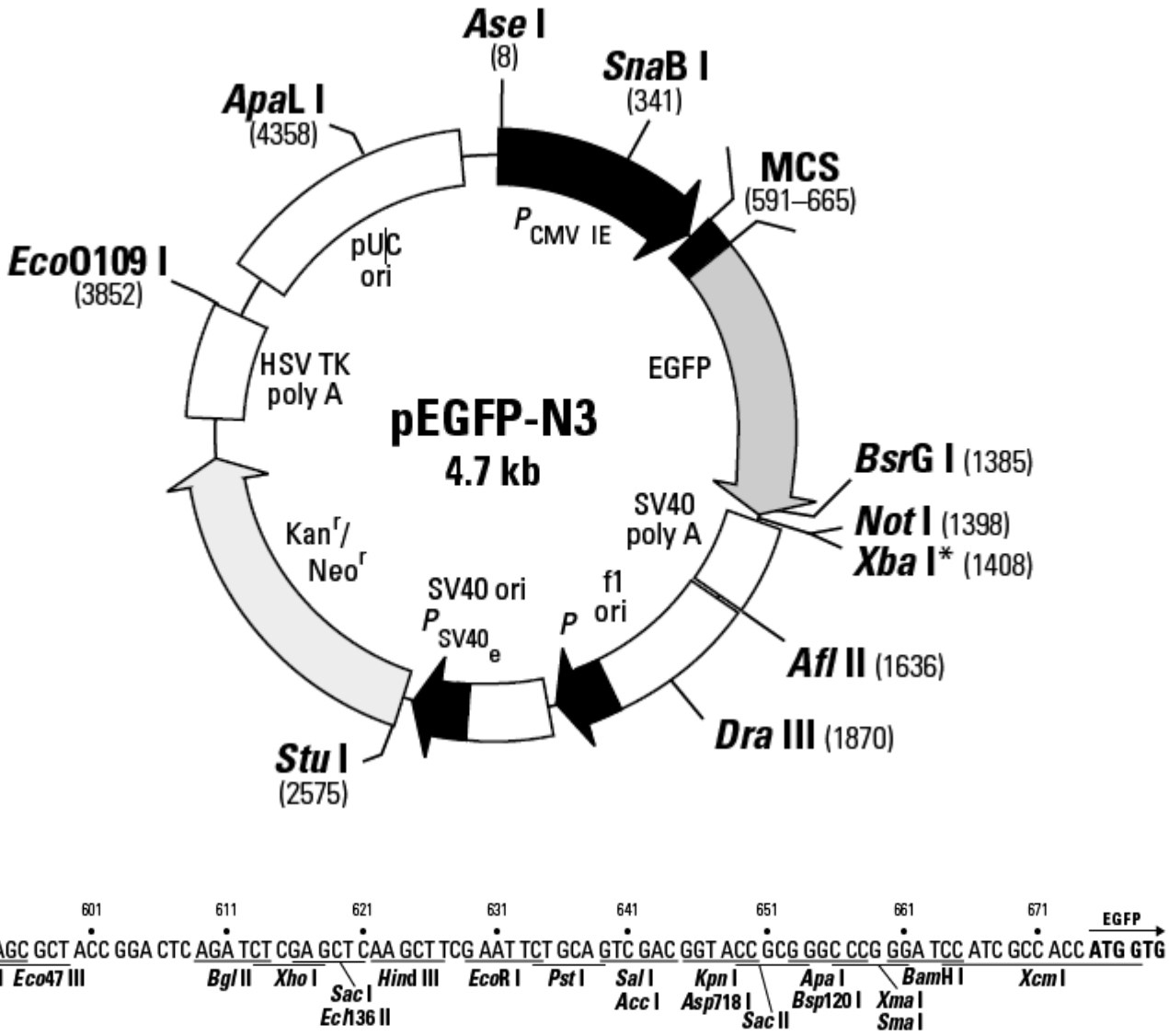

Figure 2.2 Restriction map and multiple cloning sites (MCS) of pEGFP-N3 vector (GenBank Accession #: U57609).

The rat cDNA of Cx46 and Cx43 was sub-cloned into EcoR1/BamH1 sites.

2.2.4. Whole cell homogenate preparations (WCH)

Cells were washed 3 times with cold phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 and a pH of 7.4), collected by scraping from plates and centrifuged at 4000 rpm for 5 min at 4^OC. 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 sec (3 times) and protein concentration of each sample was measured using Bio-Rad Protein Assay.

2.2.5. Western blot and densitometric analyses

Equal amounts of total protein in cell lysates were run into 10% SDS-PAGE and transferred to a nitrocellulose membrane (OPTI TRAN, Midwest Scientific, St. Louis, MO). The membrane was incubated with anti-Cx46 antibody for 6 hr at room temperature. For all other antibodies, the blot was incubated with primary antibody for overnight at 4^OC. The dilutions of the antibodies used are as follows; Anti-Cx46 antibody: 1:500, anti-Cx43 antibody: 1:8000, anti-GFP antibody: 1:2000, anti-tubulin antibody: 1:5000 and anti-β-actin antibody: 1:10000. The membrane then washed with 1 X TDN buffer (1 M NaCl, 0.2 N Tris, 0.04 M EDTA, pH 7.4) for 10 min, 3 times, and incubated with secondary anti-mouse IgG or anti-rabbit IgG (dilution 1:1500) conjugated

with horseradish peroxidase for 2 hr at room temperature. The membrane was again washed with 1 X TDN for 15 min, 4 times and then incubated with substrate SuperSignal West Femto Substrate kit (Pierce, Rockford, IL) followed by exposure to X-ray film (Midwest Scientific, St. Louis, MO).

For densitometric analysis the X-ray film was scanned and bands were digitized using UN-SCAN-IT software (Silk Scientific, Inc, Orem, Utah). The average pixel values were calculated for each Cx46 bands, normalized against average pixel values for loading control (tubulin or β-actin) and plotted in percentage (%) of control.

2.2.6. Reverse Transcriptase (RT)-PCR

RT-PCR analyses were performed by Dr. Gunjan Gakhar at Dr. T. A .Nguyen's lab, Department of Diagnostic Medicine/Pathobiology, Kansas State University. In brief, total RNA was purified from HMEC, MCF-7 and HLEC (human lens epithelial cells) using RNeasy Mini Kit (Qiagen, Valencia, CA) and RT-PCR was performed using one-step RT-PCR kit (Qiagen) according to the instruction manual with Cx46 cDNA specific primers and β-actin cDNA specific primers as control. The primers for Cx46 cDNA were 5′-CTG GCC CTG CTG GCC TTG-3′ and 5′-CCA CCA CCT GCT GAT GAC-3′. The primers for β-actin were 5′-GAA ATC GTG CGT GAC ATT AAG-3′ and 5′-CTA GAA GCA TTT GCG GTG GAC GAT-3′. For PCR, initial activation was done at 95^{O} C for 15 min, denaturation at 95^{O} C for 30 sec, annealing at 60^{O} C for 30 sec. PCR was performed at 35 cycles with final extension at 70^{O} C for 10 min. The PCR product was run into 1% agarose gel at 90V for 80 min. The gel was photographed using Fotodyne

software. The PCR products were eluted from the gel using QIAquick Gel Extraction Kit (Qiagen) and sent out for DNA sequencing at KSU-DNA sequencing facility, Department of Plant Pathology, Kansas State University for the confirmation of correct amplification of PCR products.

2.2.7. Immunohistochemical study

Immunohistochemistry was performed by Dr. Gunjan Gakhar at Dr. T. A .Nguyen's lab. Immunohistochemsitry was performed on paraffin-embedded human breast tumor tissue (Histologic type: Infiltrating ductal carcinoma; Histologic grade: Nottingham Grade 3; Tumor type: PT2, PN0, PMO with undetected lymphovascular invasion) by following standard protocol for 3, 3-diaminobenzidine (DAB) and using rabbit anti-Cx46 antibody (USBiological). In brief, human breast tissue slides were baked for 1 hr at 56 ^{O} C. Paraffin was washed off the tissue by rinsing three times in xylene for 5 min each followed by graded series of ethanol washes. Slides were washed with distilled water twice before incubating with antigen retrieval in a steamer for 20 min. Slides were incubated in 3% hydrogen peroxide and washed twice in PBS-T. Slides were blocked in horse serum for 1 hr and then rabbit anti-Cx46 (1:200, US biological) was applied for 1 hr. Slides were again washed for 15 min in PBS-T and incubated in secondary antibody (1:200) for 45 min. ABC Elite reagent was applied for 15 min before incubating with DAB. Slides were counterstained and mounted.

2.2.8. Hypoxia study

Hypoxia conditions were considered as 1% O_2, 5% CO_2 at 37^{O}C and 100% relative humidity. Hypoxia conditions were created by a Proox C21 hypoxic chamber (BioSpherix, Lacona, NY) using nitrogen and CO_2 as displacement gases. Normoxia conditions were considered as 21% O_2, 5% CO_2 at 37°C and 100% relative humidity. For hypoxia studies, 6×10^5 rabbit NN1003A cells were preincubated with DMEM low glucose media (supplemented with 10% FBS) at 21% O_2, 5% CO_2 (normoxic conditions) for 12 hr. Following this incubation, the media was replaced with DMEM low glucose complete media that was pre-equilibrated to 1% O_2. Then the cells were incubated under hypoxic conditions (1% O_2, 5% CO_2) in Proox C21 hypoxic chamber (BioSpherix) and harvested after 1–7 days. Whole cell lysates were run in 8% SDS-PAGE followed by western blot to check the expression levels of different connexins.

2.2.9. siRNA transfection

A set of four siRNAs, against Cx46, were purchased from Qiagen (Valencia, CA). The siRNAs and their target sequences are presented below. The siRNA with catalog number SI00131670 and target sequence: 5'-CGC ATG GAA GAG AAG AAG AAA-3' was able to produce the most effective gene silencing effect. This siRNA was used in all the experiments thereafter and designated as "anti-Cx46" siRNA. Negative non-silencing control siRNA (a siRNA with no homology with mammalian

genes) was also purchased from Qiagen. HLEC (human lens epithelial cells) or MCF-7 cells were cultured under normoxic conditions (5% CO_2, 21% O_2) on 60-mm dishes. 2 × 10^6 HLEC or MCF-7 cells were transfected with 512 ng of Cx46 siRNA (final concentration 10 nM) or 512 ng negative control siRNA (10nM) and 20 µl of HiPerFect transfectant reagent (Qiagen). After transfection, cells were incubated under normoxic conditions (21% O_2, 5% CO_2) and harvested at different time intervals (24 hr and 48 hr for HLEC and 12 hr, 24 hr and 48 hr, for MCF-7 cells). Whole cell lysates were analyzed by western blot.

SiRNA	Catalog Number	Target Sequence
1	SI03094203	CTG CAC ATC GTG CGC ATG GAA
2	SI00131656	ACC GGA GGA CTT GGC CAT CTA
3 (Anti-Cx46 siRNA)	SI00131670	*CGC ATG GAA GAG AAG AAG AAA*
4	SI00131663	CTG CGC GTC CCT GCT GCT CAA

Table 2.1 The siRNAs used to knock-down Cx46

2.2.10. Cell viability assay

Cell viability assay was performed using CellTiter-Blue® Cell Viability Assay kit (Promega, Madison, WI). The kit applies the fluorometric detection of resorufin

converted from resazurin (Ahmed, Gogal et al. 1994) by viable cells. The amount of fluorescence measured is directly proportional to the number of viable cells. For this assay, 3 X 10^4 HLEC or MCF-7 cells, in 100 µL of DMEM low glucose media or MEM media respectively, were seeded into each well in 96-well microtiter plates and cultured for 14 hr at 37^{O}C under normoxic conditions (21% O_2, 5% CO_2). The cells were then transfected with 10 ng (per well, final concentration 10 nM) of Cx46 siRNA or 10 ng negative nonsilencing siRNA along with 0.75 µL (per well) of HiPerFect transfectant reagent and incubated for 24 hr under normoxic conditions (21% O_2, 5% CO_2). Cells with no siRNA treatment were considered as controls. Following this incubation, the media for HLEC or MCF-7 cells were changed with 100 µl (per well) of their respective media that was equilibrated to 1% O_2. Then the cells were incubated under hypoxic conditions (1% O_2, 5% CO_2) in a Proox C21 hypoxic chamber (BioSpherix, CA) for different time intervals depending on the cell type (6 hr, 12 hr, 18 hr and 36 hr for HLEC and 12 hr and 24 hr for MCF-7 cells). Another set of 96-well plates of HLEC or MCF-7 cells with similar siRNA treatment containing 100 µl media but equilibrated to 21% O_2 were incubated under normoxic conditions (21% O_2, 5% CO_2) for the same interval of time. For the estimation of number of viable cells, 20 µL of Cell Titer-Blue reagent was added in each well for MCF-7 or HLEC incubated under hypoxic or normoxic conditions and following incubation, for 4 hr for HLEC or 3 hr for MCF-7 cells, at 37^{O}C, fluorescence was recorded at $560(5)_{Ex}/590(5)_{Em}$.

To determine the cell viability of wild-type N2A cells and N2A cells overexpressing Cx46-GFP or Cx43 GFP under hypoxic or normoxic conditions, 3 X 10^4

cells (per well of 96-well microtiter plate) were cultured in 100 µL DMEM low glucose media for 14 h at 37^{O}C under normoxic conditions (21% O_2, 5% CO_2). Then the media was replaced with 100 µL (per well) DMEM low glucose media equilibrated to 1% O_2 or 21% O_2. The cells were then incubated under hypoxic or normoxic conditions and cell viability was assessed every 4 hr for up to 24 hr. To measure the number of viable cells 20 µL of Cell Titer-Blue reagent was added in each well, incubated for 4 hr at 37^{O}C and fluorescence was recorded at $560(5)_{Ex}/590(5)_{Em}$.

2.2.11. Cell fractionation

Cell fractionation was performed using Qproteome Cell Compartment Kit (Qiagen, CA, #37502) according to the instruction manual. In brief, 5 X 10^6 MCF-7 cells were collected by scraping and washed with ice-cold phosphate-buffer saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). Cells were centrifuged at 500g for 10 min and cell pellet was lysed in 1mL ice cold extraction buffer CE1 containing protease inhibitor solution (1:100). Then, the lysate was centrifuged at 1000g for 10 min at 4^{O} C and the supernatant containing cytosolic proteins fraction was collected. The pellet was further resuspended in 1mL ice-cold extraction buffer CE2 and incubated for 30 min on a shaker. Resuspended cell pellet was again centrifuged at 6000g for 10 min and supernatant representing membrane protein fraction was collected. The nuclear proteins fractions was isolated by treating the cell pellet, obtained after above centrifugation step, with 7uL Benzonase Nuclease and extraction buffer, CE3, followed by centrifugation at 6800g for 10 min. The supernatant, thus obtained,

contained nuclear proteins. All fractions, cytosolic, membrane and nuclear proteins, were quantitated by Bio-Rad Protein Assay and analyzed by western blot.

2.2.12. Immunofluorescence study

MCF-7 cells were grown on cover-slips in a 12 well plate. When confluency reached to 70-80%, cells were fixed in 4% paraformaldehyde for 10 min followed by permeabilization with 0.05% Tritron-X100 for 30 min. Then the cells were blocked in 3% bovine albumin solution (BSA) for 2 hr at 4^{O}C. After blocking, cells were incubated with anti-Cx46 antibody (dilution 1:200) for overnight at 4^{O} C. Cells were washed 3 times with PBS buffer and incubated with secondary Alexa Fluor-568 (red color, dilution 1:200) anti-rabbit antibody for 2 hr at room temperature. The nuclei were counterstained with DAPI (blue color, dilution 1:1000). The cover-slips were then washed 3 times in cold PBS and mounted onto slides using ProLong Gold Antifade reagent. The images were taken using a Leica DMI 6000 B microscope (Leica, Deerfield, IL).

2.2.13. Human breast tumor and normal tissues

The human breast tumor tissue lysates were purchased from Protein Biotechnologies, Ramona, CA. The characterizations of human breast tumors tissues are as follows: Tumor breast tissue 1: Infiltrating Ductal Carcinoma, Grade 2, Stage IIA. T2N0M0, source: female, 42 years. Tumor breast tissue 2: Invasive Ductal Carcinoma, Grade 2, Stage IIA. T2N0M0, source: female, 42 years. The human adult normal breast

tissue lysate was purchased from Novus Biologicals, Littleton, CO. Twenty microgram of total protein of each lysate was loaded and run in 8% SDS-PAGE. The blot was probed with rabbit polyclonal anti-Cx46 antibody

2.2.14. Xenograft tumors of MCF-7 cells in Nu/Nu mice

Nu/Nu mice (strain NuFox^{n1}) were purchased from Charles River Laboratory (Wilmington, MA). Mice were implanted with 17 β-estradiol (1.7 mg / pellet) one week before the injection of 1×10^7 MCF-7 breast cancer cells subcutaneously into the inguinal region of mammary fat pad. Cell viability of MCF-7 cells was performed before the injection. Mice were observed for any change in behavior, appearance or weight. Two weeks after MCF-7 cell injection, tumors were injected with 7.5 µg of anti-Cx46 siRNA or negative non-silencing siRNA or no siRNA (control) every 48 hr for minimum of 10 days to maximum of 18 days. Depending on the size of each tumor the siRNAs were injected directly at 2 or 3 different locations. The tumor size was measured in 2 dimensions by a caliper every other day before injection. The tumor volume was estimated by the formula: tumor volume = a (b^2)/2, where a and b are the tumor length and width, respectively, (Gieseg, Man et al. 2004) in mm. The tumor size measured before first siRNA injection is considered Day 0 measurement. After 10 days of first injection, 2 anti-Cx46 siRNA treated, 1 negative non-silencing siRNA treated and 1 control (no siRNA) tumors were dissected out. The size of two anti-Cx46 siRNA treated tumors became almost undetectable at Day 12. Three anti-Cx46 siRNA treated tumors were dissected out at Day 16. The rest of the anti-Cx46 siRNA treated tumors were isolated from euthanized mice at Day 18. Tumors were dissected out, homogenized and

lysed in RIPA buffer. The lysates were sonicated for 10 sec for 3 times. Whole tumor tissue lysates were quantitated by Bio-Rad Protein Assay and analyzed by western blot.

2.2.15. Statistical analysis

For statistical analysis “Origin” software (Microcal Software Inc., Northampton, MA) was used. The level of significance (see “*” in figure legends) was considered at $p < 0.01$ or $p < 0.001$ using paired *t* test analyses. All data are presented as mean ± s.e.m. of at least 3 independent experiments.

2.3. Results

2.3.1. The effect of hypoxia on connexin isoforms present in lens

The Cx46 is highly expressed in the fiber cell region which is located at the hypoxic center of the lens. Therefore, I first determined the effect of low oxygen on different connexin isoforms expressed in lens cells. For this purpose, rabbit lens epithelial NN1003A cells were incubated under hypoxic conditions (1% O_2, 5% CO_2) as well as under normoxic conditions (21% O_2, 5% CO_2). We found that the Cx46 protein level was elevated during 4–7 days of hypoxia in rabbit NN1003A lens epithelial cells (Figure 2.3). Interestingly, increase in Cx46 protein was concomitant with decrease in Cx43 protein level during 4-7 days of low oxygen treatment, whereas, Cx50 protein level was unchanged (Figure 2.3). However, normoxia treatment (21% O_2, 5% CO_2) had no effect on expression level of any of the connexins.

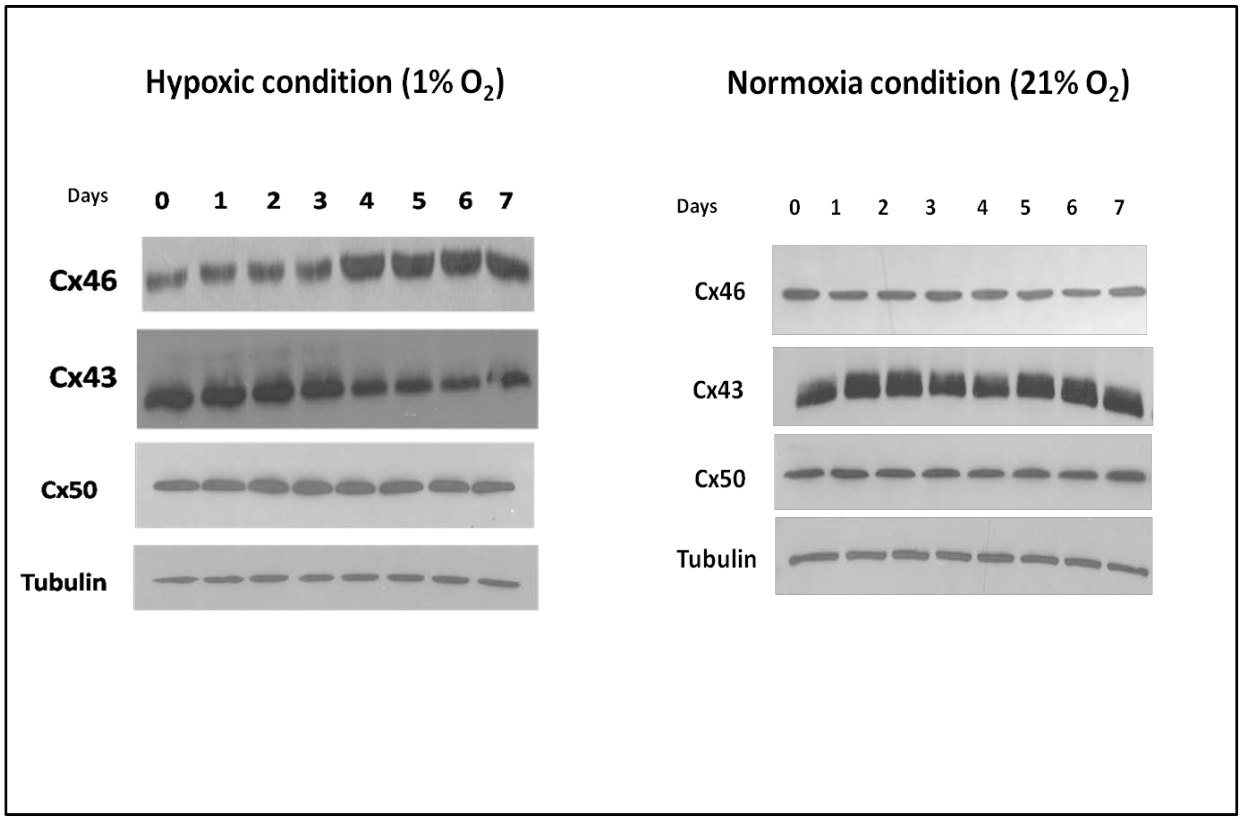

Figure 2.3 The upregulation of Cx46 protein in response to 1% O_2 (hypoxia) in rabbit lens epithelial NN 1003A cells.

Cells were subjected to hypoxia or normoxia and harvested at 0–7 days. Connexin protein levels were analyzed by western blot using antibody against Cx46, Cx43 and Cx50. Tubulin blot acted as a loading control.

