THE ROLE OF CONNEXIN43 IN GLIOMA MOTILITY

By

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ABSTRACT

Gap junctions, proteinaceous channels which directly link the cytosol of adjacent cells and allow the passage of ions and small molecules, are formed from the hexameric oligomerization of connexin subunits. We are interested in the role of Connexin43 (Cx43), the most abundant isoform expressed in astrocytes, in glioma motility. To achieve this objective, we have isolated a C6 subclone endogenously expressing high levels of Cx43 (C6-H) and have employed *in vitro* wound healing and transwell assays to evaluate cellular motility. When compared to parental C6 cells in which Cx43 is expressed at low levels (C6-L), the C6-H subclones were more motile. To deduce whether Cx43 was indeed responsible for the observed differences in motility, the C6-H cells were retrovirally infected with Cx43 shRNA to stably knock down Cx43 expression. Coincident with the knockdown of endogenous Cx43, a decrease in motility and invasion was observed. As gap junction intercellular communication (GJIC) was also decreased, motility assays were conducted in the presence of gap junction inhibitors to evaluate the contribution of GJIC to cell motility. Because no significant differences in motility could be detected upon blocking GJIC, C6 cells exogenously expressing full length or truncated Cx43 were subjected to the aforementioned motility assays to expose alternate mechanisms of Cx43-mediated motility. Cells expressing full length Cx43 exhibited increased motility while cells expressing the truncated form of Cx43 did not. Our results indicate that downregulation of Cx43 decreases motility in C6 glioma cells and suggest that the carboxy terminus plays an important role in Cx43-mediated motility.

Keywords: Gap junction, Connexin43, C6 cells, Glioma, Motility, Invasion, RNAi.

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LIST OF ABBREVIATIONS

AM	Acetoxymethyl
ANOVA	Analysis of Variance
APC	Adenomatous Polyposis Coli
ARP2/3	Actin-Related Protein 2/3
BMP(s)	Bone Morphogenic Protein(s)
BRMS1	Breast Cancer Metastasis Suppressor 1
C-	Carboxy
DBT 2	Delayed Brain Tumor
DIC	Differential Interference Contrast
DMEM	Dulbecco's Minimal Essential Medium
CBX	Carbenoxolone
CCN	Cysteine-Rich61/Connective Tissue Growth Factor/Nephroblastoma
Cdc42	Cell Division Cycle 42
CK1	Casein Kinase 1
CMV	Cytomegalovirus
CMV43	Mice in which Cx43 is overexpressed by the CMV promoter
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
CNTFsRα	CNTF soluble receptor alpha
Cx(s)	Connexin(s)
Cx43	Connexin43
Cx43KO	Cx43 knockout
DADS	Diallyl Disulfide
DBMA	Dimethylbenzanthracene

DIC	Differential Interference Contrast
EGFR	Epidermal Growth Factor Receptor
EMT	Endothelial-to-Mesenchymal Transition
ER	Endoplasmic Reticulum
ERK	Extracellular Signal Related Protein Kinase
F-	Filamentous
G-	Globular
GAPDH	Glyseraldehyde-3-phosphate dehydrogenase
GBM	Glioblastoma Multiforme
GCV	Glanciclovir
GFP	Green Fluorescence Protein
GJIC	Gap Junction Intercellular Communication
GZA	Glycyrrhizic Acid
HRP	Horse Radish Peroxidase
HSPC300	Heat Shock Protein C300
HSVtk	Herpes Simplex Thymidine Kinase
LIMK	LIM kinase
MAPK	Mitogen Activated Protein Kinase
MMP	Matrix Metalloprotease
mRNA	Messenger RNA
N-	Amino
Nap125	Nck-associated protein 125
N-Cad	Neural Cadherin, N-Cadherin
NC	Neural Crest

NF1/2	Neurofibromatosis 1/2
NOV	Nephroblastoma Overexpressed
PAGE	Polyacrylamide Gel Electrophoresis
p120ctn	p120 catenin
PET	Polyethylene Terephthalate
PDGF	Platelet-Derived Growth Factor PDGFR
PDGFR	Platelet-Derived Growth Factor Receptor PDGFR
PKA	Protein Kinase A
PKC	Protein Kinase C
PTC	Papillary Thyroid Carcinoma
RIPA	Radioimmune Precipitation Lysis Buffer
RNA	Ribonucleic Acid
SH2	Src-Homology 2
shRNA	Short Hairpin RNA
siRNA	Short Interfering RNA
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline containing Tween 20
TP53	Tumor Protein53
TSC2	Tuberous Sclerosis Complex-2
VASP	Vasodilator-Stimulated Phosphoprotein
WASP	Wiskott-Aldrich Syndrome Protein
WAVE	WASP Family Verprolin Homologous Protein
WHO	World Health Organization
ZO-1	Zona Occludens-1

DEDICATION

THIS WORK IS DEDICATED TO MY GRANDAD, THOMAS BATES, AND TO HIS WORDS OF WISDOM:

'BE GREEDY TO LEARN' AND 'EVERYTHING BECOMES A MEMORY'

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CHAPTER 1 INTRODUCTION

1.0 Motility

Cell motility is an essential process for unicellular and multicellular organisms alike as it underlies processes fundamental for survival. In addition to its role in development and physiological processes, such as inflammation and wound repair, cell motility plays a key role in pathophysiology of tumorigenesis.

1.1 Directional vs. Random motility

The distinction between chemotaxis or chemorepulsion, directional movement along or against a concentration gradient of signal molecules, and chemokinesis, non-directional movement triggered by uniformly distributed signal molecules, enables the deconvolution of the highly complex biological process we generalize as cell motility (