2.3.2. Cx46 protects human lens epithelial cells from hypoxia-induced death

As Cx46 protein was increased under hypoxia, I speculated whether lens epithelial cells are hypoxia-resistant due to the Cx46 protein. I downregulated Cx46 protein in human lens epithelial cells (HLEC) using selective siRNA (Figure 2.4) and

investigated the effect of Cx46 downregulation on cell viability under hypoxic conditions (1% O_2, 5% CO_2). A set of four siRNAs (see Methods and Materials for details) was used separately to knock down Cx46 with the purpose of identifying the most potent siRNA for using in subsequent experiments (Figure 2.4A). As seen in the figure, the siRNA 3 (or anti-Cx46 siRNA) was able to produce maximum Cx46 protein knockdown as determined by western blot (Figure 2.4A). HLEC, treated with the anti-Cx46 siRNA, showed about 85% selective knockdown of Cx46 protein expression after 24 hr and 48 hr of transfection (Figure 2.4B and D). The action of the anti-Cx46 siRNA was specific as it did not result in knocking down another connexin, Cx50, which shares considerable sequence similarity with Cx46. Cells transfected with negative non-silencing siRNA (negative control) showed no alteration in Cx46 protein level (Figure 2.4C).

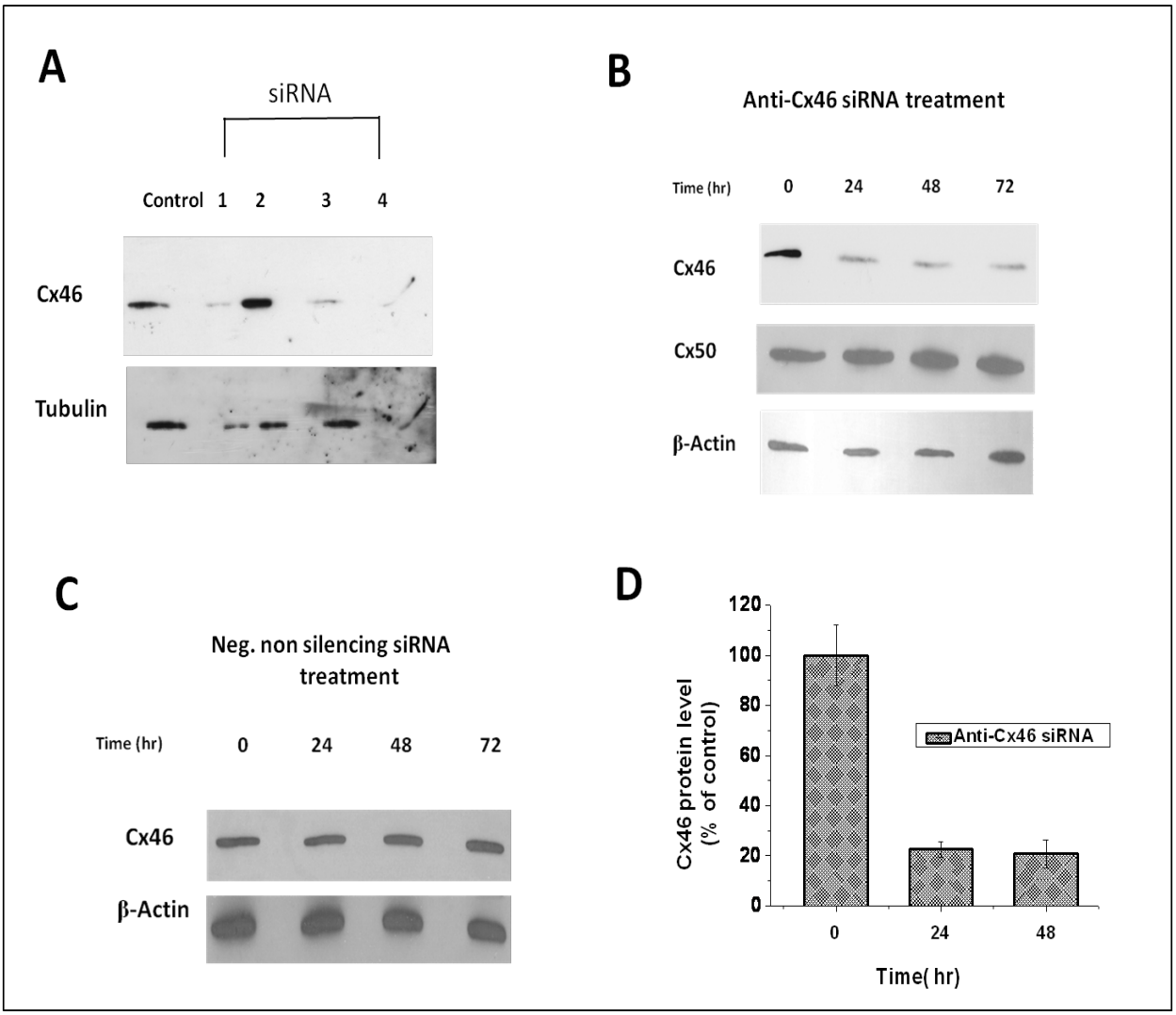

Figure 2.4 siRNA knockdown of Cx46 in HLEC.

(A) Western blot analyses of Cx46 protein in HLEC (2×10^6) control (untransfected) or transfected with 512 ng siRNA 1,siRNA 2, siRNA 3 and siRNA 4 (final concentration 10 nM) for 24 hr. siRNA 3 or anti-Cx46 siRNA showed maximum knockdown of Cx46 protein. **(B)** Western blot analyses of Cx46 and Cx50 protein in HLEC (2×10^6) transfected with 512 ng anti-Cx46 siRNA (final concentration 10 nM) or **(C)** 512 ng negative nonsilencing siRNA for 0-72 hr. Cx50 blot was done to show specific action of the siRNA. **(D)** Densitometric analyses showed effective knockdown (more than 85%) of Cx46 was achieved using anti-Cx46 siRNA. Cx46 and tubulin (control) bands were digitized using UN-SCAN-It software. The average pixel value for each Cx46 band was

normalized against average pixel value for corresponding tubulin band and plotted in % of control.

Next, I analyzed the effect of Cx46 knockdown on HLEC viability under hypoxic conditions (1% O_2, 5% CO_2). HLEC, with Cx46 knocked-down, showed significant reduction in cell viability under hypoxia (Figure 2.5A). Anti-Cx46 siRNA treated HLEC viability was reduced to 23% at 6 hr, 38% at 12 hr and 42% at 18 hr compared with untreated (control) or non-silencing siRNA treated cells (Figure 2.5A) under low oxygen. Interestingly, Cx46 downregulation had no effect on HLEC viability under normoxic conditions (Figure 2.4B) which suggested that Cx46 provides protections to lens cells only against hypoxia-induced death.

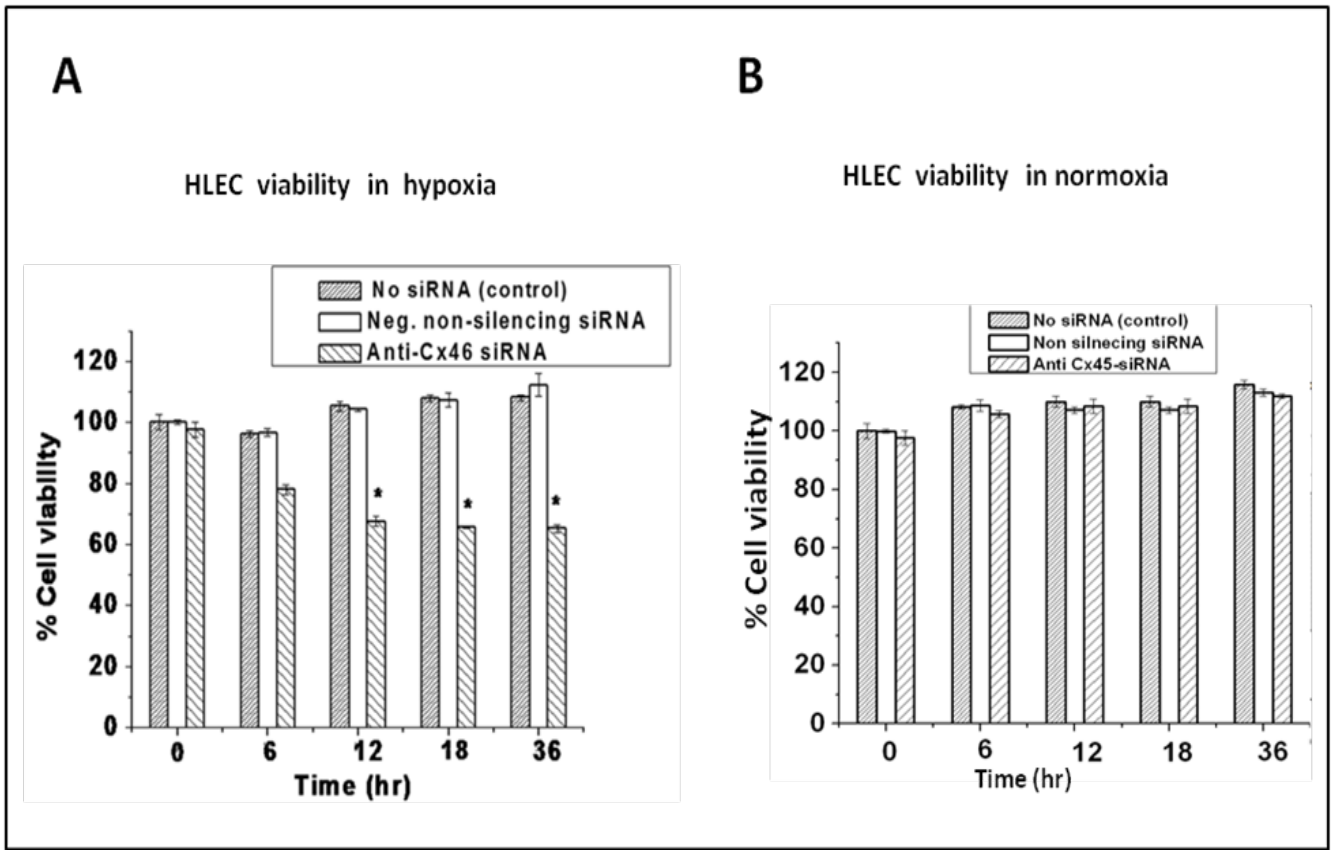

Figure 2.5 Cx46 downregulated human lens epithelial cells (HLEC) are susceptible to hypoxia induced death.

A total of 3×10^4 HLEC (per well of 96-well microtiter plate) were treated with 10 ng (per well, final concentration 10 nM) of anti-Cx46 siRNA or non-silencing siRNA (10 nM) or no siRNA (control) and preincubated for 24 hr under normoxic conditions (21% O_2, 5% CO_2). Following that the cells were incubated under hypoxia **(A)** or normoxia **(B)** and cell viability was measured by fluorometric resazurin reduction method at 6, 12, 18 and 36 hr intervals. Data are represented as mean ± s.e.m of 3 independent experiments. Asterisk indicates significant statistical difference ($p < 0.01$) between indicated data and controls (cell viability at 12, 18 and 36 hr for no-siRNA or negative control siRNA treatment).

2.3.3. Overexpression of Cx46 increases the cell viability of hypoxia-susceptible neuronal N2A cells in hypoxic conditions

To further establish the role of connexin46, Cx46 and Cx43 were stably overexpressed as a GFP tagged fusion protein in hypoxia-sensitive murine neuronal N2A cells and cell viability was assessed under hypoxic conditions. N2A cells are gap junction deficient. They do not express endogenous Cx46 or Cx43 proteins (Figure 2.6A). N2A cells transfected with Cx43-GFP or Cx46-GFP expressed fusion proteins at predicted molecular weight of ~71 kDa and 73 kDa, respectively (Figure 2.6B) as determined by western blot using anti-GFP antibody. These N2A cells overexpressing Cx46-GFP or Cx43-GFP were incubated under hypoxic conditions (1% O_2, 5% CO_2) or normoxic conditions (21% O_2, 5% CO_2) and cell viability was assessed by fluorometric resazurin reduction method. Wild-type N2A cells were hypoxia sensitive as they began to die after 4 hr under hypoxic conditions and cell viability was reduced by 40% at 12 hr and almost by 90% at 24 hr (Figure 2.7A). The cell viability of N2A cells overexpressing Cx43-GFP showed the same pattern as wild-type N2A cells with cell viability reducing significantly during 4–24 hr time period at 1% O_2 (Figure 2.7A). But interestingly, N2A cells overexpressing Cx46-GFP remained viable to a considerable extent even after 12 hr at 1% O_2. The cell viability of these cells was only reduced by 3% at 12 hr and 51% at 24 hr at 1% O_2. No reduction in cell viability was observed for wild type, Cx46-GFP or Cx43-GFP stably transfected cells under normoxic conditions (Figure 2.7B). Taken together, the results suggested that Cx46 can confer protection to a hypoxia sensitive cell while another connexin, Cx43, cannot.

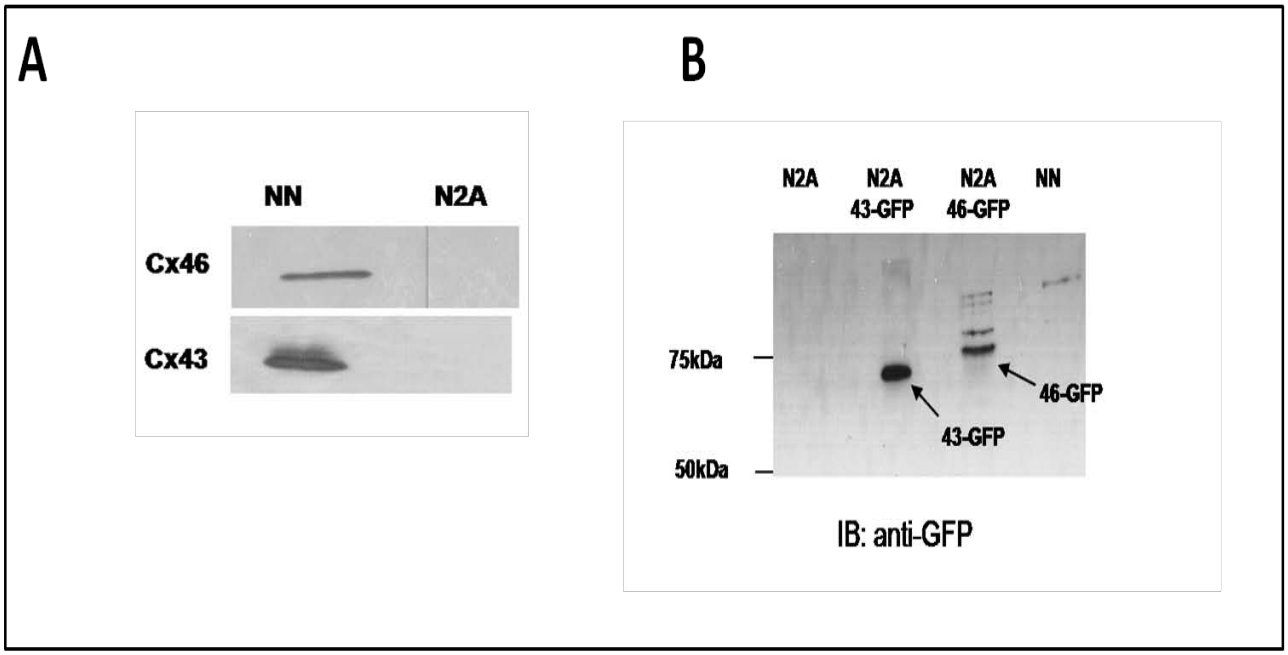

Figure 2.6 The endogenous and exogenous expression of Cx46 and Cx43 proteins in N2A cells

(A) Western blot analyses of Cx46 and Cx43 in N2A cells. 20 µg of total protein was loaded in each lane. Rabbit lens epithelial NN1003A cell lysate was also loaded as positive control for anti-Cx46 and anti-Cx43 antibodies. **(B)** Western blot analyses of stable overexpression of Cx46-GFP and Cx43-GFP proteins in neuronal N2A cells. The blot was probed with antibody against GFP. Stably transfected N2A cells overexpressing Cx46-GFP or Cx43-GFP were generated by transfecting cells with plasmid encoding Cx46-GFP or Cx43-GFP and then growing the cells for 6 weeks in the presence of G418 antibiotic.

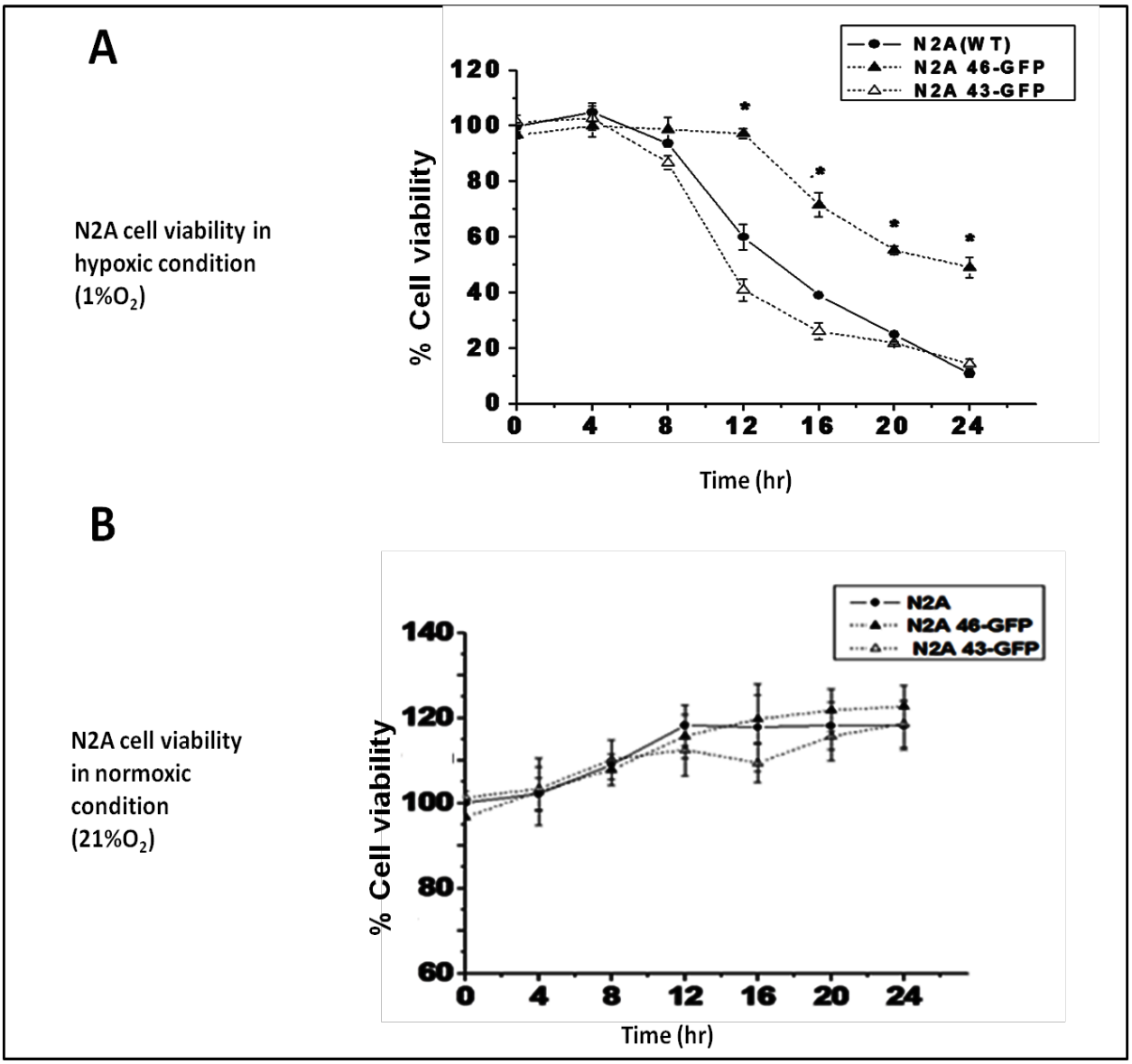

Figure 2.7 Overexpression of Cx46 in hypoxia sensitive neuronal N2A cells confers survival in hypoxia (1% O_2).

A total of 3 × 10^4 wild-type N2A cells or N2A cells, stably overexpressing Cx46 or Cx43, were incubated under **(A)** hypoxic conditions (1% O_2, 5% CO_2) and **(B)** normoxic conditions (21% O_2, 5% CO_2). The viability was assessed every 4 hr for up to 24 hr by fluorometric resazurin reduction assay. The data are plotted as mean ± s.e.m of three independent experiments. Asterisk indicates the statistical significance ($p < 0.01$) between indicated data and control (N2A-WT).

2.3.4. Cx46 expression in breast cancer cells and breast tumor tissues

Tissues, other than lens, reported to express Cx46 protein are lung adenoma and lung alveolar cells (Abraham, Chou et al. 1999; Avanzo, Mesnil et al. 2006), Schwann cells(Chandross 1998) and bone osteoblastic cells (Koval, Harley et al. 1997). Here I showed, for the first time, that human breast cancer cells and human breast tumor tissues express Cx46 protein. Expression of Cx46 protein was detected in MCF-7 human breast adenocarcinoma cells as determined by western blot analyses; however, in normal human mammary epithelial cells (HMEC) the expression was not seen (Figure 2.8A). In contrast, Cx43 protein was strongly expressed in HMEC and weakly expressed in MCF-7 cells. The expression of Cx46 was further confirmed by RT-PCR which showed the presence of Cx46 mRNA in MCF-7 cells but not in HMEC (Figure 2.8B). Next, I investigated the expression of Cx46 protein in human breast tumor tissue. Indeed, Cx46 protein was present in premetastatic breast tumor as determined by immunohistochemistry (Figure 2.8C). In addition, immunoblot analyses showed that the Cx46 protein was highly expressed in human tumor tissues as compared with normal breast tissue (Figure 2.8D).

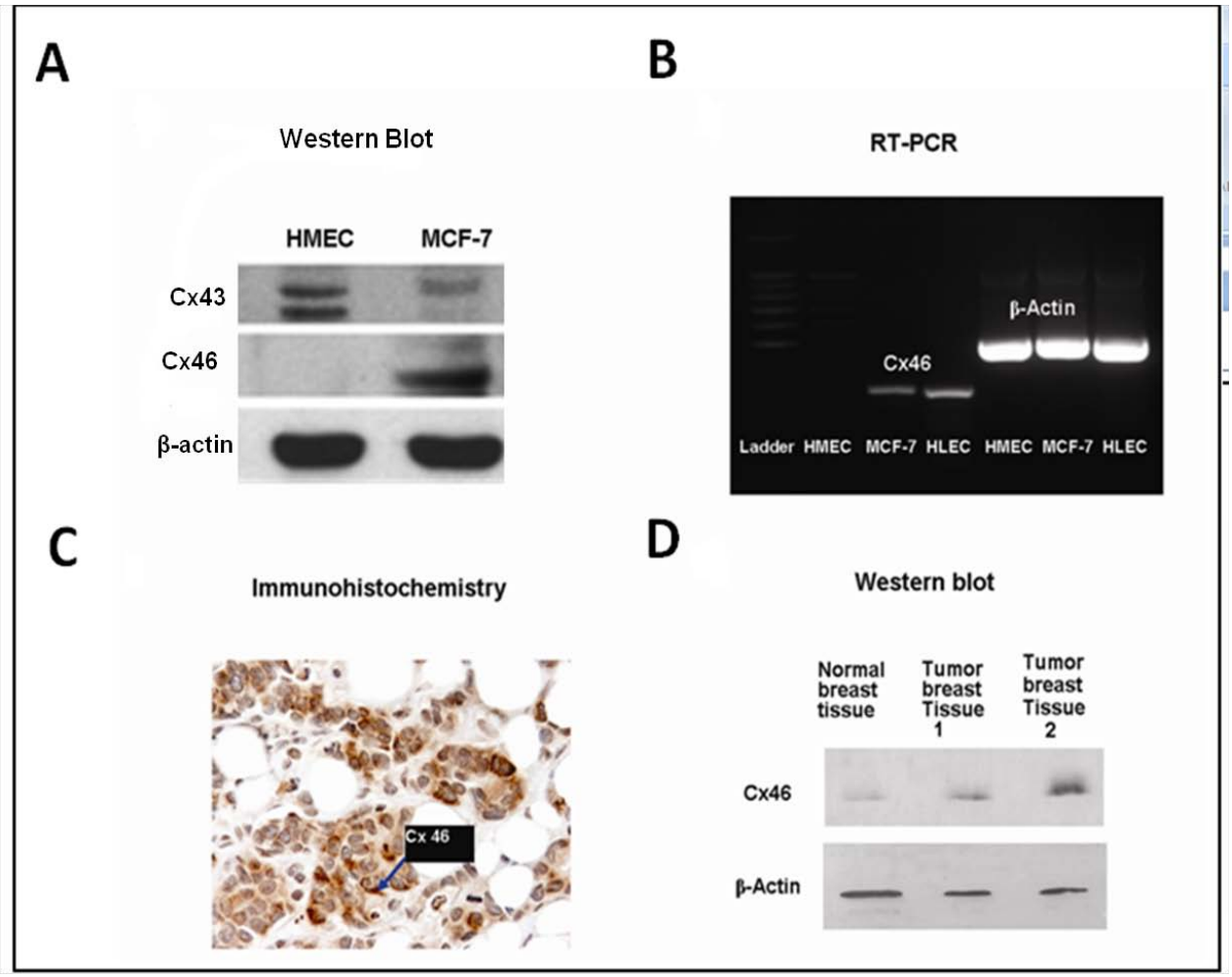

Figure 2.8 Cx46 is expressed in human breast cancer cells and human breast tumors.

Breast cancer cell MCF-7 express Cx46 protein as determined by western blot **(A)** and RT-PCR study **(B).** HMEC, Human mammary epithelial cells. **(C)** Immunohistochemistry showing the expression of Cx46 protein in premetastatic breast tumors. The blue arrow indicates Cx46 staining. The characterization of this tumor breast tissue is infiltrating ductal carcinoma, Grade 3, T2NOMO. **(D)** western blot analyses showing Cx46 protein is upregulated in premetastatic breast tumor tissue. Tumor breast tissue 1: Infiltrating Ductal Carcinoma, Grade 2, Stage IIA. T2N0M0, source: female, 42 years; Tumor breast tissue 2: Invasive Ductal Carcinoma, Grade 2, Stage IIA. T2N0M0, source: female, 54 years. [The experiments that generated (A), (B) and (C) of this figure were

carried out at Dr. T. A. Nguyen's lab in Department of Diagnostic Medicine/Pathobiology, Kansas State University.]

I also determined the subcellular localization of Cx46 protein in MCF-7 cells. Immunofluorescence study with Cx46 antibody showed staining mostly at the cytoplasmic region (perinuclear region) (Figure 2.9A). The presence of Cx46 gap junction plaques at the cell-cell interfaces was not detected (Figure 2.9A). To confirm this, I performed subcellular fractionation of MCF-7 cells to isolate cytosolic, membrane and nuclear proteins. These fractions were then analyzed by western blot using anti-Cx46 antibody that showed the presence of majority of Cx46 protein in the cytosolic fraction (Figure 2.9B). The membrane fraction also showed the presence of Cx46 immunoreactive band. Since the membrane fraction contained plasma membrane and endoplasmic reticulum (ER) membrane proteins (Figure 2.9B), therefore, the presence of Cx46 at plasma membrane could not be ruled out.

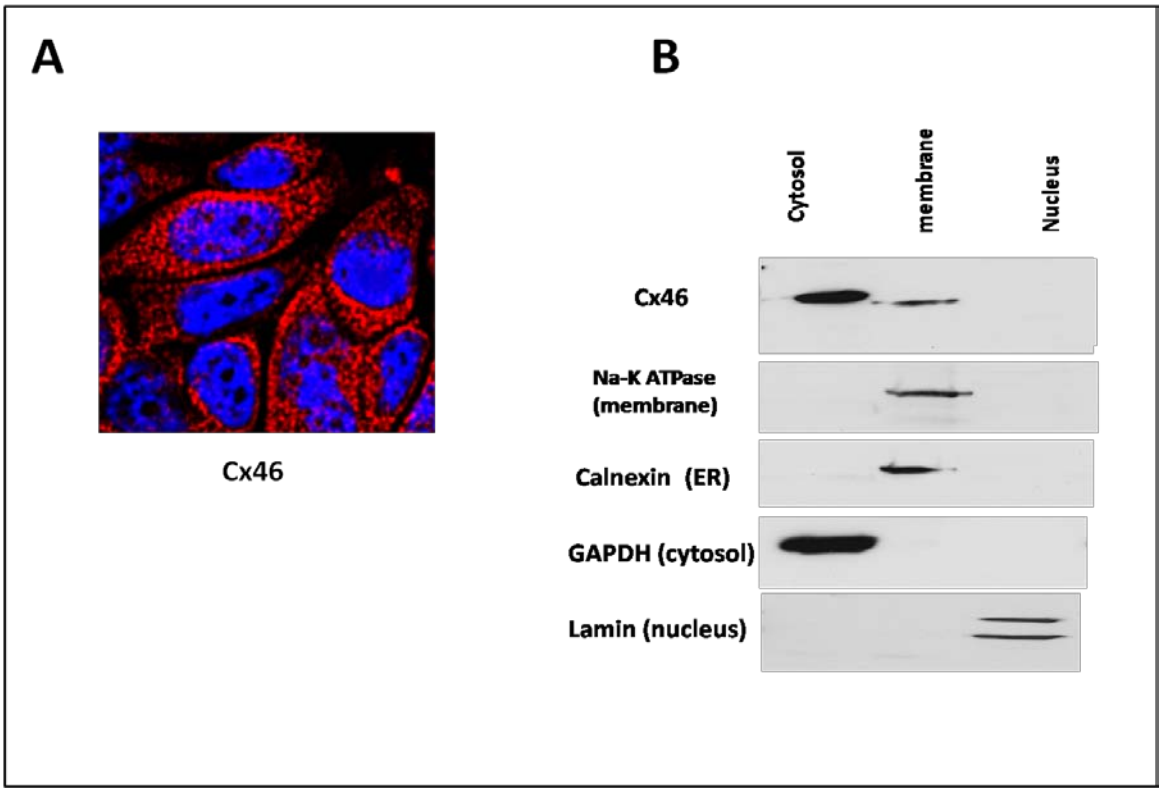

Figure 2.9 Localization of Cx46 protein in MCF-7 cells.

(A) Immunofluorescence analysis of MCF-7 cells stained with anti-Cx46 antibody (red) and nuclear stain DAPI (blue).

(B) Western blot analyses of Cx46 protein of different subcellular compartments of MCF-7 cells. Cells were fractionated into cytosolic, membrane and nuclear compartments using Qproteome Cell Compartment Kit. Equal amounts of total protein, from each fraction, were subjected to SDS-PAGE followed by western blots. The blots were also probed with antibodies against a plasma membrane marker protein (Na^+-K^+ ATPase), endoplasmic reticulum marker protein (Calnexin), cytosolic marker (GAPDH), and nuclear marker (Lamin) to show that proper fractionation was achieved.

2.3.5. Downregulation of Cx46 in MCF-7 cells increases hypoxia-induced cell death

The presence of Cx46 in MCF-7 cells and absence in HMEC led us to hypothesize that breast cancer cells and breast tumors also use Cx46, as an adaptation to hypoxia, to survive and grow. To confirm this, Cx46 protein was downregulated in MCF-7 cells using anti-Cx46 siRNA (Figure 2.10A) to more than 60% and 80% (Figure 2.10B) after 24 hr and 48 hr of transfection, respectively. Downregulation of Cx46 remarkably reduced the MCF-7 cell viability to approximately 34% at 12 hr and 40% at 24 hr under hypoxic conditions (1% O_2, 5% CO_2) as compared with control (no siRNA) or negative non-silencing siRNA treated cells (Figure 2.11). As also seen in the case for HLEC, the downregulation of Cx46 had no effect on MCF-7 cell viability under normoxia (Figure 2.11). These data clearly demonstrated that human breast cancer cells, MCF-7, also utilize Cx46 to survive against death caused by hypoxia.

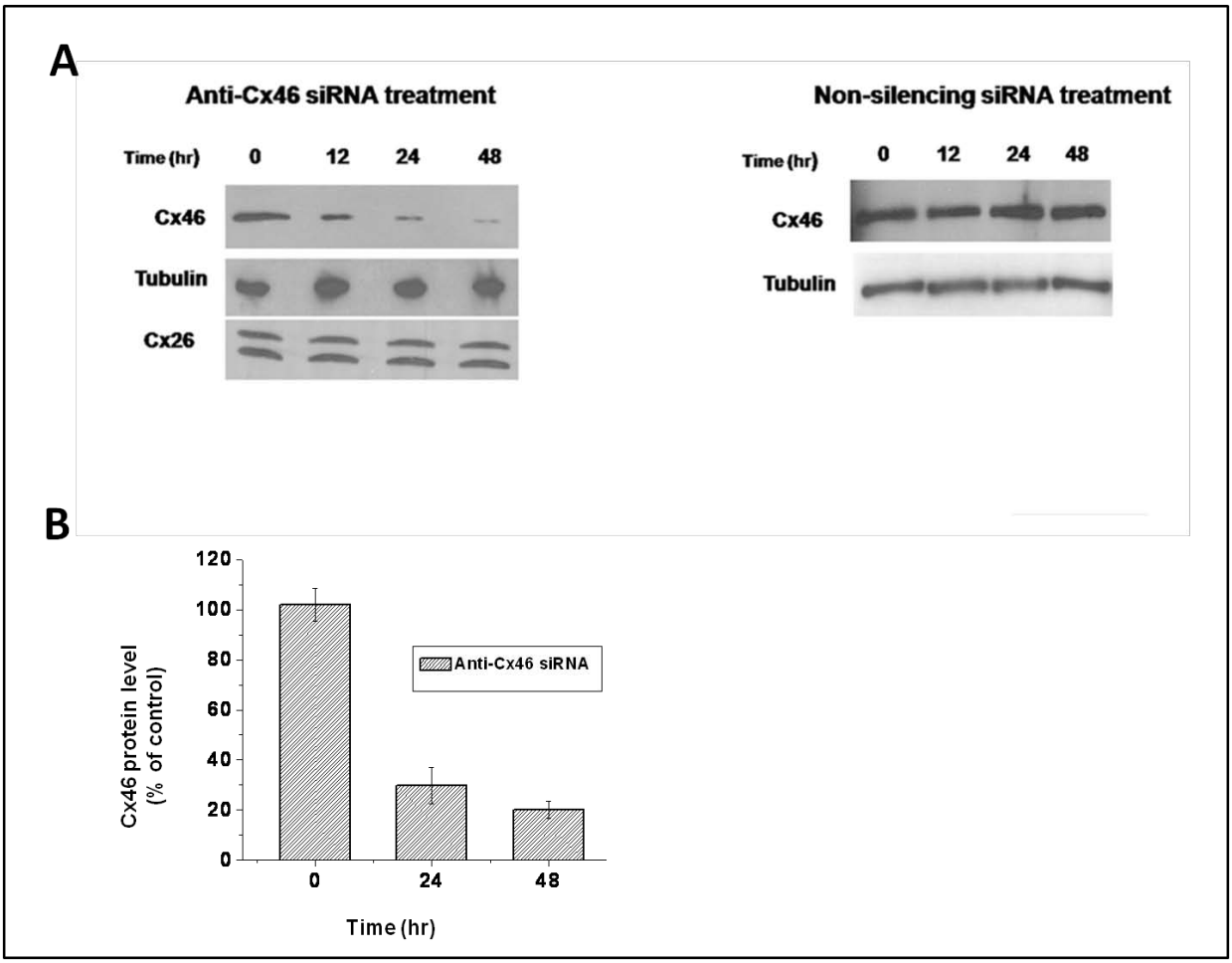

Figure 2.10 siRNA mediated downregulation of Cx46 in MCF-7 cells.

(A) A total of 2 × 10^6 MCF-7 cells were transfected with 512 ng anti-Cx46 siRNA (final concentration 10 nM) or negative non-silencing siRNA (10nM). Level of knockdown was determined by western blots using anti-Cx46 antibody at 12, 24 and 48 hr after transfection. The same blots were probed with anti-tubulin antibody to show equal loading of protein in each lane. Cx26 blot was done to show the absence of any nonspecific action of anti-Cx46 siRNA in MCF-7 cells for another connexin protein. **(B)** Densitometric analyses shows maximum knockdown was achieved between 24 and 48 hr time period.

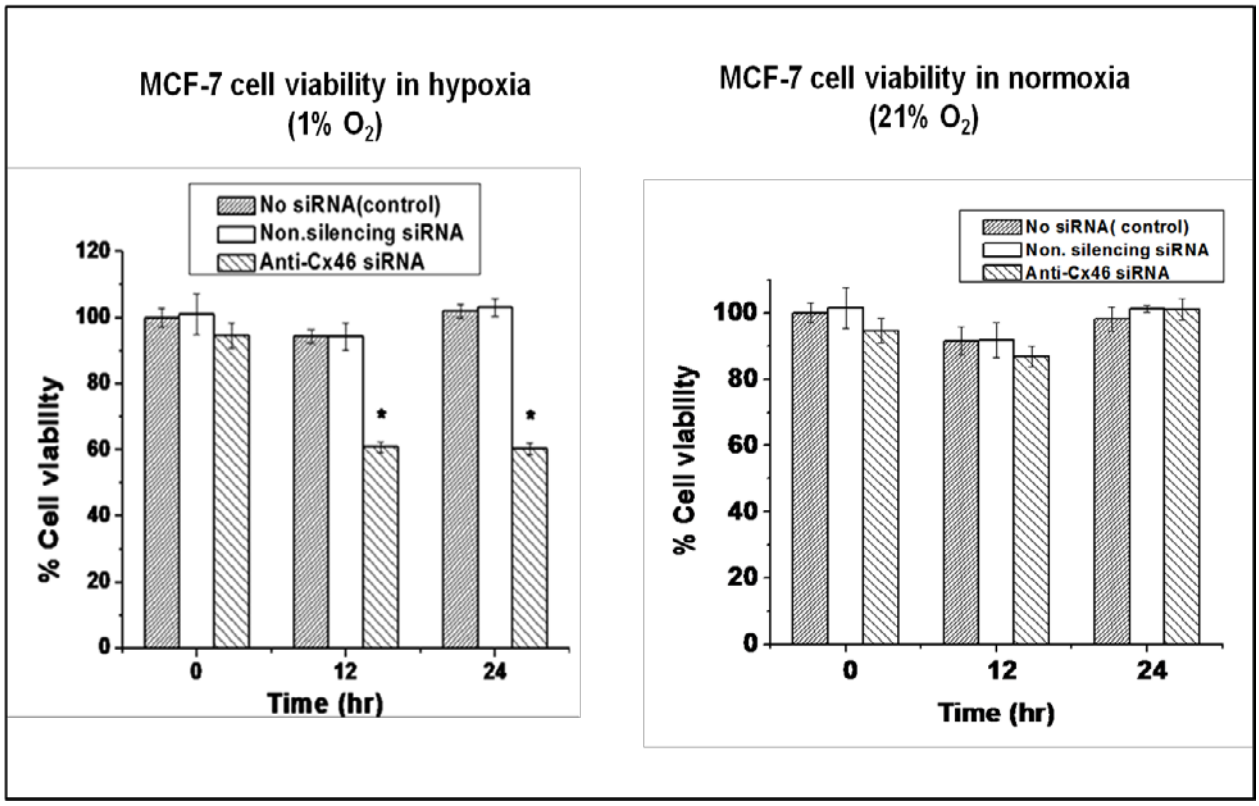

Figure 2.11 Downregulation of Cx46 in MCF-7 cells decreases cell viability under hypoxic conditions.

3×10^4 MCF-7 cells (per well of 96 well microtiter plate) were preincubated with 10 ng (per well, final concentration 10 nM) negative non-silencing siRNA or anti-Cx46 siRNA for 24 hr at normoxic conditions (21% O_2, 5% CO_2) and then cells were kept at hypoxic conditions (1% O_2, 5% CO_2) or normoxia. Cell viability was assessed at 12 hr and 24 hr. The data are represented as mean ± s.e.m of three independent experiments. The asterisk indicates significant statistical difference ($p < 0.01$) between indicated data and controls (cell viability at 12 and 24 hr for no-siRNA or negative control siRNA treatment).

2.3.6. Downregulation of Cx46 inhibits the growth of MCF-7 tumor xenografts in nude mice

To further investigate the function of Cx46 as a hypoxia survival factor, I carried forward our work to an *in vivo* human tumor-bearing xenograft nude mouse system. I asked whether Cx46 plays a similar and significant hypoxia-protective role in the growth of human breast tumors *in vivo*. Estrogen stimulated immunodeficient Nu/Nu mice were injected with 1×10^7 MCF-7 cells into the inguinal region of mammary fat pad to develop tumor of human origin. Two weeks after MCF-7 cells injection, newly formed tumors were directly injected with 7.5 µg of anti-Cx46 siRNA (*n* = 10 tumors) or negative non-silencing siRNA (*n* = 7 tumors) or no siRNA (control, *n* = 7 tumors) every 48 hr for a minimum of 10 to maximum of 18 days. Tumor size was measured every alternate day before siRNA injection and hence the tumor size measured before first injection was considered as Day 0 (measurement). I found that after 10 days of siRNA injection (total of 5 siRNA injections with injection every 48 hr), all the tumors with no siRNA (*n* = 7 tumors) or nonsilencing (*n* = 6 tumors) siRNA treatment increased in size with time, with an average increase of about 426% and 482%, respectively, when compared to Day 0 (before first injection; Figure 2.12). No notable reduction in size of control (no siRNA) or nonsilencing siRNA injected tumors was observed even after 18 days (Table 2.2 and 2.4). However, anti-Cx46 siRNA treatment inhibited the tumor growth significantly with average increase of only about 30% (compared to Day 0) after 10 days of injection (Figure 2.12-2.14). Any noteworthy increase in the size of anti-Cx46 siRNA treated tumors was not noticed from Day 10 to Day 18 (Table 2.3).

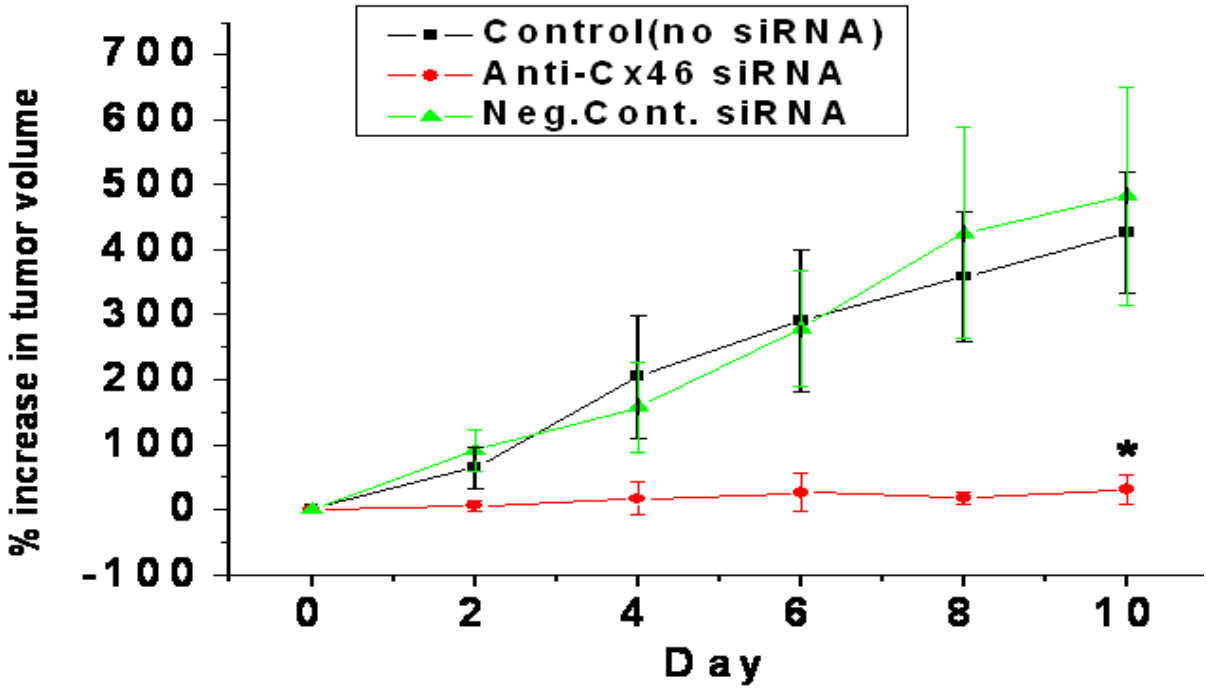

Figure 2.12 siRNA mediated downregulation of Cx46 in mouse tumor xenografts inhibits the growth of breast cancer tumors *in vivo*.

Mice were implanted with 17ß-estradiol (1.7 mg/pellet) followed by the injection of 1 × 10^7 MCF-7 cells subcutaneously into the inguinal region of mammary fat pads to develop breast tumors. After 2 weeks, animals were injected with 7.5 µg Cx46 siRNA (*n* = 10 tumor) or 7.5 µg negative control siRNA (*n* = 7 tumors) every 48 hr for 10 days. The siRNAs were injected directly at 2–3 different locations of each tumor. Animals with no siRNA treatment represent control (*n* = 6 tumor). The tumors size was measured in two dimensions by a caliper every alternate day before injection. The results after 10 days (total 5 injections) show a significant decrease in tumor growth of anti-Cx46 siRNA treated mice. Data are plotted as mean ± s.e.m of two different independent experiments. Asterisk indicates statistical difference ($p < 0.001$) between anti-Cx46 siRNA treated tumors and control (no siRNA) tumors.

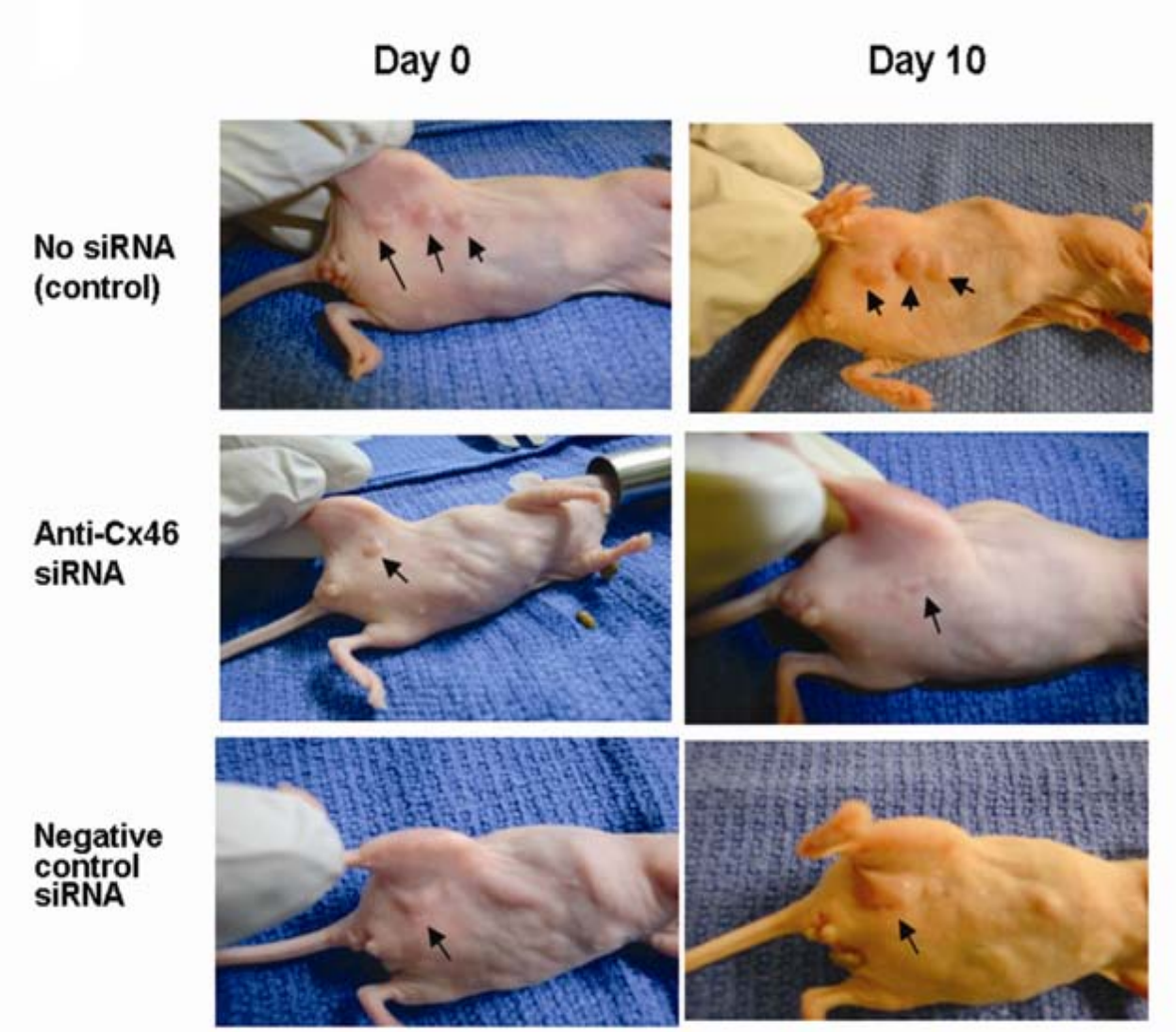

Figure 2.13 The images of control (no treatment), anti-Cx46 siRNA and negative control siRNA treated MCF-7 tumor-bearing xenograft mice at Day 0 and Day 10. The reduction of tumor size at Day 10 is observed only after anti-Cx46 siRNA treatment.

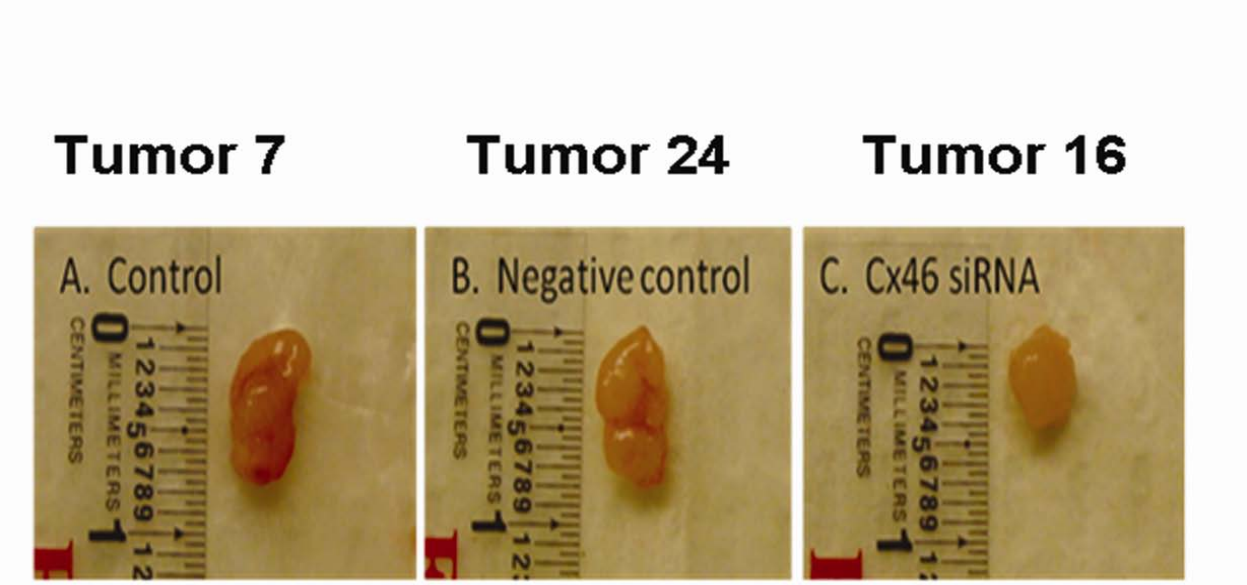

Figure 2.14 The gross images of control (no siRNA), or nonsilencing siRNA treated (negative control) or anti-Cx46 siRNA treated xenograft tumors in Nu/Nu mice.

Mice were euthanized and tumors were isolated after 10 days of siRNA treatment. The images were taken by Dr. T. A. Nguyen from Department of Diagnostic Medicine/Pathobiology, Kansas State University.

Control (no siRNA) injected tumors	Day 0 Volume (mm^3)	Day 2 Volume (mm^3)	Day 4 Volume (mm^3)	Day 6 Volume (mm^3)	Day 8 Volume (mm^3)	Day 10 Volume (mm^3)	Day 12 Volume (mm^3)	Day 14 Volume (mm^3)	Day 16 Volume (mm^3)	Day 18 Volume (mm^3)
Tumor 1	30.62	40	48	60.75	75	90.75	98.31	117	117	137.3 (E)
Tumor 2	18	21.43	32	56.25	75.62	90.75	90.75	98.31	98.31	126.75 (E)
Tumor 3	15.75	15.75	28	45.56	62.5	62.5	75.62	75.62	75.62	90.75 (E)
Tumor 4	40	83.18	137	126	147.87	147.87	147.87	147.87	147.87	
Tumor 5	15.75	18	30.62	36.75	56	87.5	93.75	100	106.25 (E)	
Tumor 6	12.5	17.18	27	39.81	42.87	60	60(E)			
Tumor 7	21.4	73.5	182	224	224	224(E)				

Table 2.2 The volume (mm^3) of no siRNA injected (control) tumors measured every alternate day. E = Euthanized.

Anti-Cx46 siRNA injected tumors	Day 0 Volume (mm^3)	Day 2 Volume (mm^3)	Day 4 Volume (mm^3)	Day 6 Volume (mm^3)	Day 8 Volume (mm^3)	Day 10 Volume (mm^3)	Day 12 Volume (mm^3)	Day 14 Volume (mm^3)	Day 16 Volume (mm^3)	Day 18 Volume (mm^3)
Tumor 8	48	48	56	69	75	149	147	147	159(E)	
Tumor 9	18	18	21.43	21.43	21.43	15.75	15.75 (E)			
Tumor 10	105.87	126	168.75	171.5	144	144	144	48	40(E)	
Tumor 11	69	69	69	69	62.5	56	56	50	50	28(E)
Tumor 12	14	14	18	13.5	9.37	9.37	9.37 (E)			
Tumor 13	56	56	62.5	62.5	62.5	105.6	105.6	105.6	105.6(E)	
Tumor 14	–	–	–	33.75	49	49	49	49	49	49(E)
Tumor 15	–	–	–	21.43	15.75	15.75	15.75	15.75	15.75	15.75 (E)
Tumor 16	45.5	32	32	68	56.25	62.5 (E)				
Tumor 17	21.43	32	32	32	32	32 (E)				

Table 2.3 The volume (mm^3) of anti-Cx46 siRNA injected tumors measured every alternate day.

The anti-Cx46 siRNA was injected directly into the MCF-7 xenograft tumors every 48 hr and each tumor size was measured prior to siRNA injection. Hence the tumor size measured prior to first injection was considered as day 0 (measurement). Tumor 14 and tumor 15 appeared at Day 6 and hence Day 6 was considered as Day 0 for these two tumors. E = Euthanized.

Negative non-silencing injected tumors	Day 0 Volume (mm^3)	Day 2 Volume (mm^3)	Day 4 Volume (mm^3)	Day 6 Volume (mm^3)	Day 8 Volume (mm^3)	Day 10 Volume (mm^3)	Day 12 Volume (mm^3)	Day 14 Volume (mm^3)	Day 16 Volume (mm^3)	Day 18 Volume (mm^3)
Tumor 18	60	75	90	105.6	105.6	105.6	116	122.5	147	171(E)
Tumor 19	18	50	50	50	50	50	50	50(E)		
Tumor 20	40	50.62	75.62	75.62	75	90.75	98.31.	98.31	105.87	105.87 (E)
Tumor 21	11	40.5	81	81	90	116.2	116.2 (E)			
Tumor 22	32	69	116.2	116.2	134.75	134.75	134.75	134.75	208(E)	
Tumor 23	–	–	–	15.75	24.5	32	40	40	62.5	108(E)
Tumor 24	13.5	13.5	32.5	112.5	192	192(E)				

Table 2.4 The volume (mm^3) of negative non-silencing siRNA injected tumors measured every alternate day.

The non-silencing siRNA was injected directly into the MCF-7 xenograft tumors every 48 hr and each tumor size was measured prior to siRNA injection. The tumor size measured prior to first injection was considered as day 0 (measurement). Tumor 23 appeared at Day 6 and hence Day 6 was considered as Day 0 for this particular tumor. E = Euthanized.

To confirm that the inhibition of anti-Cx46 siRNA treated tumor growth is due to Cx46 downregulation, I analyzed the tumors by western blot. Effective knockdown of Cx46 protein was only seen in tumors injected with anti-Cx46 siRNA (Figure 2.15). Of the ten anti-Cx46 siRNA injected tumors, six tumors (tumors 16 and 17 at Day 10; tumor 10 at Day 16; tumors 11, 14 and 15 at Day 18) presented reduced level of Cx46 protein (Figure 2.15), two tumors (tumors 8 and 13 at Day 16) showed no reduction in Cx46 protein expression (Figure 2.15 B) and two tumors (tumors 9 and 12, Table 2.3) became too small to be analyzed by western blot. We also measured Cx26 protein level in all tumors to investigate any nonspecific effect of the siRNA but no change in Cx26 protein level was observed. This suggested that the decreased tumor growth in xenograft mice resulted due to selective knockdown of Cx46 by anti-Cx46 siRNA.

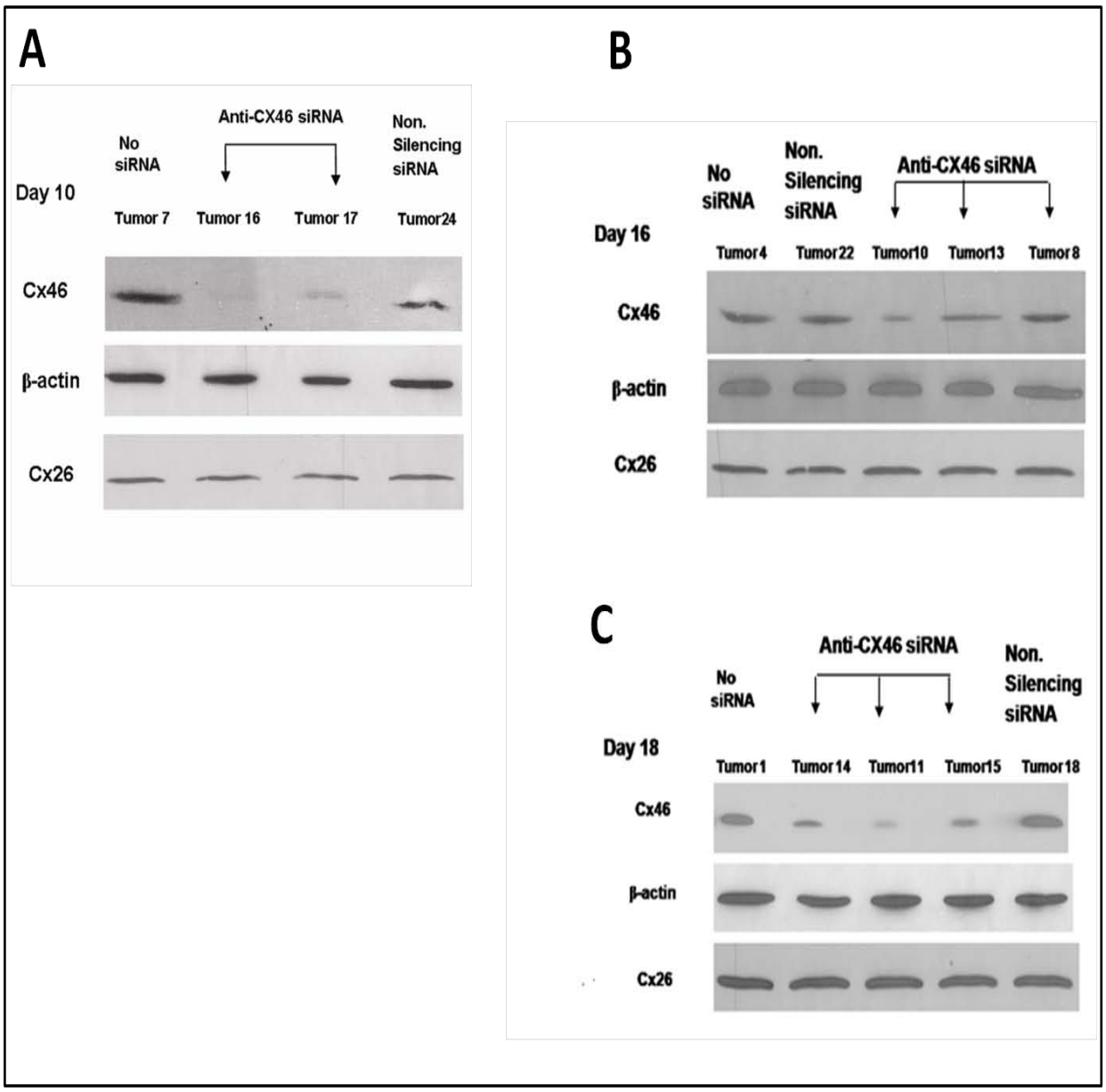

Figure 2.15 Western blot analyses show knockdown of Cx46 in xenograft tumors by anti-Cx46 siRNA.

The tumors were dissected out at (A) Day 10, (B) Day 16 or (C) Day 18. Tumors were harvested and Western blots were performed on whole tissue lysates using rabbit anti-Cx46 antibody. The same blots were reprobed with anti-Cx26 antibody to show the specificity of anti-Cx46 siRNA action *in vivo*.

2.4. Discussion

In the present study, I demonstrated that Cx46 helps cells to survive under hypoxia. Several studies have shown that the oxygen concentration inside the lens and in breast tumor, at early stage, can go as low as 1% (McNulty, Wang et al. 2004; Holekamp, Shui et al. 2005; Holekamp, Shui et al. 2006; Shui, Fu et al. 2006). Therefore, I chose 1% O_2 for hypoxia treatment. I observed an upregulation of Cx46 protein in HLEC under hypoxic conditions at 4–7 days but no change in protein expression was found under normoxia treatment (Figure 2.3). HLEC are naturally hypoxic cells as they survive for weeks at 1% O_2. But when Cx46 was downregulated (by siRNA), the HLEC viability reduced significantly even after 12 hr under hypoxia (Figure 2.4-2.5). This indicates that lens epithelial cells survive under hypoxia by utilizing Cx46 protein function.

If HLEC are able to survive in hypoxia because of Cx46 protein, then overexpression of Cx46 should confer resistance to a hypoxia susceptible cell that lacks endogenous Cx46. Indeed, I observed that overexpression of Cx46 in hypoxia susceptible murine neuronal N2A cells significantly increased the cell viability under hypoxic conditions (Figure 2.7A). However, no such effect on N2A cell survival at 1% O_2 was observed when another connexin, Cx43, was overexpressed. This suggests that Cx46 can also help cells other than in lens to survive in hypoxic condition and this effect is specific to Cx46 as Cx43 overexpression did not show similar effect.

To date, the gap junction connexins, Cx26, Cx32 and low level of Cx43 have been detected in breast cancer MCF-7 cells. Here, for the first time, I show that human breast cancer cells and human breast tumors also express Cx46 (Figure 2.8). Both Cx46 mRNA and protein were detected in breast cancer MCF-7 cells but not in normal HMEC (Figure 2.8A and 2.8B). Moreover, higher expression of Cx46 protein was observed in human breast tumor tissues than in normal breast tissues (Figure 2.8D) raising the possibility that Cx46 may be used as a marker in breast oncogenesis. Immunofluorescence staining with anti-Cx46 antibody showed that Cx46 predominantly localized to the cytoplasmic compartment at perinuclear region of the MCF-7 cells (Figure 2.9A). This observation was further confirmed by cell fractionation-western blot analyses that showed the presence of higher intensity Cx46 protein band in cytosolic fraction (Figure 2.9B). However localization of Cx46 in the plasma membrane could not be ruled out as membrane fraction, that contained both plasma membrane and ER membrane proteins, also showed Cx46 band.

Our results clearly demonstrated that Cx46 functions to protect MCF-7 cells against hypoxia-induced death. MCF-7 cells with downregulated Cx46 protein showed significant decrease in cell viability at 1% O_2 (Figure 2.11). However, downregulation of Cx46 had no effect on cell viability under normoxia treatment (Figure 2.11). Like in HLEC, Cx46 may be involved only in hypoxia-signaling pathways in breast cancer cells. The function of Cx46 in human breast tumor formation was investigated, *in vivo*, in human tumor xenograft-bearing nude mice. The tumor of human origin in nude mice was generated by injecting MCF-7 cells into inguinal region of mammary fat pads. As

levels of Cx46 protein were reduced maximally after 48 hr of anti-Cx46 siRNA transfection in MCF-7 cells *in vitro*, I injected anti-Cx46 siRNA directly into the MCF-7 xenograft tumors every 48 hr. Consistent with the result of decreased cell viability of MCF-7 cells *in vitro* under hypoxia, dramatic inhibition of tumor growth was also observed in MCF-7 xenograft tumors (Figure 2.12), *in vivo,* treated with anti-Cx46 siRNA even for 18 days. Thus Cx46 appeared to play a significant role in early breast tumor development.

I achieved significant knockdown of Cx46 protein *in vivo* by injecting siRNA directly into the xenograft tumors in nude mice. The anti-Cx46 siRNA treated xenograft tumors that were associated with reduced tumor growth (tumors 10, 11, 14, 15, 16 and 17) presented low levels of Cx46 protein (Figure 2.15). Two anti-Cx46 siRNA treated tumors (tumors 8 and 13) manifested increased tumor growth and showed no significant reduction in Cx46 protein level. The relationship of Cx46 knock down level with tumor size clearly suggests that downregulation of Cx46 protein accounted for the reduced growth of xenograft tumors.

The breast cancer tumor progression is generally believed to be associated with loss of gap junctional intercellular communication (GJIC). The decreased communication of tumor cells with neighboring normal cells due to defective intercellular gap junctions has been shown to be beneficial for tumor progression. Therefore, the other gap junction connexins, Cx43, Cx26 and Cx32, expressed in breast tissue, are generally deemed tumor suppressors. Loss of Cx43 is correlated with breast tumor

progression and restoration of Cx43 protein level promoted controlled growth and less proliferation of cancer cells (Hirschi, Xu et al. 1996; Laird, Fistouris et al. 1999; Qin, Shao et al. 2002). In contrast, our studies identify a unique gap junction protein, Cx46, which is upregulated and acts as a proto-oncogene in early breast cancer tumors

Due to its observed cytoplasmic localization, Cx46 possibly helps breast tumor to adapt and survive in the hypoxic environment by mechanisms other than GJIC. Though, in most cases, functions of connexin proteins have been shown to be through their ability to form channels; evidence of connexin activity regardless of gap junction communication is increasing. In breast cancer MDA-MB-435 cells, Cx26 prevents tumorigenesis by a communication independent mechanism (Kalra, Shao et al. 2006). When a GJIC-incompetent variant of Cx26 was overexpressed in MDA-MB-435 cells, the cell proliferation and anchorage-independent growth was reduced. In addition, this communication defective Cx26 variant was also able to inhibit cell migration and invasion by decreasing the level of β1 integrin and reducing the activity of matrix metalloproteinase-9 (MMP-9) while increasing the tissue inhibitors of MMP-1 (TIMP-1) activity. Another study, done by Conklin et al (Conklin, Bechberger et al. 2007)), also reported that an anticancer drug, genistein, upregulates Cx43 and inhibits MDA-MB-231 cell proliferation independent of GJIC.

The mechanism of Cx46 action needs further investigation. It seems plausible that Cx46 mediates its action by putative binding partners that function in the hypoxia-survival mechanisms. It is well established that connexins interact with several kinases

such as protein kinase A (PKA), protein kinase C (PKC), mitogen activated protein kinase (MAPK) and src kinase. Cx46 shares a significant sequence homology with Cx43 with the C-terminal region being unique. Cx46 has a 191 amino acid long C-terminal end, exposed to the cytoplasm, that has conserved phosphorylation and binding sites for PKC, CK-1 (the casein kinase-1), AKT kinase and MAPK. A recent study in our lab shows that C-terminal deletion mutant of Cx46 is unable to protect N2A cells against hypoxic cell death (Takemoto Lab, unpublished data). This indicates that C-terminal tail region of Cx46 is required for its hypoxia protective function. Further studies with mutant Cx46 proteins that are defective in phosphorylation or binding of the above mentioned proteins are currently underway to shed some insight, in the future, into how Cx46 protects cells from hypoxia.

I also speculate that another possible way Cx46 favors tumor growth is by inducing Cx43 protein degradation in breast tumors. It is well-established that Cx43 is downregulated in breast tumor and, when overexpressed, has a tumor suppressive role. In contrast to Cx43 expression, I found Cx46 to be highly expressed in breast tumor tissues when compared with the normal breast tissue. In addition, I have also found that, in response to hypoxia, Cx46 protein expression is strongly increased with concomitant reduction of Cx43 protein levels in HLEC. Further support for this speculation comes from another related study, in our lab; on retinoblastoma xenograft tumors that showed that the downregulation of Cx46 (by siRNA) was associated with inhibition of tumor growth and interestingly, an increase in Cx43 protein levels. How

Cx46 upregulation causes the degradation of Cx43 has been presented in Chapter 3 of the thesis.

Our data also provide evidence that the presence of Cx46 and its function as a hypoxia-critical-factor can be targeted for the treatment of breast cancer. This is the first report to utilize a natural hypoxic tissue (lens) to understand the complexity of an adaptive hypoxic tissue such as solid tumor for their survival strategies involving a novel gap junction protein. Our findings also revealed that the local administration of siRNA can mediate effective knock down of this target protein with minimal nonspecific effects and prevent tumor formation. These results will have a high impact on breast cancer therapeutics in designing an entirely new line of drugs to control and potentially eliminate early breast cancers while at the early hypoxic stage.

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CHAPTER 3 - Overexpression of connexin46 causes connexin43 degradation by ubiquitin-proteasome dependent pathway in rabbit lens epithelial NN1003A cells.

3.1. Introduction

Gap junctions are intercellular membrane channels that maintain direct intercellular communication through the exchange of ions, small molecules and cellular metabolites between neighboring cells (Goodenough, Goliger et al. 1996; Harris 2001; Goldberg, Valiunas et al. 2004). Gap junction channels are formed at the basolateral surfaces of two cells and directly connect their cytoplasms (Saez, Berthoud et al. 2003). Gap junction channels are composed of a transmembrane family of proteins called connexins (Laird 1996; Laird 2006). So far, there are 21 connexin isoforms that have been identified in human (Laird 2006). Gap junction mediated intercellular communications are involved in the regulation of important cellular processes including cellular homeostasis, cell growth and differentiation, morphogenesis and cell death (Loewenstein 1979). Loss of gap junctions has been found to be associated with malignant transformation (Loewenstein 1979; Trosko and Ruch 1998).

Connexins have rapid turnover rates with very short half-lives ranging from 1.5-4 hr in the different cell types studied (Fallon and Goodenough 1981; Traub, Look et al. 1989; Crow, Beyer et al. 1990; Laird, Puranam et al. 1991). This short half-life of connexin is very unusual for integral membrane protein whose half-lives are expected to be long (>20h) (Herve, Derangeon et al. 2007).The reason for the rapid turnover rate is

not well documented, however it is assumed that connexins are continuously degraded and synthesized to increase or decrease the gap junctional intercellular communication (GJIC) as per cell's physiological requirement. Connexins can respond to several extrinsic and intrinsic factors such as trans-junctional voltage, pH, carcinogenic chemicals and phosphorylation to control gap junctional intercellular communication (GJIC) (Bennett and Verselis 1992; Wang, Lee et al. 2001; Bukauskas and Verselis 2004). Among the connexin family members, connexin43 (Cx43) is most widely expressed and has been shown to regulate gap junction permeability, assembly and disassembly by phosphorylation at its C-terminal cytoplasmic tail (Solan and Lampe 2009).

The degradation of connexin can occur by both lysosomal and proteasomal pathways (Larsen and Hai 1978; Laing and Beyer 1995; Laing, Tadros et al. 1997; Lampe and Lau 2000; Musil, Le et al. 2000; Qin, Shao et al. 2003). Lysosomal pathways have been implicated to be involved in the degradation of internalized gap junctions. However, the proteasomal pathway for connexin degradation is not well characterized. In general, degradation via the proteasome involves, at first, polyubiquitin conjugation of target protein by the sequential action of three classes of enzymes, ubiquitin-activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3). Next, the ubiquitin-conjugated substrate protein is recognized and degraded by multisubunit protease complex 26S proteasome (Hershko and Ciechanover 1992; Ciechanover 1998; Lecker, Goldberg et al. 2006). There is increasing evidence that the ubiquitin-proteasomal pathway plays distinct roles in the turnover of connexins. Several

studies have shown that treatment with proteasomal inhibitors results in increases in Cx43 protein levels in several cell lines (Laing and Beyer 1995; Laing, Tadros et al. 1997; Qin, Shao et al. 2003). Proteasome is also required for the endoplasmic reticulum–associated degradation (ERAD) of misfolded connexins during synthesis (VanSlyke, Deschenes et al. 2000; VanSlyke and Musil 2002). The proteasome is also believed to play a role in the process of internalization of Cx43 from plasma membrane (Leithe and Rivedal 2004).

As discussed in Chapter 1, vertebrate lens is a transparent organ consisting of three perceivable zones of cells, an outer layer of epithelial cells at the anterior surface, an intermediate layer of differentiating fiber cells (DF), and a core of closely-packed mature fiber cells (MF). Outer epithelial cells are differentiated to form inner fiber cells in the lens differentiation process that is accompanied with a loss of organelles and induction of crystallin proteins in the fiber cell cytoplasm (Donaldson, Kistler et al. 2001; Bassnett 2002; Wang, Garcia et al. 2004). Ocular lens endogenously expresses three connexin isoforms Cx43 (α1), Cx50 (α8), and Cx46 (α3) (Donaldson, Kistler et al. 2001; Berthoud and Beyer 2009). These three connexins play a major role in the proper lens development and differentiation. The connexins show differential spatial distribution within the lens. The lens epithelium primarily contains Cx43 and Cx50 (Musil, Beyer et al. 1990; TenBroek, Johnson et al. 1994; Dahm, van Marle et al. 1999; Rong, Wang et al. 2002), whereas differentiating and mature fiber cells abundantly express Cx46 and Cx50 (Paul, Ebihara et al. 1991; White, Bruzzone et al. 1992). During lens epithelial-to-fiber cell differentiation Cx43 is substantially downregulated and Cx46 is significantly

upregulated. This change in connexin isoform expression is crucial for maintaining connexin mediated communications in lens. However, the underlying molecular mechanism of Cx46 upregulation and Cx43 downregulation during lens epithelial-to-fiber cells transition is largely unknown.

The reciprocal expression of Cx46 and Cx43 is also observed in breast tumorigenesis. Cx43 is widely regarded as a tumor suppressor protein. The expression of Cx43 is downregulated in breast tumors (Lee, Tomasetto et al. 1992; Laird, Fistouris et al. 1999; Shao, Wang et al. 2005), when compared to normal breast tissues, and, when re-expressed, Cx43 reverses the tumorigenic phenotype (Hirschi, Xu et al. 1996; Qin, Shao et al. 2002). In chapter 2, I have shown that, in contrast to Cx43 protein expression, the Cx46 is strongly expressed in breast cancer MCF-7 cells and human breast tumor tissues.

In the present study, I demonstrated how Cx46 upregulation is associated with Cx43 degradation. Using an overexpression system of rabbit lens epithelial NN1003A cells, I find that overexpression of Cx46 causes a decrease in Cx43 protein levels. The Cx43 degradation, due to the overexpression of Cx46, is mediated by the ubiquitin-proteasome pathway as determined by the observations that (i) treatment with only proteasome inhibitors restores the Cx43 protein level and (ii) there is an increase in ubiquitin conjugation of Cx43, in Cx46 overexpressing cells. I also provide evidence that the C-terminal tail region of Cx46 is required to induce degradation of Cx43. Thus, our

study shows that Cx46 has a novel function in Cx43 downregulation in addition to its conventional role as a gap junction protein.

3.2. Material and Methods

3.2.1. Antibody

Mouse anti-GFP antibody was purchased from Clontech (Mountain View, CA). Rabbit anti-Cx43-CT (against C-terminal) and mouse anti-β-actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Anti-Cx50 antibody was purchased from Zymed-Invitrogen (Carlsbad, CA) and anti-ubiquitin antibody was purchased from Calbiochem (EMD Biosciences, OH). Mouse anti-N-terminal-Cx43 (anti-Cx43-NT) and mouse anti-Cx43-IF1 (used in immunofluorescence studies) antibodies were purchased from Fred Hutchinson Cancer Center (Seattle, WA). Rabbit polyclonal anti-Cx46 antibody was purchased from US Biological (Swampscott, MA).

3.2.2. Cell culture

NN1003A rabbit lens epithelial cells were cultured in Dulbecco's modified Eagle's medium (DMEM, low glucose; Invitrogen, CA) supplemented with 10% fetal bovine serum and 50 µg/mL gentamicin, 0.05 U/mL penicillin, and 50 µg/mL streptomycin, pH 7.4 at 37^{O}C in an atmosphere of 90% air and 10% CO_2. All the experiments were done at the confluency level of 70-80% of NN1003A cells.

3.2.3. Cloning and transfection

To generate NN1003A cells, stably overexpressing Cx46 or Cx50, the cDNA of rat Cx46 (NCBI Reference Sequence: NM_024376.1) or rat Cx50 (NCBI Reference Sequence: NM_153465.2) was sub-cloned into pEGFP-N3 vector (Clontech, CA) and transfection was carried out using Lipofectamine^{TM} 2000 (Invitrogen, CA), as described in chapter 2. The transfected cells were grown in the presence of 1 mg/ml G418 for 3 weeks for the selection of positive colonies The positive colonies were then isolated and grown in media containing G418 (1 mg/ml) thereafter

The cDNA of rat Cx43 (NCBI Reference Sequence: NM_012567.2) was also sub-cloned into the pEGFP-N3 vector. To generate the Cx46 mutant protein with deleted C terminus tail region (Cx46-dCT, deletion in residues 232-416), the truncated cDNA of rat Cx46 was also sub-cloned into pEGFP-N3. The rat Cx46 cDNA was also sub-cloned in pQE-TriSystem vector (Qiagen, CA) to get Cx46-His (-His tagged) protein. These plasmids were used in transient transfection experiments.

All transient transfections were carried out at 70-80% confluency of cells and using Lipofectamine^{TM} 2000. The expression of Cx43-GFP, Cx46-GFP, Cx50-GFP and Cx46-dCT-GFP were checked by western blot analyses using anti-GFP antibody. The expression of Cx46-His fusion protein was verified by immunofluorescence analysis using anti-His antibody

3.2.4. Whole cell homogenate preparations (WCH)

Cells were washed 3 times with cold phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic and a pH of 7.4), collected by scraping from plates and centrifuged at 4000 rpm for 5 min at 4^{O}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 sec (3 times) and protein concentration of each sample was measured using Bio-Rad Protein Assay.

3.2.5 Reverse Transcriptase (RT)-PCR

Total RNA was isolated from NN1003A wild type cells and cells stably or transiently overexpressing Cx46-GFP or GFP (empty vector) using RNeasy Mini Kit (Qiagen, CA) and the RT-PCR was carried out using SuperScript^{TM} III First- Strand RT-PCR kit (Invitrogen, CA) according to the manufacturer's protocol. The primers for Cx43 cDNA were 5'-GAT GAG CAG TCT GCC TTT CGT -3' and 5'-CGT TGA CAC CAT CAG TTT GG-3'. The primers for β-actin were 5'-GAA ATC GTG CGT GAC ATT AAG-3' and 5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG GC-3'. For PCR, initial activation was done at 94^{O}C, followed by denaturation at 94^{O} C for 1 min, annealing at 30^{O} C for 1 min and extension at 72^{O}C for 30 sec. The number of cycles for PCR was 20 or 25. The PCR products were run in 1.2 % agarose gel. DNA bands, at expected

position, were eluted and sent for sequencing to confirm correct amplification of the PCR products.

3.2.6. Plasma membrane fractionation

The fractionation of plasma membrane proteins from untransfected or transfected NN1003A cells was carried out using Qproteome^{TM} Plasma Membrane Protein (Qiagen, CA) kit according to the instruction manual. In brief, 1 X 10^7 cells were collected by a cell scraper, centrifuged and resuspended in 2 ml Lysis Buffer PM. Then the cells were centrifuged again at 450g for 5 min and cell pellets were resuspended in 500 µL Lysis Buffer PM containing protease inhibitor solution (1:100). Lysis Solution PL (2.5 µL) was then added to the resuspended cells and, following an incubation period for 15 min at 4^{O}C, cells were disrupted using a 21 gauge needle and syringe. The cell lysates were then centrifuged at 12000g for 20 min to remove the intact cells, cell debris and nuclei. The supernatants that contained cytosolic proteins, microsomes, Golgi vesicles and plasma membranes were incubated with 20 µL of Binding Ligand PBL, a ligand that is specific for the molecules on the plasma membrane, for 1 hr at 4^{O}C. The ligand-plasma membrane complexes were then precipitated using Strep-Tactin Magnetic Beads and washed with Wash Buffer PW. The plasma membrane proteins were next eluted with Elution Buffer PME. The eluted fractions were quantitated by Bio-Rad Protein Assay and analyzed by western blot. Western blot analysis was performed as described in Chapter 2.

3.2.7. Immunofluorescence study

Untransfected control cells and GFP or Cx46-GFP stably overexpressing NN1003A cells were seeded on cover-slip in a 12 well plate. When confluency reached to 80%, cells were fixed in 4% paraformaldehyde, for 10 min, followed by permeabilization with 0.05% Tritron-X100 for 30 min. Then the cells were washed in PBS and blocked in 3% bovine serum albumin solution (BSA) for 2 hr at 4^{O}C., The untransfected control cells were stained with rabbit anti-Cx46 antibody (dilution 1:200) to visualize endogenous Cx46. The cells were then treated with mouse anti-Cx43 antibody (Cx43-IF1, dilution 1:100) for overnight at 4^{O}C. Cells were washed 3 times with PBS buffer and incubated with secondary Alexa Fluor-568 (red color) anti-mouse or Alexa Fluor-488 (green color) anti-rabbit antibodies for 2 hr at room temperature. For some experiments, the nuclei were also stained with DAPI (blue color, dilution 1:1000). The cover-slips were then washed 3 times in cold PBS, and mounted onto slides using ProLong Gold Antifade reagent (Invitrogen, CA). The images were taken using a Leica DMI 6000 B microscope (Leica, Deerfield, IL) or a Nikon C1 scanning confocal microscope. The Cx46-GFP or GFP proteins were visualized under the microscopes by green fluorescence only (without immunostaining).

For immunofluorescence studies involving transiently transfected cells, NN1003A cells were seeded on cover-slips in a 12 well plate one day before the transfection. The transfection was carried out using Cx46-GFP plasmid or Cx46-dCT-GFP (C-terminal tail deletion mutant of Cx46) and Lipofectamine 2000. The immunostaining of the cells were performed as described above.

3.2.8. Immunoprecipitation study

Cells, stably overexpressing GFP or Cx46-GFP or Cx50-GFP and untransfected (control), were grown in 100 mm dishes to approximately 90% confluency. NN1003A cells were also transiently transfected with Cx50-GFP plasmid for 36 hr. All the cells (untransfected, stable or transient) were harvested and total cell lysates were made as described previously. The total protein concentration in the whole cell lysates were quantified by Bio-Rad Protein Assay. To pre-clear the whole cell lysates, 0.25 µg of rabbit non-specific IgG and 20 µL of protein A/G-Agarose beads (Santa Cruz Biotechnology, CA) were added to the 750 µg of each whole cell lysate. The agarose beads-IgG complex was removed by centrifugation at 3,000 rpm and the supernatants were incubated with 5 µg of rabbit anti-Cx43CT antibody (against C-terminal) for overnight at 4^{O}C. After the overnight incubation, the supernatants were further incubated with 20 µL of protein A/G-Agarose beads for 4 h at 4^{O}C. The beads were collected by centrifugation at 3,000 rpm, washed 3 times with 1X RIPA buffer (Cell Signaling Technology, MA) and the samples were boiled for 5 min in 2x electrophoresis sample buffer (Santa Cruz Biotechnology, CA). The samples were then analyzed by western blot with anti-ubiquitin and anti-Cx43 antibodies. For a positive control for anti-ubiquitin antibody, a commercially available polyubiquitin chain (ubiquitin ladder, Affinity Research Products, UK) was used. In this chain, ubiquitin is polymerized through lysine 48.

3.2.9. Proteasome and lysosome inhibitor

Cells were incubated with proteasome inhibitors, ALLN (N-Acetyl-Leu-Leu-Nle-CHO, 100µM) or MG-132 (*N*-[(Phenylmethoxy) carbonyl]-L-leucy l-*N*-[(1*S*)-1-formyl-3-methylbutyl]-L-leucinamide, 10µM) or lysosome inhibitors, leupeptin (200 µM) or choloroquine (200 µM) for 4 hr. The cells were harvested and analyzed by western blot. The inhibitors were purchased from Sigma-Aldrich (St. Louis, MO).

3.3.10. Statistical analysis

For statistical analysis “Origin” software (Microcal Software Inc., Northampton, MA) was used. 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 ± s.e.m. of at least 3 independent experiments.

3.3. Results

3.3.1. Overexpression of Cx46 causes a decrease in Cx43 protein levels

In the ocular lens, Cx46 is mainly expressed in the fiber cell region. To determine the role of Cx46, we stably overexpressed Cx46 as a GFP tagged fusion protein in rabbit lens epithelial NN1003A cells and analyzed the effect of Cx46 overexpression on other connexins. The cells, stably overexpressing Cx46-GFP, showed the expression of fusion protein as bands around 75 kDa as determined by western blot using antibodies against GFP (Figure 3.1A). Interestingly, stable overexpression of Cx46 decreased the Cx43 protein levels of both non-phosphorylated

(Po) and phosphorylated forms (P1 and P2). In SDS-PAGE, Cx43 usually form three bands depending on the phosphorylation status of the residues. The fastest migrating band represents nonphosphorylated form (termed as Po) and the other two slower migrating forms represent phosphorylated forms (termed as P1 and P2). Stable overexpression of Cx46 reduced the Cx43 protein levels to 50% when compared to untransfected or GFP transfected cells (Figure 3.2B). However Cx50 protein level was not changed due to the stable overexpression of Cx46 (Figure 3.2). Consistent with the results obtained for stably transfected cells, transient transfection of Cx46-GFP also showed a reduction in Cx43 protein levels at 36 hr and 48 hr after transfection.

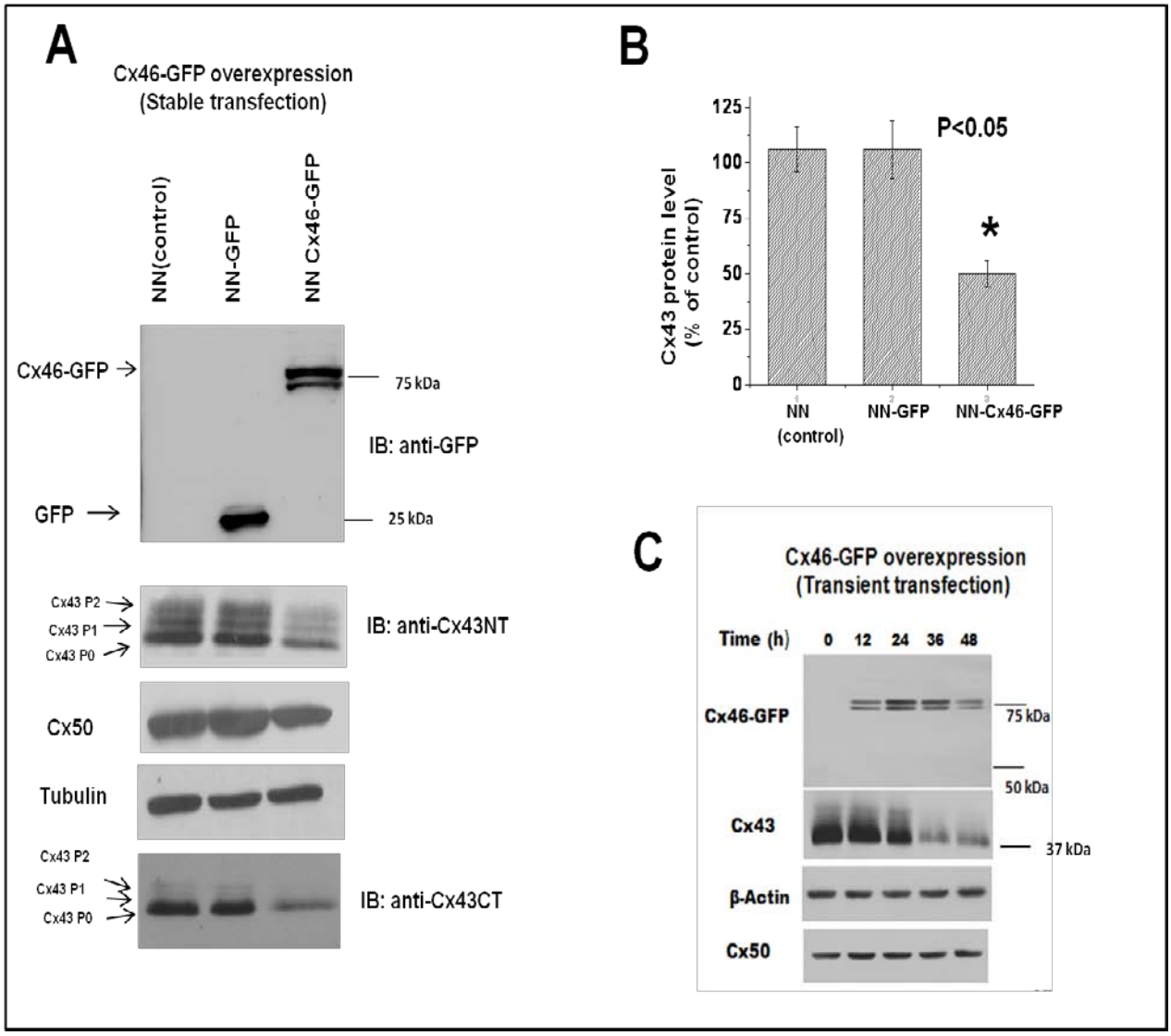

Figure 3.1 Overexpression of Cx46 causes reduction in Cx43 protein levels in NN1003A cells.

(A) Western blot analyses of Cx46-GFP, Cx43, Cx50 and tubulin in control (untransfected) cells and in cells stably overexpressing GFP or Cx46-GFP. 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-GFP antibody to detect Cx46-GFP expression. The blots were also probed with anti-Cx43CT (against C-terminal) and anti-Cx43NT (against N-terminal) antibodies to detect Cx43.

(B) Densitometric analyses show significant reduction of Cx43 protein levels in Cx46-GFP stably transfected NN1003A cells. The bands representing Cx43 and tubulin were

digitized by UN-SCAN-It gel software. The average pixel values were calculated for each Cx43 band, normalized against average pixel values for loading control (tubulin) and then plotted in % of control. Data are represented as mean ± s.e.m of three independent experiments. Asterisk indicates significant statistical difference ($P<0.05$) between indicated data and control (untransfected cells). (C) Cells were transiently transfected with plasmid encoding Cx46-GFP. Cell lysates were made at 0, 12, 24, 36 and 48 hr after transfection and analyzed by western blot using antibodies against , GFP, Cx43 (anti-Cx43 CT), Cx50 and β-Actin.

3.3.2. Cx46 localization in NN1003A cells

Next, I determined the intercellular localization of Cx46 in the stably transfected cells by immunofluorescence study. Green fluorescence of Cx46-GFP expression was predominantly detected to an intracellular perinuclear compartment (Figure 3.2A). Immunofluorescence signal of Cx46-GFP localization was not observed at the plasma membrane region. The localization of Cx46 to the intercellular compartments was also detected in NN1003A cells transiently transfected with Cx46-GFP plasmid (Figure 3.2B).

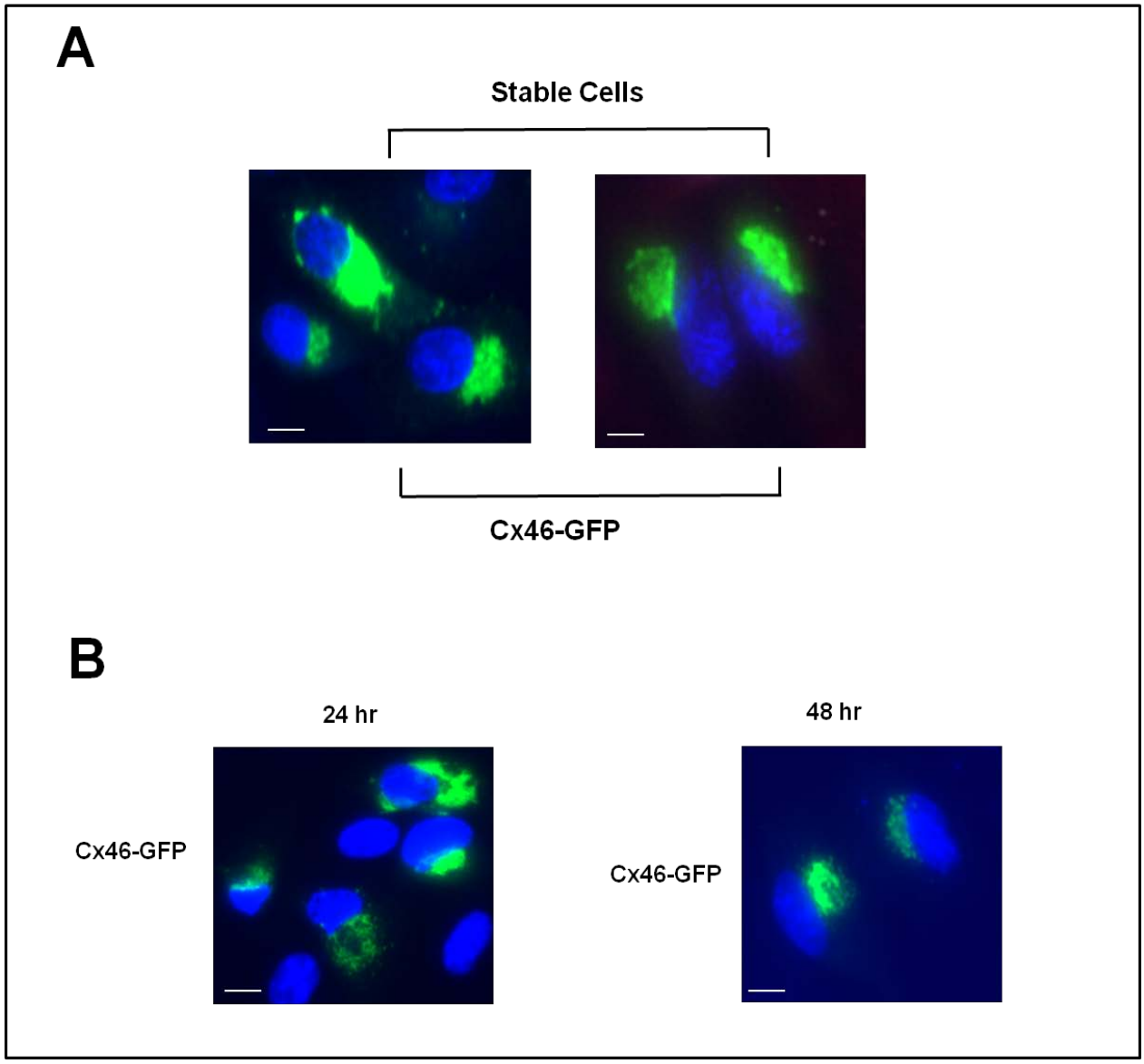

Figure 3.2 Localization of Cx46-GFP in stable (A) or transiently transfected NN1003A cells (B).

NN1003A cells stably overexpressing Cx46-GFP were generated by transfecting cells with rat Cx46 cDNA cloned in pEGFP-N3 vector and then selecting cells in the presence of G418 antibiotic. Cells were also transiently transfected with the plasmid encoding Cx46-GFP for 24 hr and 48 hr. All cells were fixed with paraformaldehyde and stained with DAPI to visualize nucleus. The images were taken using a Leica DMI 6000 B fluorescence microscope. Bar, 20 μm.

To further confirm the localization of Cx46, untransfected, stable or transiently transfected cells were fractionated to isolate plasma membrane proteins. Western blot analyses with antibodies against GFP or Cx46 detected no immunoreactive bands that corresponded to endogenous Cx46 or Cx46-GFP protein in the plasma membrane fraction of untransfected or transfected NN1003A cells (Figure 3.3A). In all the preparations of plasma membrane fractions, the marker for plasma membrane (Na^+-K^+ ATPase) was detected but not the marker for cytosol (Glyceraldehyde 3-phosphate dehydrogenase, GAPDH) or endoplasmic reticulum (Calnexin), confirming the purity of the fractions (Figure 3.3A).

The GFP protein has a molecular weight of 27 kDa. Therefore I speculated whether GFP tagging at C-terminal of Cx46 prevents it from reaching the cell surface. To investigate this, I transfected cells with plasmid encoding Cx46-His (Histidine-tagged) and examined the localization of Cx46 –His fusion protein in order to see if tagging with a smaller molecule affects the localization of Cx46. Immunofluorescence studies, using anti-His antibody, also demonstrated the localization of Cx46 predominantly to the intercellular compartments (Figure 3.3C). To further demonstrate that GFP-tagging does not affect the localization of connexin proteins in NN1003A cells, cells were transiently transfected to express Cx43-GFP. Using western blot analysis, Cx43-GFP fusion protein (around 70 kDa) was detected in plasma membrane fractions isolated from Cx43-GFP overexpressing NN1003A cells (Figure 3.3B). These data clearly suggested that GFP tagging had no influence on the Cx46 transportation to the cell membrane. It has also been previously shown that C-terminal tagging of connexin

does not alter membrane transport (Paemeleire, Martin et al. 2000; Thomas, Jordan et al. 2005).

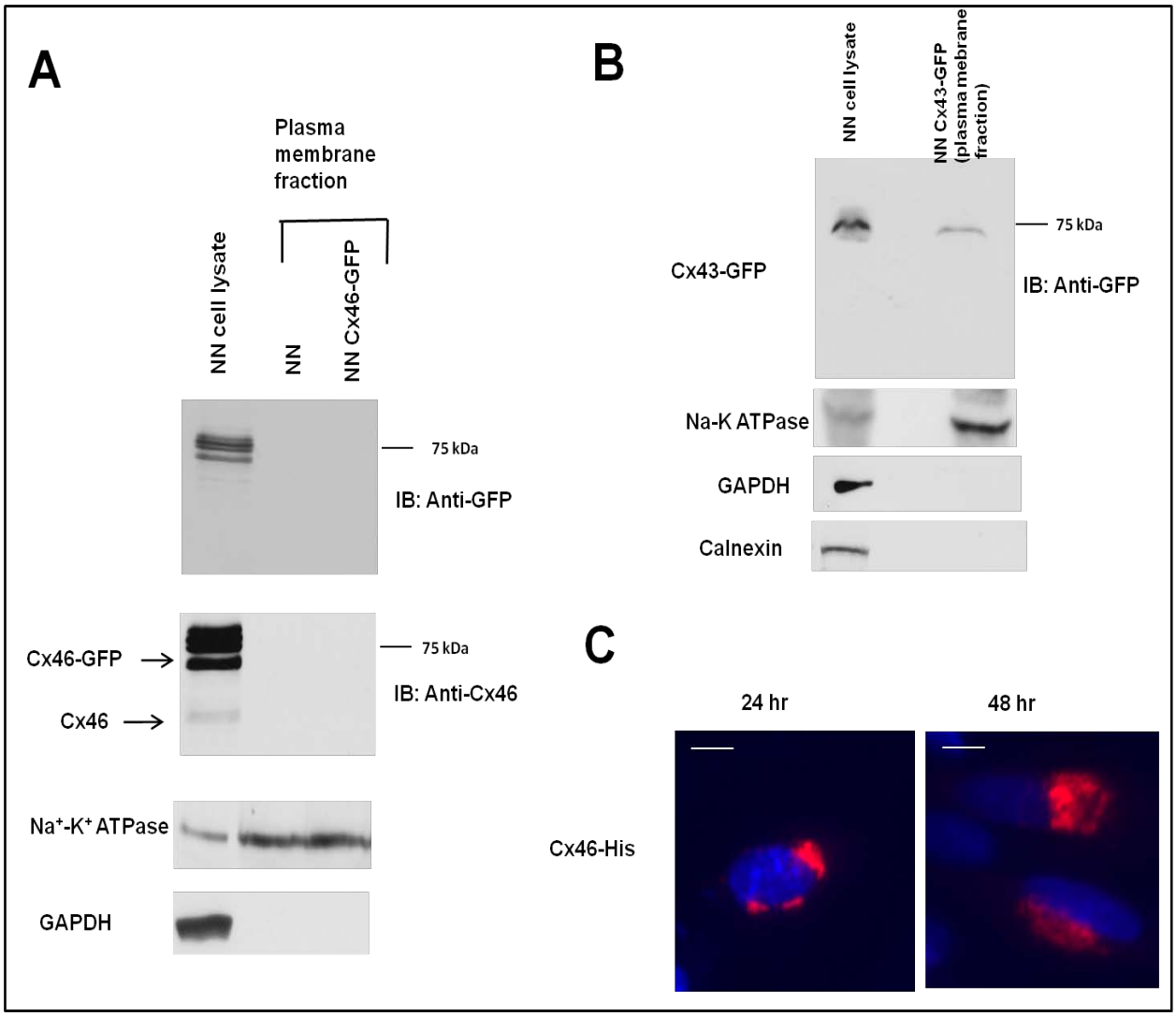

Figure 3.3 Cx46 protein is predominantly localized to the intercellular compartments in lens NN1003A cells.

(A) Western blot analyses were performed for Cx46 (with anti-GFP or anti-Cx46) on equal amounts of total protein from lysates of Cx46-GFP expressing cells (stable transfection) and plasma membrane protein fractions from untransfected (NN) or Cx46-GFP stably overexpressing 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. **(B)** Western blot analyses

show the localization of Cx43 in the plasma membrane of Cx43 GFP expressing cells. Cells were transiently transfected with plasmid encoding Cx43-GFP for 24 hr. Then the cells were fractionated into plasma membrane protein fraction. Equal amounts of total protein of lysate and plasma membrane fraction of Cx43-GFP expressing cells were subjected to western blot analyses with antibodies against GFP, $Na^{+-}K^{+}$ ATPase and GAPDH and an ER marker (calnexin). **(C)** The localization of Cx46-His protein (Histidine-tagged) in NN1003A cells. Cells were transfected with plasmid encoding Cx46-His. After 24 and 48 hr of transfection, cells were fixed, permeabilized and stained with anti-Cx43 antibody (red) and DAPI. Images were taken using a Leica DMI 6000 B fluorescence microscope. Bar, 20 μm.

3.3.3. Overexpression of Cx46 does not alter Cx43 message level

Next, we investigated whether Cx43 degradation was pre or post-transcriptionally initiated in Cx46 overexpressing NN1003A cells. To check this, reverse transcription (RT)-PCR was carried out to detect the Cx43 transcript levels in cells stably or transiently overexpressing Cx46-GFP. The results of the RT-PCR experiments are shown in Figure.3.4. The overexpression of Cx46 did not cause a change in the Cx43 message level indicating the occurrence of post-transcriptional regulation of Cx43.

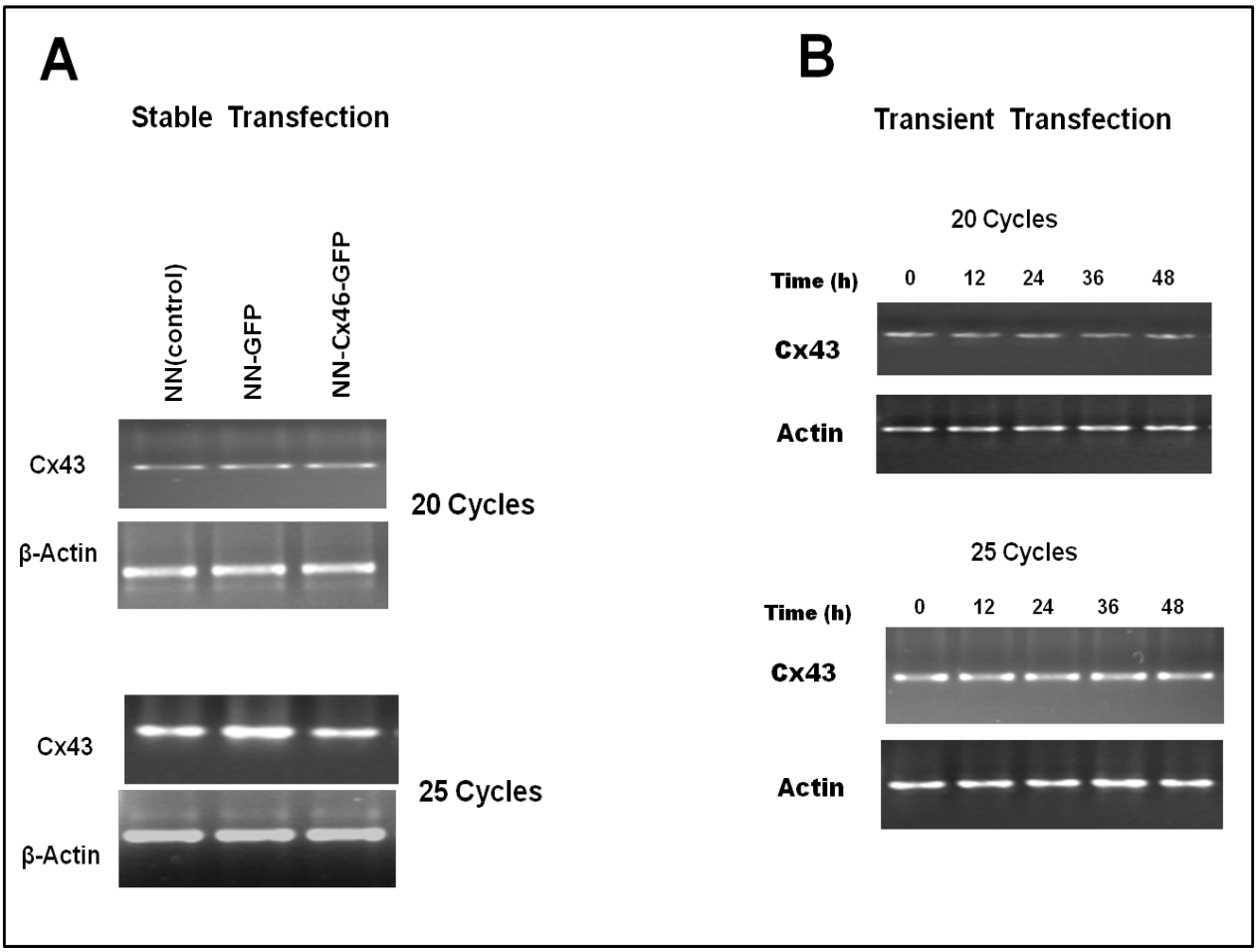

Figure 3.4 RT-PCR analyses of Cx43 mRNA levels in NN1003A cells overexpressing Cx46.

Total RNA was isolated from **(A)** stably or **(B)** transiently transfected cells followed by RT-PCR using specific primers for Cx43 cDNA and β-actin cDNA (internal control). For transient transfection, total RNA was isolated at 0, 12, 24, 36 and 48 hr after transfection with plasmid encoding Cx46-GFP. The numbers of cycles of PCR are given in the figure.

3.3.4. Overexpression of Cx50 protein has no effect on Cx43 protein level.

Another connexin protein expressed in lens is connexin50 (Cx50). Cx50 is expressed in lens epithelial as well as in fiber cells. Therefore I examined the effect of

overexpression of Cx50 on Cx43 protein levels. The cells were stably or transiently transfected with plasmid encoding Cx50-GFP for 12, 24, 36 and 48 h. Overexpression of Cx50 had no effect on Cx43 protein levels at 12-48 hr after transfection (Figure 3.5, left). Stable overexpression of Cx50-GFP also showed the similar effect with no change in Cx43 protein levels (Figure 3.5, right).Collectively, the above results suggested that Cx50, unlike Cx46, was unable to induce Cx43 degradation.

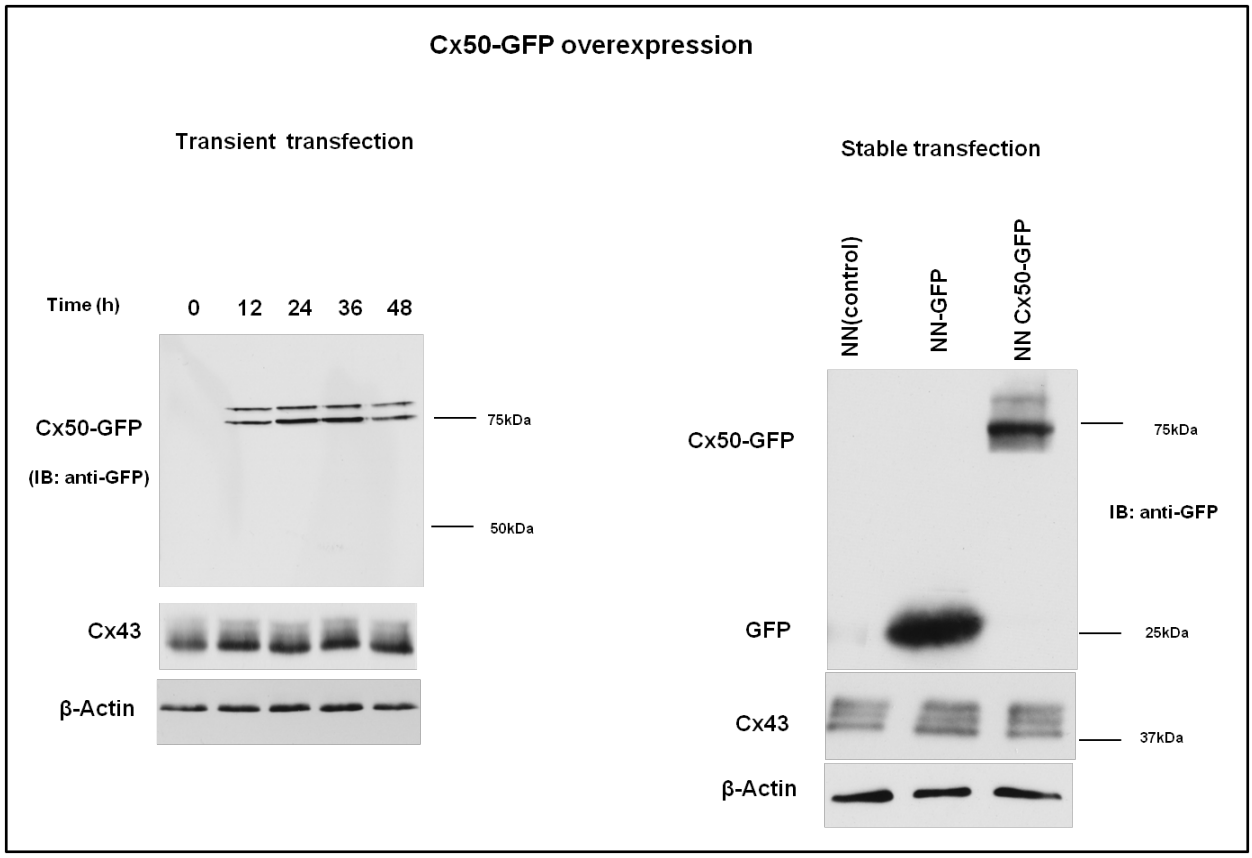

Figure 3.5 Overexpression of Cx50 does not cause a decrease in Cx43 protein levels.

NN1003A cells were transiently (left) or stably (right) transfected with plasmid encoding Cx50-GFP. Cell lysates were prepared and equal amounts of total protein were run in to SDS-PAGE followed by western blot. The Cx50-GFP and Cx43 proteins were detected by probing the blots with anti-GFP and anti-Cx43 antibodies, respectively.

3.3.5. Treatment with proteasome inhibitors restores Cx43 protein level while lysosomal inhibitors have no effect

Next I investigated the pathway that is involved in the degradation of Cx43 upon Cx46 overexpression. Cx43 degradation have been reported to be mediated by both lysosomal and proteasomal pathways. To identify which proteolytic degradation pathway is involved, I treated stably transfected cells with a series of inhibitors for both lysosome and proteasome and analyzed their effect on Cx43 degradation by western blot. Treatment with the lysosome inhibitor, leupeptin, did not cause a change in the Cx46-induced loss of Cx43 (Figure 3.6A, lane 4). Treatment with another lysosome inhibitor, chloroquine, or delivery vehicle DMSO showed the same result as no change in the reduced levels of Cx43 was observed (Figure 3.6B-C, lane 4). However, when stably transfected cells were incubated with proteasomal inhibitor, ALLN (N-Acetyl-Leu-Leu-Nle-CHO), Cx43 protein levels were not reduced (Figure 3.7A). Treatment with ALLN restored all three forms of Cx43 protein (Cx43-P0, P1 and P2) to the levels of Cx43 protein in control untransfected cells (Figure 3.7A-B). The restoration of Cx43 protein levels, due to the addition of the proteasome inhibitor, was further confirmed by treating the cells with another proteasomal inhibitor MG132. Expectedly, the Cx46-induced loss of Cx43 was strongly counteracted in Cx46-GFP expressing NN1003A cells (Figure 3.7A). Together, these results suggested that the proteasome was the major site where Cx43 was degraded due to overexpression of Cx46.

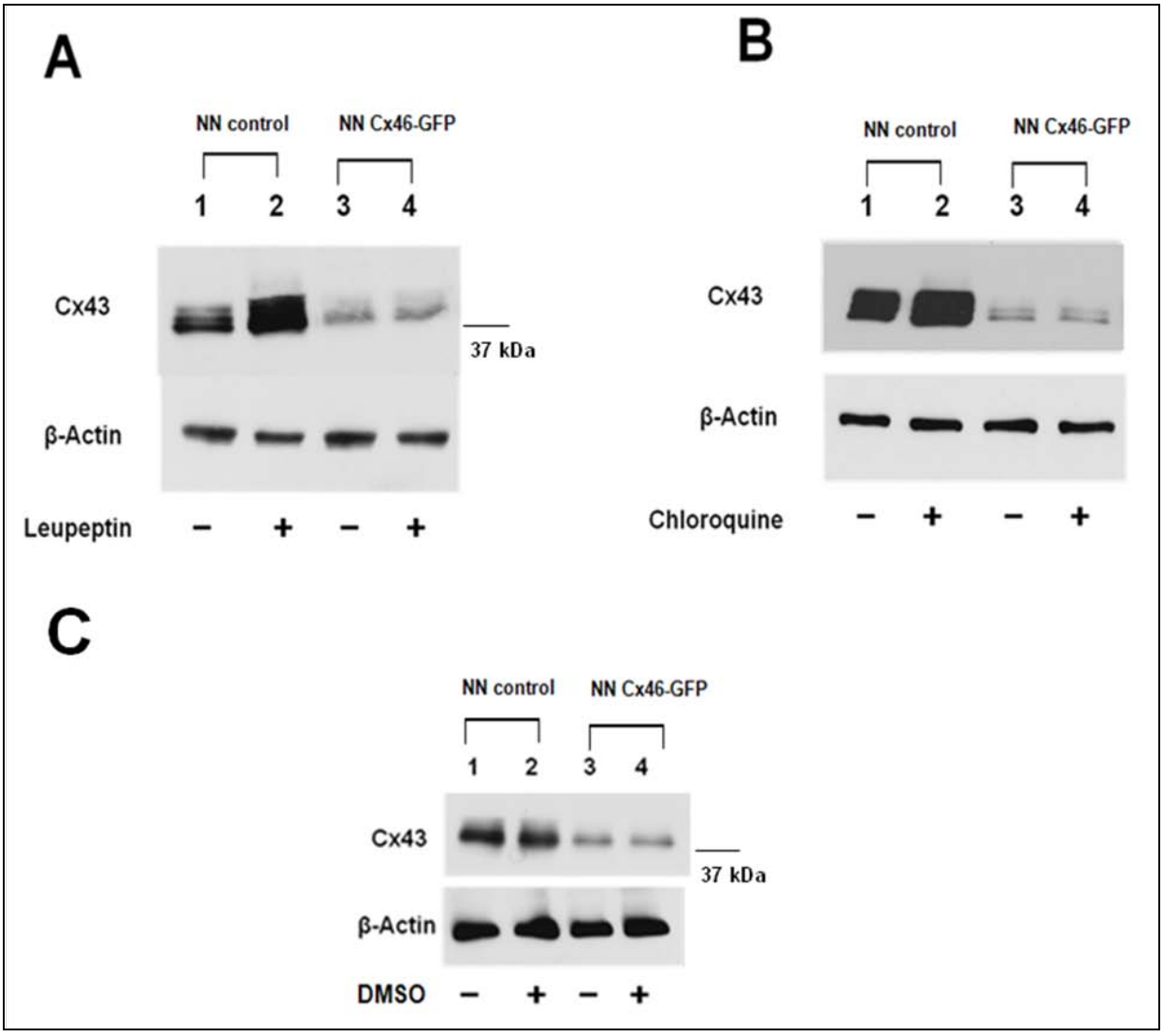

Figure 3.6 Treatment with lysosome inhibitors has no effect on Cx46-induced degradation of Cx43.

Untransfected control and Cx46-GFP stably transfected cells were treated with lysosome inhibitors (A) leupeptin (200 uM) or (B) chloroquine (200 uM) or (C) vehicle (DMSO) for 4 hr. Cell lysates were prepared and equal amounts of total protein were analyzed by western blot using antibody against Cx43 (anti-Cx43 CT) and β-actin (loading control). Lanes 1 and 2: Untransfected (control) cells. Lanes 3 and 4: Cx46-GFP stably transfected cells.

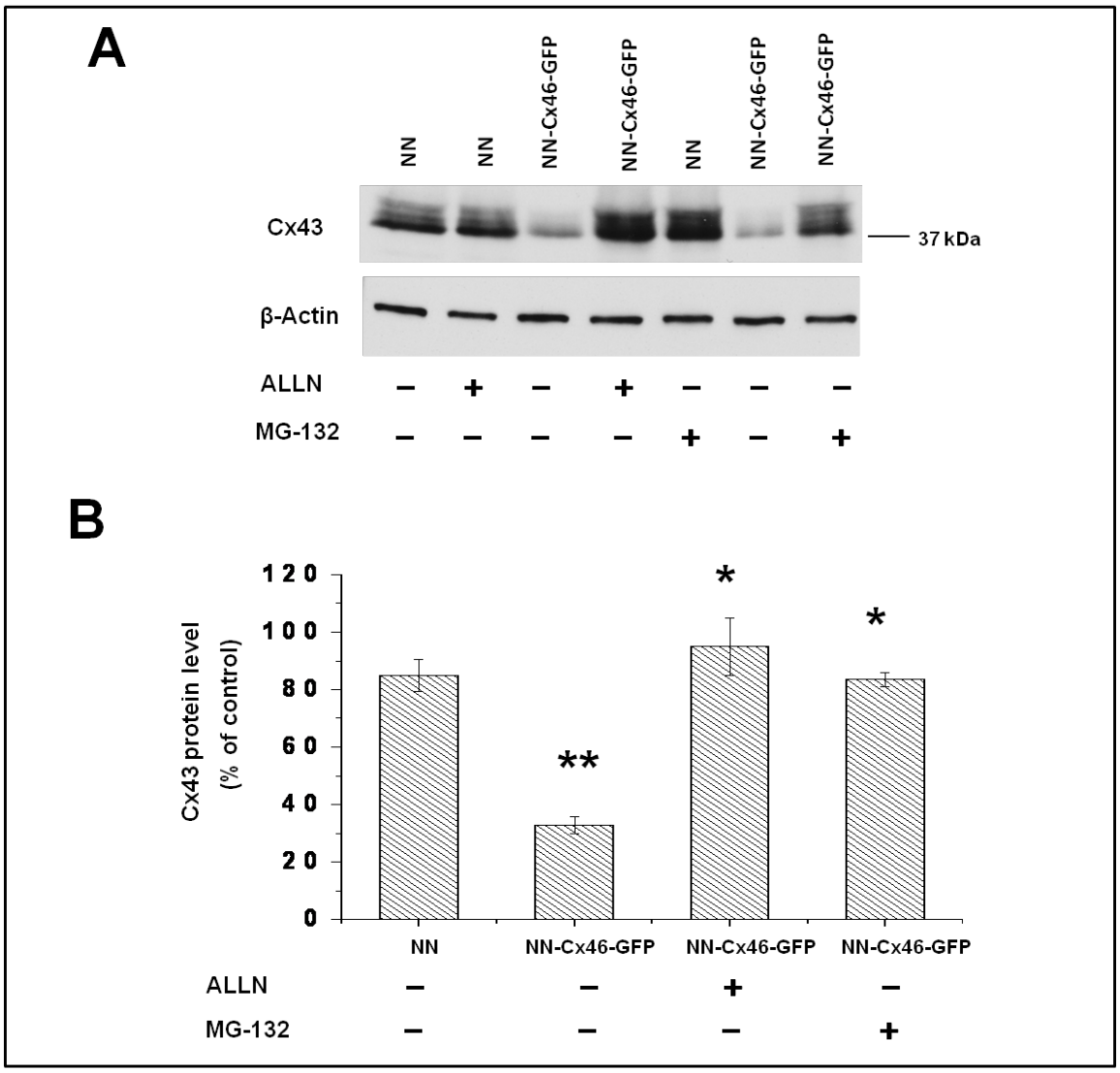

Figure 3.7 Treatment with proteasomal inhibitors counteracts Cx46-induced loss of Cx43.

(A) Control or Cx46-GFP stably transfected NN1003A cells were treated with proteasomal inhibitors, ALLN (100uM) or MG-132 (10uM), for 4 hr. Cell lysates were prepared and equal amounts of total cell protein in each cell lysate were analyzed by western blot with antibodies against Cx43 (anti-Cx43 CT) and β-actin (loading control)

(B) Densitometric analyses show that the treatment with ALLN or MG-132 restored Cx43 protein levels in Cx46-GFP expressing cells. Cx43 and β-actin bands were digitized by UN-SCAN-It gel software. The average pixel value was calculated for each Cx43 band, normalized and plotted in % of control (β-actin). Asterisk (*) indicates

significant statistical difference (P<0.05) between indicated data and Cx46-GFP expressing untreated cell (double asterisk **)

3.3.6. Overexpression of Cx46 increases Cx43 ubiquitination

Proteasomal degradation may involve direct proteolysis or ubiquitin-mediated degradation of targeted protein. Binding of ubiquitin has been previously shown to serve as an important signal in the regulation of Cx43 localization and turnover (Leithe and Rivedal 2004; Leithe, Kjenseth et al. 2009). To determine if Cx46-induced Cx43 degradation is initiated by ubiquitin conjugation, first, Cx43 was immunoprecipitated from untransfected or GFP or Cx46-GFP stably transfected cells using anti-Cx43 CT antibody. Immunoprecipitated samples were then analyzed for the presence of ubiquitinated Cx43 by western blot using anti-ubiquitin antibody. As a positive control, anti-ubiquitin antibody was able to recognize purified ubiquitin ladder (polymerized ubiquitin chain through lysine 48) (Figure 3.8B). In non-transfected (control) and GFP (only) transfected cells, a faint ladder of ubiquitin bands of high molecular weight (between 50-75 kDa regions), characteristic of polyubiquitinated Cx43, was observed (Figure 3.3A, lanes 2 and 3). I also wanted to investigate whether Cx50 overexpression has any effect on Cx43 ubiquitination level. Stable or transient overexpression of Cx50 showed the presence of similar ubiquitin ladder with faint intensities (Figure 3.3A, lanes 4 and 5). But interestingly, cells stably overexpressing Cx46-GFP demonstrated a ladder of ubiquitin bands with strong intensities at the same region of the blot (Figure 3.3A, lane 6). However, in the negative control sample, where cell lysate made from Cx46-GFP expressing cells was immunoprecipitated with a rabbit non-specific IgG,

ubiquitin bands were not detected (Figure 3.3A, lane1). These data indicated that Cx46 overexpression caused an increase in the ubiquitination of Cx43.

To further confirm that the presence of ubiquitin ladders in the immunoprecipitated samples represented actual ubiquitinated-Cx43 and did not result because of nonspecific interactions of the antibody, I preincubated anti-ubiquitin antibody with the purified ubiquitin ladder (Figure 3.3B) and used this neutralized antibody to stain ubiquitinated Cx43 in anti-Cx43 antibody immunoprecipitated samples on the western blot. The anti-ubiquitin antibody binds to purified ubiquitin ladder and therefore would not be available to bind ubiquitinated Cx43 on the western blot. Indeed, the ladder of bands (over 50 kDa region), characteristics of ubiquitinated Cx43 in Cx46-GFP expressing cells, was not seen when anti-ubiquitin antibody was preincubated with the purified ubiquitin ladder (Figure 3.8C). The band at 50 kDa region was probably resulted because of cross reactivity of the secondary antibody with IgG. Collectively, these data suggested that the overexpression of Cx46 induced ubiquitin binding to Cx43 leading to Cx43 degradation by the proteasome pathway.

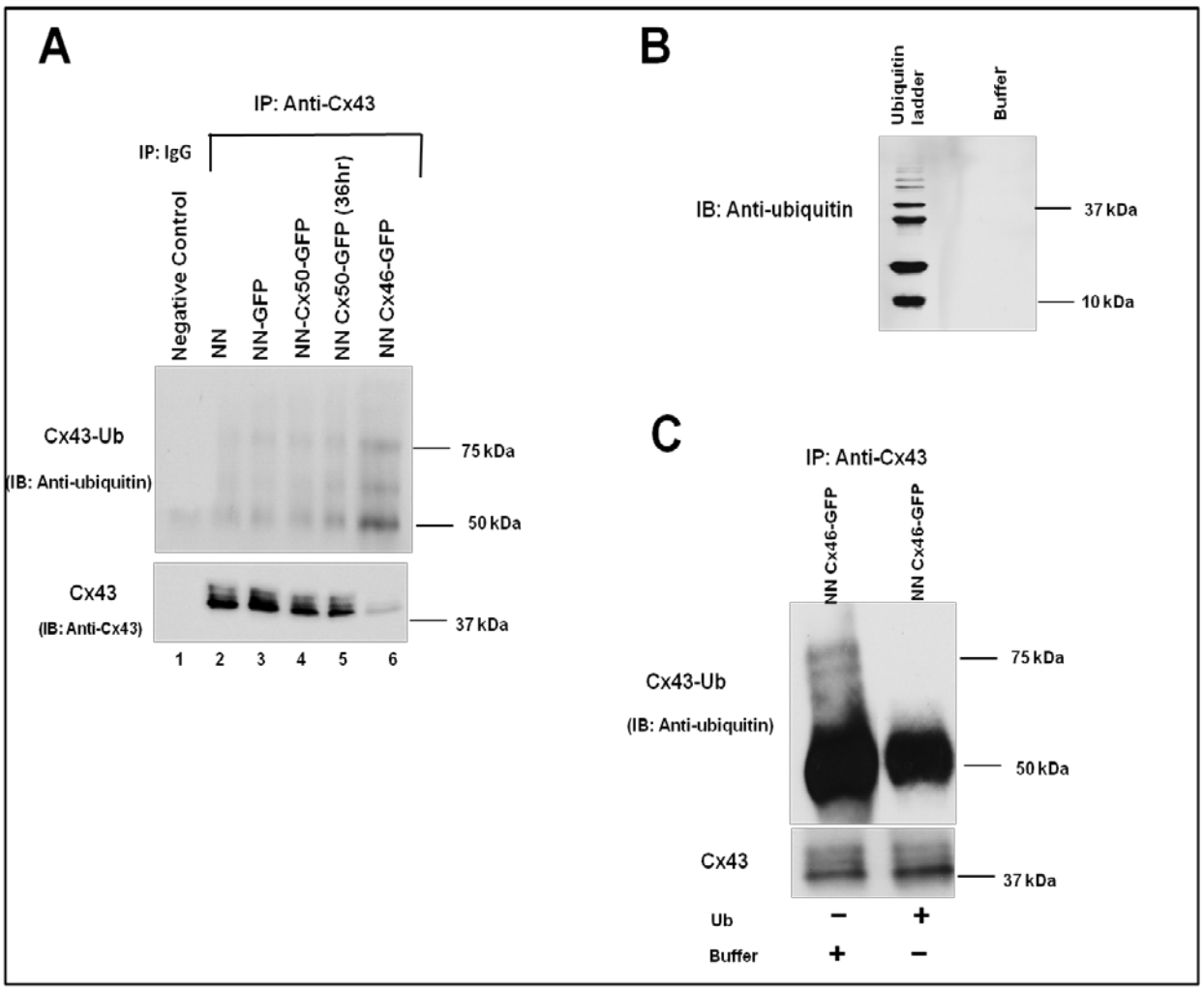

Figure 3.8 Overexpression of Cx46 increases ubiquitination of Cx43.

(A) Lysates from untransfected (NN) cells (lane 2) or GFP (lane 3), Cx50-GFP (lane 4), Cx46-GFP (lane 6) stably transfected or Cx50-GFP transiently transfected cells (36 hr, lane 5) were prepared and subjected to immunoprecipitation by rabbit anti-Cx43 antibody (anti-Cx43 CT). Equal amounts of immunoprecipitated samples were loaded into 8% SDS-PAGE. Ubiquitinated Cx43 were detected by immunoblotting using anti-ubiquitin antibody. As a negative control, cell lysate, made from Cx46-GFP expressing cells, was immunoprecipitated with a rabbit non-specific IgG. The same blot was stripped and reprobed with anti-Cx43 antibody (anti-Cx43CT) to show equal loading of immunoprecipitates. A band observed at 50 kDa region in the negative control sample was probably resulted from cross reactivity of the secondary antibody to IgG. **(B)** As a positive control of anti-ubiquitin antibody, a commercially available purified ubiquitin

ladder (ubiquitin is polymerized through lysine 48) was subjected to 15 % SDS-PAGE. Western blot was performed and probed with anti-ubiquitn antibody. **(C)** Anti-Cx43 (anti-Cx43CT) immunoprecipitate samples were made from Cx46-GFP expressing cells and subjected to SDS-PAGE and analyzed by western blotting with anti-ubiquitin antibody that was preincubated with the ubiquitin ladder or buffer. The Cx43-ubiqtuin bands were not detected when anti-ubiquitin antibody was preincubated with ubiquitin ladder.

3.3.7. Cx46 colocalizes with Cx43 in Cx46-GFP expressing stable cells

To further explore the mechanism involved in the Cx46-induced loss of Cx43, I investigated whether Cx46 interacts with Cx43 in stably transfected cells by imuunofluorescence-confocal microscope study. Staining NN1003A control (untransfected) or GFP transfected cells with anti-Cx43 antibody (red) showed Cx43 gap-junctional staining at cell-cell interfaces (Figure 3.9). However Cx43 gap junctions had disappeared in Cx46-GFP stably transfected cells and Cx43 staining was only observed in the cytoplasm. In these cells, green signal representing Cx46-GFP was mainly distributed in the intercellular compartments with no apparent membrane signal being visible (Figure 3.9). The merged figure showed co-localization of Cx46 and Cx43 (yellow) to the intercellular compartments. However, staining NN1003A control cells with anti-Cx46 (Figure 3.9 a) and anti-Cx43 antibody showed no significant co-localization between endogenous Cx46 and Cx43. These data suggested that Cx46, only upon overexpression, co-localized with Cx43.

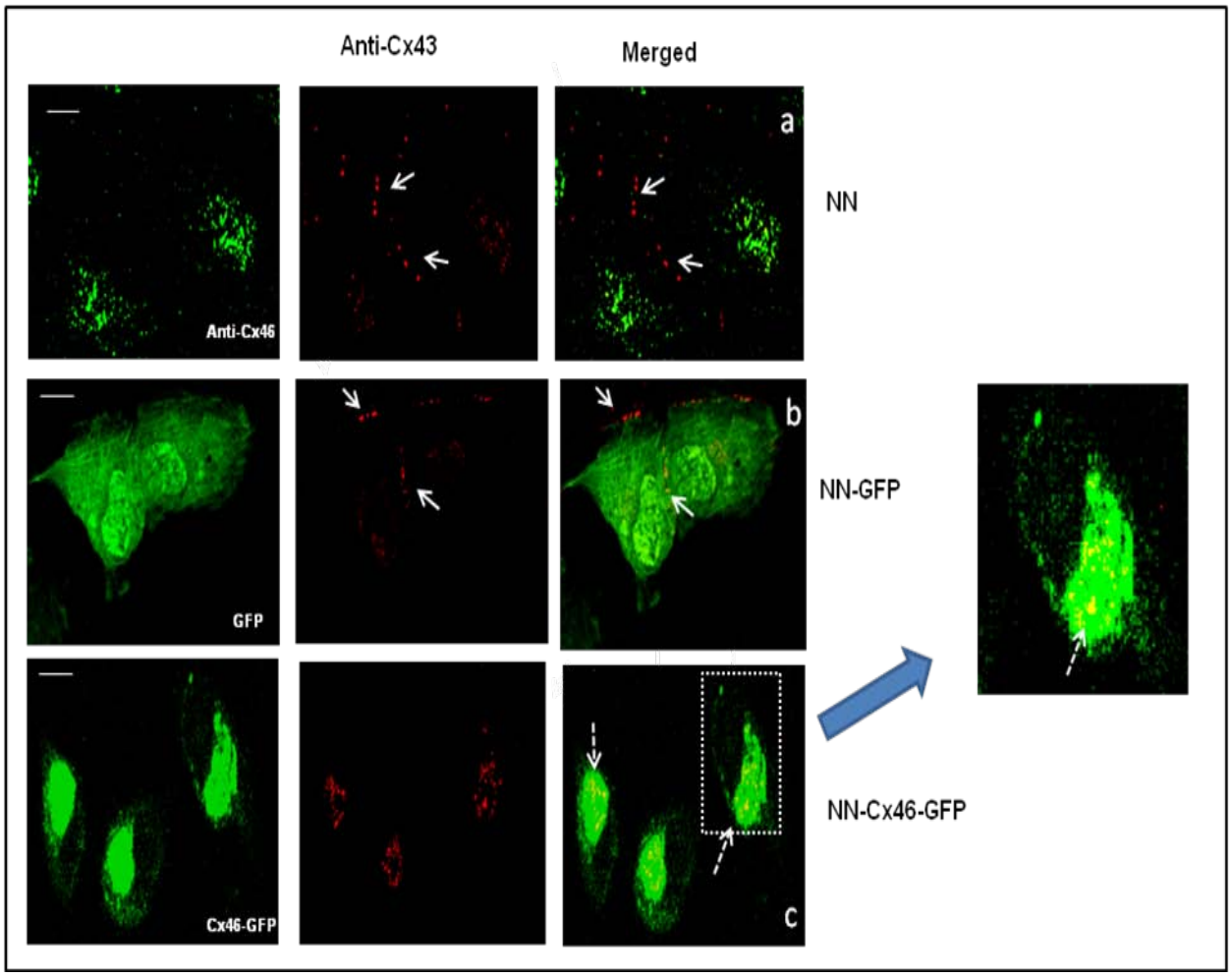

Figure 3.9 Cx46 colocalizes with Cx43 in stably transfected cells.

GFP (b) or Cx46-GFP stably (c) transfected NN1003A cells were fixed in paraformaldehyde, permeabilized and stained with anti-Cx43 (red). The untransfected control cells were also fixed, permeabilized and stained with anti-Cx46 (to visualize endogenous Cx46, green) and anti-Cx43 antibodies. The images were taken using a Nikon C1 scanning confocal microscope. The overexpression of GFP (b) or Cx46-GFP (c) was visualized by green fluorescence only. White solid arrows indicate Cx43 gap junctions in the image. Broken white arrows indicate co-localization (yellow) of Cx46 and Cx43 in cells stably overexpressing Cx46-GFP. The inset (white) has been enlarged (blue arrow) where co-localization is more evident.

3.3.8. Carboxyl (C-) terminal tail of Cx46 is required for Cx43 degradation

Next, we asked which part of the Cx46 protein is required to induce Cx43 degradation. Like other connexins, Cx46 also has four transmembrane domains with a short N-terminal and relatively long C-terminal domain within the cytoplasm. Amino acid sequence alignments of rat Cx46 and Cx50 shows significant sequence similarity with the highest variability observed at the C-terminal tail region (Figure 3.11). Since Cx50 overexpression was not found to mediate Cx43 degradation I speculated whether the C-terminal tail region (amino acid 225-416) is the active part of Cx46 that induces Cx43 degradation. To test this hypothesis I transfected NN003A cells with plasmid encoding Cx46 GFP tagged C-terminal tail deletion (residues 232-416) mutant (Cx46-dCT-GFP, Figure 3.10 A) and analyzed the effect of overexpression of mutant Cx46 protein on Cx43 protein levels (Figure 3.10B). Cx46-dCT-GFP was expressed at predicted molecular weight of ~50 kDa as determined by western blot using anti-GFP antibody (Figure 3.10B). As seen in the figure 3.10B, overexpression of Cx46 deletion mutant did not induce Cx43 degradation at 24-48 hr after transfection. This was further confirmed by immunofluorescence-confocal microscope studies where Cx43 gap junctions were observed in the cells overexpressing C-terminal Cx46 deletion mutant (Figure 3.10C). In these cells, cytoplasmic localization of Cx43 or co-localization with Cx46 was not seen (Figure 3.10C). These data clearly suggested that deletion of C-terminal tail rendered Cx46 mutant ineffective to co-localize and degrade Cx43.

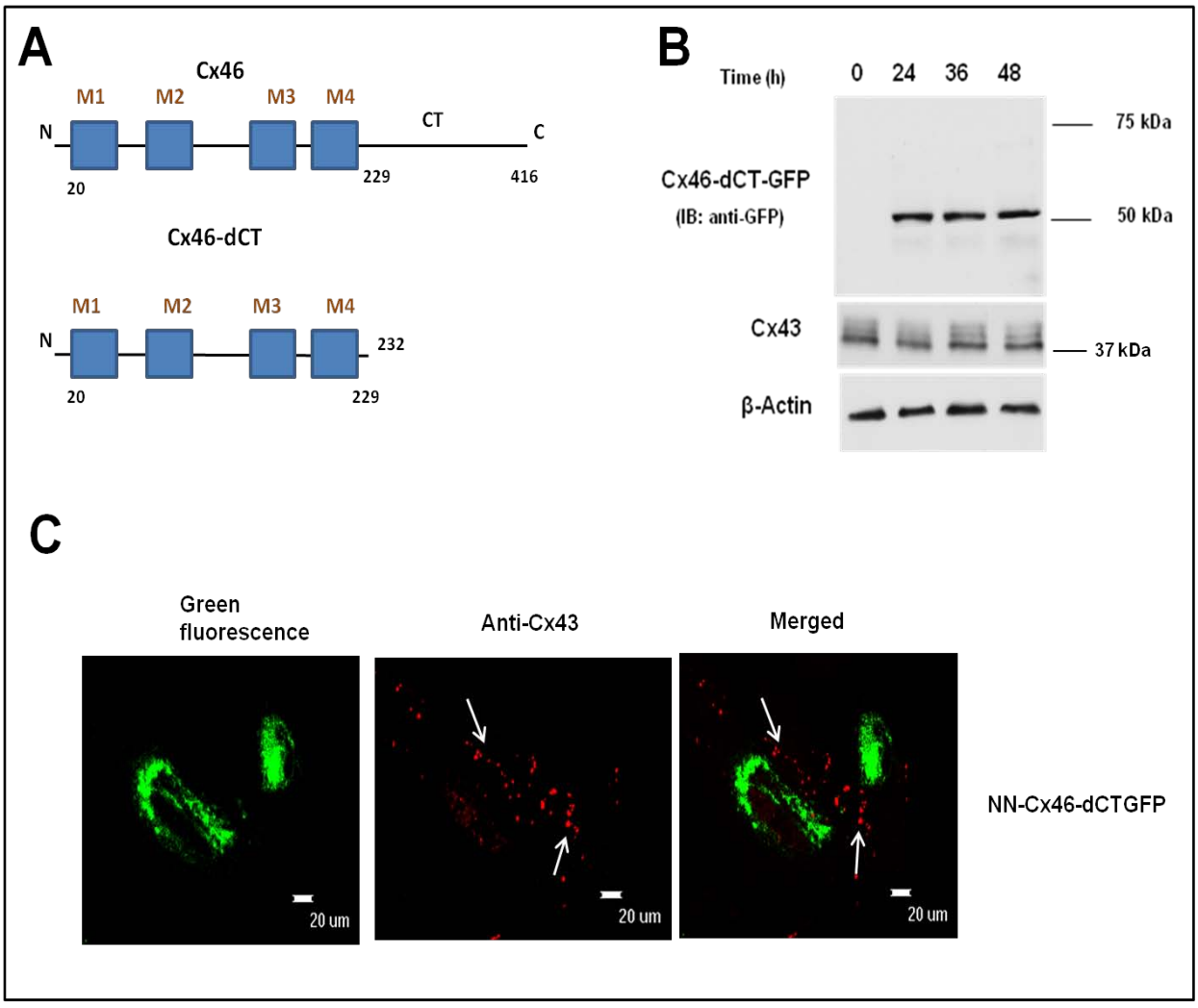

Figure 3.10 The C-terminal region of Cx46 is required to induce Cx43 degradation. **(A)** Schematic diagram of wild type Cx46 and C-terminal (CT) deletion (amino acid 225-416) Cx46 mutant (Cx46-dCT). The cDNA corresponding to Cx46-dCT was sub-cloned into pEGFP-N3 vector to get Cx46-dCT-GFP **(B)** Overexpression of Cx46 deletion mutant (Cx46-dCT) does not induce Cx43 degradation. Western blot analyses of Cx46-dCT mutant (GFP tagged) and Cx43 in untransfected cells (0hr) or in cells transiently transfected with plasmid encoding Cx46 deletion mutant (Cx46-dCT-GFP). The expression of Cx46-dCT-GFP was confirmed by probing the blot with anti-GFP antibody. The blot was also probed with anti-Cx43 CT and β-Actin antibodies.

(C) Immunofluorescence-confocal images of Cx43 and Cx46-dCT-GFP in NN1003A cells. Cells were transfected with plasmid encoding Cx46-dCT-GFP for 36 hr. Cells

were fixed, permeabilized and stained with anti- Cx43 antibody. The images were taken using a Nikon C1 scanning confocal microscope.

3.4. Discussion

In this study I demonstrated a novel role of gap junction Cx46 protein in the regulation of turnover of another gap junction protein Cx43. Using a well-characterized, long-term lens epithelial cell culture overexpressing Cx46, I found Cx46-induced degradation of Cx43 (Figure 3.1). I confirmed reduction in the levels of all three forms of Cx43 (nonphosphorylated P0 and phosphorylated P1 and P2) protein by western blotting with antibodies against C-terminal and N-terminal region (Figure 3.1 A). The cleavage of Cx43 C-terminal fragments has been reported to occur in lens fiber cells, therefore, antibody against N-terminal region of Cx43 was also used to confirm degradation (Mathias, White et al. 2010). Our data also showed that the protein expression of another major lens connexin, Cx50, was not altered upon Cx46 overexpression (Figure 3.1 A-C). In addition, overexpression of Cx50 protein did not induce degradation of Cx43 (Figure 3.5). Collectively, these indicate that only Cx46, when overexpressed, can stimulate Cx43 degradation which another connexin, Cx50, cannot do.

The reduction of Cx43 protein due to the Cx46 overexpression is not pre-transcriptionally initiated as Cx43 mRNA level was not changed as determined by RT-PCR experiments. (Figure 3.4) Therefore, I investigated the proteolytic pathway that is involved in the degradation. Several studies have reported previously that Cx43 are

degraded via both lysosome and proteasome. The lysosomal pathway was shown to be involved in the degradation of Cx43 when it is internalized from cell membrane (Berthoud, Minogue et al. 2004; Leithe and Rivedal 2004). Treatment with protease inhibitors such as leupeptin, which inhibits cathepsin B, H and L (Leithe and Rivedal 2004), and chloroquine, which is another well know lysosome inhibitor(Gonzalez-Noriega, Grubb et al. 1980; Qin, Shao et al. 2003; Marques, Pereira et al. 2004), showed no effect in the degradation of Cx43 in Cx46-GFP expressing cells (Figure 3.6). But, the treatment with a proteasome inhibitor, ALLN, strongly counteracted Cx46-induced Cx43 degradation (Figure 3.7). ALLN is widely used for proteasome inhibition studies (Griscavage, Wilk et al. 1996; Hughes, Ortmann et al. 1996; Laing, Tadros et al. 1998; Musil, Le et al. 2000), however, ALLN can also inhibit some non-proteasomal enzymes such as calpains and cathepsins (Boswell, Le et al. 2009). I therefore treated the cells with another very potent and widely used proteasome inhibitor, MG132 (Mailhes, Hilliard et al. 2002; Leithe and Rivedal 2004; Leithe and Rivedal 2004; Alexandrova, Petrov et al. 2008). The effect of MG132 treatment was identical, as seen with ALLN, where Cx43 protein levels were restored to normal in Cx46-GFP stably transfected cells (Figure 3.7).

Conjugation of ubiquitin serves as a signal for targeting protein to the proteasome for degradation. The ubiquitin-proteasome pathways mediates the degradation of several proteins that are localized to the different compartments of cells and are involved in signal transduction or other cellular processes (Hershko and Ciechanover 1998). Cx43 has also been previously reported to undergo

polyubiquitination under stress conditions. Tumor-promoting protein kinase C (PKC)-activator, phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), has been shown to cause Cx43 ubiquitination and subsequent proteasomal degradation (Leithe and Rivedal 2004). Therefore I looked into the Cx43 ubiquitination status in the cells overexpressing Cx46 by co-immunoprecipitation analyses. Interestingly, an increase in Cx43 ubiquitination was only found in Cx46-GFP expressing cells (Figure 3.8). Together, these results suggest that Cx46-induced degradation of Cx43 is mediated by the ubiquitin-proteasome pathway while the lysosome does not appear to be involved.

In this study, I observed that Cx46 is mainly localized to the intercellular compartments of rabbit lens epithelial NN1003A cells (Figure 3.2). Even upon overexpression, localization of Cx46 was not detected in the plasma membrane, as determined by immunofluorescence-confocal microscope study (Figure 3.2) and western blot analyses of plasma membrane protein fractions (Figure 3.3A). Cx46 was shown to form gap junction channels in lens fiber cells which are differentiated from epithelial cells (Mathias, White et al. 2010). Our lab has also found endogenous Cx46 protein localization to the intercellular compartments in human lens epithelial cells (HLEC) (Satyabrata Das thesis, 2010) in culture. In the previous chapter, I have observed a similar Cx46 localization in breast cancer MCF-7 cells. The intercellular localization of Cx46 was also shown in bone osteoblastic (Koval, Harley et al. 1997) and lung alveolar cells (Abraham, Chou et al. 1999). Conventional techniques such as immunofluorescence and western blot do not completely rule out the possibility of membrane localization of Cx46. Connexins have rapid turnover rates with half-lives of

few hours. Therefore, if Cx46 is rapidly internalized after being delivered to the plasma membrane it would not be detectable at the cell surface by conventional indirect immunofluorescence. Nonetheless, since membrane localization was not appeared, it is unlikely that overexpression of Cx46 induced Cx43 degradation by facilitating its internalization from the plasma membrane. Our study also showed that Cx43 co-localized with Cx46 in only stably transfected cells at intercellular region (Figure 3.9). However endogenous Cx46 was not found to co-localize with Cx43 (Figure 3.9 a). These results suggest that Cx46, when endogenously expressed is not involved in Cx43 turnover, but, when overexpressed, can interact with Cx43 possibly at intercellular compartments and induce its degradation by ubiquitin-proteasome pathway.

Cx50 is another connexin that is highly expressed in lens. As mentioned before, though Cx46 is upregulated and Cx43 is downregulated during lens epithelial to fiber cell differentiation, Cx50 level remains unchanged. I found that, unlike Cx46, overexpression of Cx50 did not induce Cx43 degradation. Amino acid sequence-alignment shows that Cx46 shares high sequence similarity with Cx50. Considerable sequence variability is observed only at the C-terminal region of the two proteins (residues 230-416 for Cx46 and residues 230-440 for Cx50) (Figure 3.11). The functional properties of connexins are regulated by the post-translational modifications of their C-terminal tail region which shows little sequence homology among different connexin isoforms. Phosphorylation of Cx43 at S-262 has been reported to regulate the inhibition of DNA synthesis in primary cardiomyocytes (Dang, Jeyaraman et al. 2006). C-terminal region of Cx32 has been shown to be involved in the inhibition of malignant

phenotypes of human metastatic renal cell carcinoma cell line (Caki-1) mainly through the inactivation of Src signaling (Fujimoto, Sato et al. 2005). In the present study, I have shown that the C-terminal tail of Cx46 is required to induce Cx43 downregulation. Cx43 protein levels were not reduced in the cells expressing Cx46 C–terminal tail deletion mutant (Cx46-dCT). The localization of the Cx46-dCT mutant protein was similar to the wild type Cx46, predominantly to the intercellular compartments, but, mutant protein did not co-localize with Cx43. Collectively, these results also indicate that the Cx46 C-terminal tail is required for co-localization with Cx43 to mediate degradation by the ubiquitin-proteasome pathway.

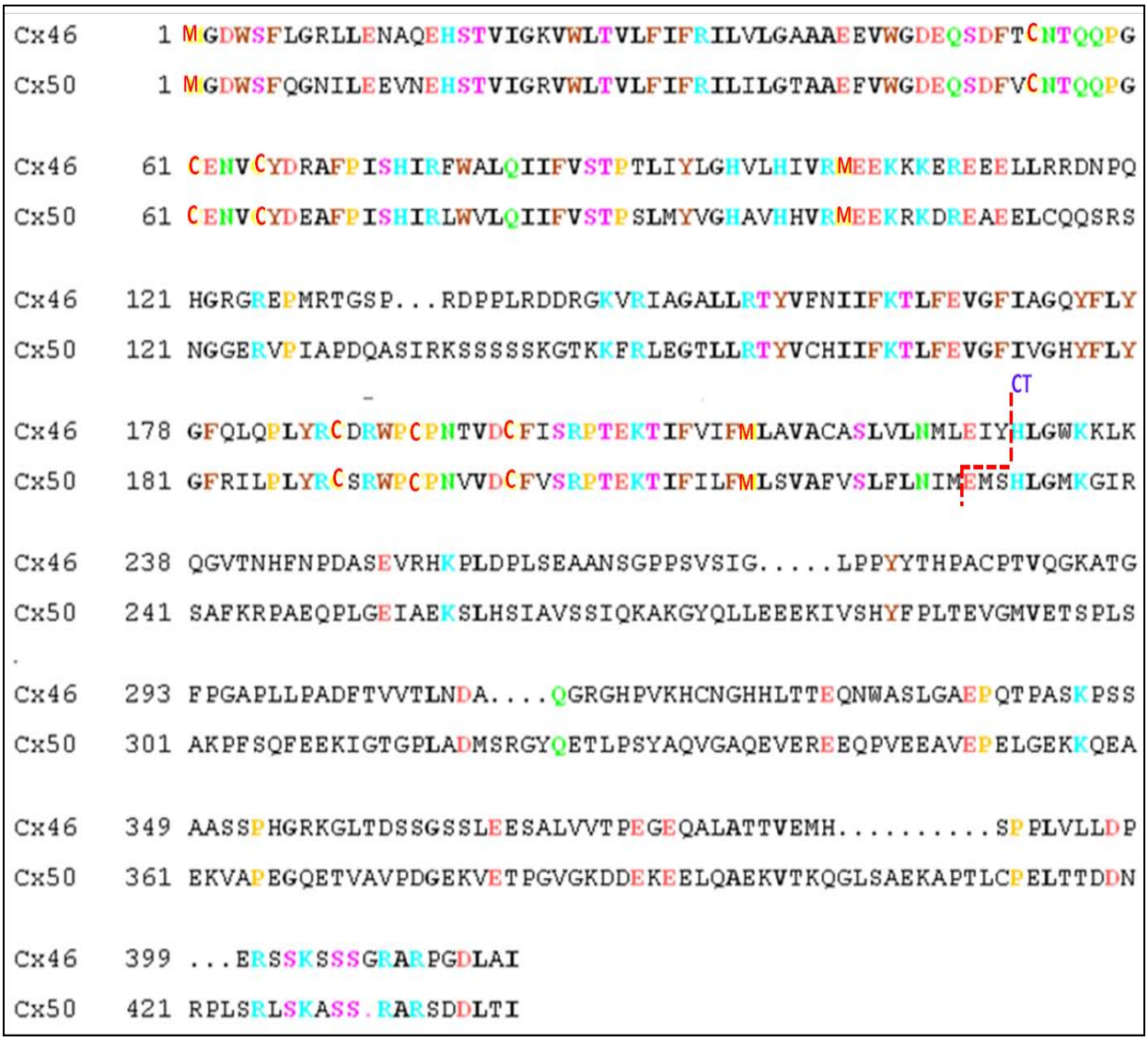

Figure 3.11 Protein sequence alignment of rat Cx46 and Cx50.

The amino acid residues that are present in both Cx46 and Cx50 are given in colors or bold black .The red line (dotted) represents the start of C-terminal region The NCBI reference sequences are NP_077352.1 for Cx46 and NP_703195.2 for Cx50.

Cx43 has been shown previously to be ubiquitinated by the action of the enzyme Nedd4, an E3 ubiquitin ligase (Leykauf, Salek et al. 2006; Girao, Catarino et al.

2009). Nedd4 mediates ubiquitin-dependent degradation of target proteins that have PY motif (PPxY) (Ingham, Gish et al. 2004). Cx43 has a Nedd4 binding site (PY motif) at its C-terminal region ($S^{282}PPGY^{286}$) that binds to Nedd4 which is recruited by the MAPK dependent pathway (Figure 3.10). The C-terminal consensus PY motif of Cx43 contains a nearby lysine residue ($S^{282}PPGYK$**L**V^{289}). It is speculated that when Nedd4 binds to Cx43 at the C-terminal region, it ubiquitinates the nearby lysine (Lys 288) and targets Cx43 for proteasomal degradation. Nedd4 has been found to co-localize with Cx43 both at plasma membrane and intercellular compartments. Cx46 has a PY motif ($^{274}LPPYY^{279}$) at its C-terminal end but the nearby lysine residue is missing whereas Cx50 lacks any such analogous binding site for Nedd4. Therefore, I speculate that in Cx46-GFP expressing cell, Cx46 binds to Nedd4 and delivers it to Cx43 during when it co-localizes with Cx43 in the intercellular compartments. The enhanced interaction of Nedd4 with Cx43 results in increased ubiquitination and degradation of Cx43 by the ubiquitin-proteasome pathway This could also explain why Cx50 or Cx46-dCT-GFP overexpression does not cause Cx43 degradation as they do not have the binding motif for Nedd4. The wild-type Cx46-GFP contains binding motif for Need4 ($^{274}LPPYY^{279}$) but it is not degraded as it lacks the nearby critical lysine residue for ubiquitnation. Further studies with Cx46 mutants, defectives in Nedd4 binding, are required to investigate the above hypothesis.

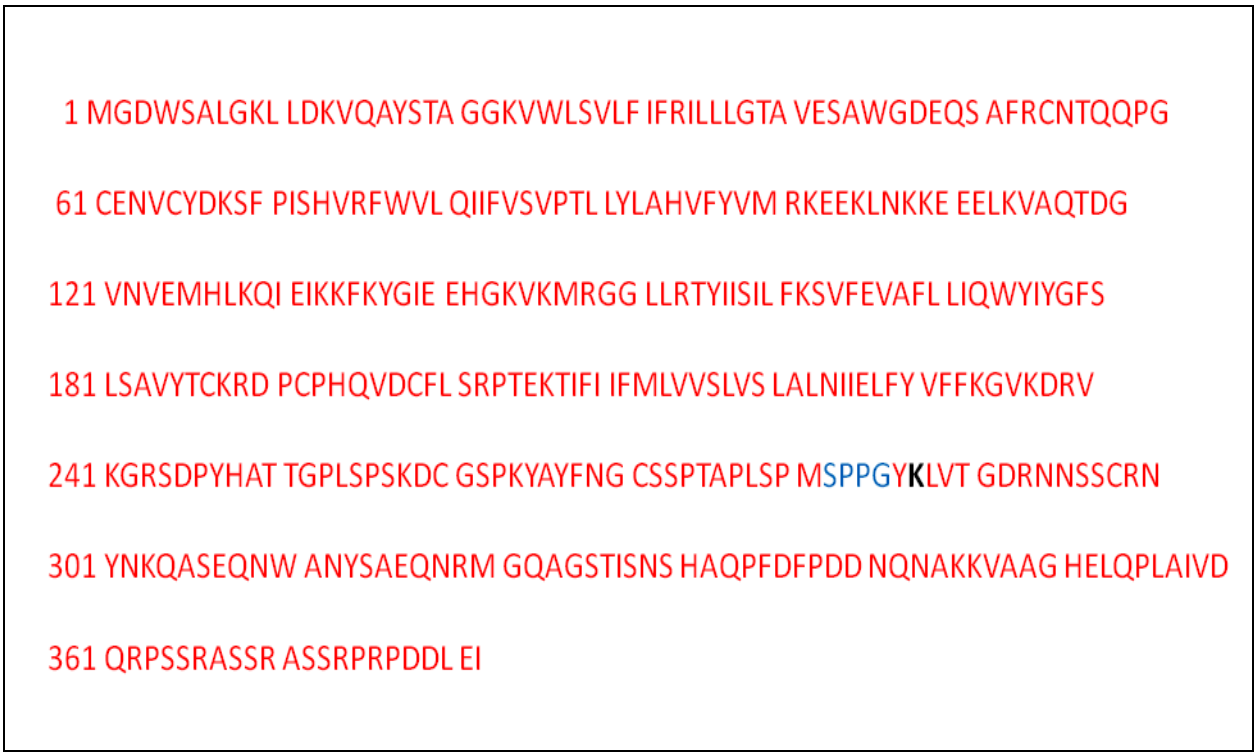

Figure 3.12 The protein sequence of rat Cx43 protein (NCBI Reference Sequence: NP_036699.1).

The Nedd4 binding site has been shown in blue and critical lysine residue has been shown in black.

Our results will help gain a better knowledge of the lens epithelial-to-fiber cell differentiation and the role that connexins play in this process. Lens differentiation process has been shown to involve several growth factors such as fibroblast growth factors (FGF), Wingless (Wnt), transforming growth factor-Beta (TGF-β) proteins (de Iongh, Lovicu et al. 2001; Lyu and Joo 2004), as well as several cell signaling pathways, including mitogen activated protein kinase (MAPK) signaling (Wang, Stump et al. 2009). However the role of connexins in the lens differentiation process is largely elusive. Our study indicates that Cx46 might play a major role in lens differentiation by enhancing

Cx43 degradation. Our lab has also found that the over expression of Cx46, in addition to inducing Cx43 degradation, elicits morphological changes in lens epithelial cells that resemble fiber cells (Takemoto lab, unpublished data). Our results will also help understand the connexins mediated regulation of breast tumorigenesis. As mentioned in the previous chapters, Cx43 has anti-tumor properties and expression of Cx43 is downregulated in breast tumors, the molecular mechanism of which is not clearly known. In Chapter 2 of this dissertation, I have shown that Cx46 is upregulated in breast tumors and favors tumor growth. Therefore I speculate that Cx46 promotes breast tumor growth by inducing the degradation of the tumor suppressor protein Cx43.

In conclusion our data indicate that overexpression of Cx46 induces the degradation of another connexin, Cx43. Cx46, upon overexpression, co-localizes with Cx43. I also provide evidence that the C-terminal tail of Cx46 is essential for co-localization and Cx46-induced degradation of Cx43. The ubiquitination of Cx43 is strongly increased in Cx46-GFP expressing cells and proteasomal inhibitors counteract the Cx43 degradation Therefore Cx46-induced Cx43 degradation is likely to be mediated via the ubiquitn-proteasome pathway.

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Chapter 4- Summary

Breast cancer is the second most common form of cancer in women and one of the leading causes of cancer deaths in white, black, Asian/Pacific Islander, and American Indian/Alaska Native women. Therefore breast cancer tumorigenesis has been a subject of intense research for the last 10 years. In breast carcinogenesis among the most studied genes are those for hypoxia. Hypoxia induces cell-death of normal cells but breast tumors use hypoxia as an adaptation to grow and survive under nutrient deprived environment. Breast tumor is an adaptive hypoxic tissue but lens is a naturally hypoxic tissue in the animal body. Whether these two different tissues use the same survival strategies to escape from hypoxia-induced cell death has been largely unanswered. In the current studies, I have compared these two tissues to answer these questions.

In lens Cx46 protein is abundantly expressed in the fiber cell region which is located at the very hypoxic center. Initially, I observed high levels of Cx46 protein in human breast cancer MCF-7 cells relative to normal breast epithelial cells (HMEC).The occurrence of Cx46 expression in the region of low oxygen led us to hypothesize that both lens and breast tumors use Cx46 protein as a means to survive under the adverse environment of hypoxia. The role of Cx46 was studied by the use of RNA interference (RNAi) technology. Knock-down of Cx46, by a selective siRNA, resulted in increased hypoxia-induced cell death of both human lens epithelial cells (HLEC) and breast cancer MCF-7 cells probably by the mechanisms independent of Cx46 gap junction

activity. Hypoxia-specific role of Cx46 was also observed in hypoxia-sensitive connexin deficient neuronal N2A cells where exogenous expression of Cx46 rendered the cell hypoxia-resistant. The function of Cx46 in human breast tumor formation was further investigated, *in vivo*, in human tumor xenograft-bearing nude mice. The tumor of human origin in nude mice was generated by injecting human MCF-7 cancer cells into the inguinal region of mammary fat pads. Direct injection of the anti-Cx46 siRNA into the tumor inhibited tumors volume significantly, suggesting a possible role of Cx46 in breast tumor growth.

Among the members of connexin family of proteins, Cx43 is most extensively studied and regarded as a tumor suppressor. Several studies have shown that the expression of Cx43 is downregulated in breast tumors as compared to normal breast tissues. Therefore, Cx43 is reciprocally expressed when compared with Cx46 in breast tumors. This reciprocal relationship of Cx43 and Cx46 expression is also seen in lens during differentiation. In lens epithelial cells Cx43 is highly expressed whereas Cx46 has low expression. However in the lens fiber cells, which are differentiated from the epithelial cells, Cx43 is downregulated and Cx46 is abundantly expressed. In last part of my dissertation work, I looked into how Cx46 is associated with Cx43 degradation using rabbit lens epithelial cells. Overexpression of Cx46 caused a decrease in only protein levels of Cx43 while mRNA levels were not reduced. This Cx46-induced degradation of Cx43 was independent of its gap junction activity and completely counteracted by inhibitors of proteasome. The overexpression of Cx46 also increased the ubiquitin conjugation of Cx43, thereby suggesting that degradation is mediated by the proteasome. The C terminal deletion mutant of Cx46, when overexpressed, did not

reduce the Cx43 protein levels indicating the-C-terminal region, which has least sequence similarity among various domains of different connexins, is the part of Cx46 that is required to mediate degradation of Cx43.

In summary, I have established the novel functions of a protein Cx46, as survival factor in hypoxic cell death and as the regulator of turnover of another connexin, Cx43. These findings will help gain a better knowledge of processes such as breast tumorigenesis and lens differentiation.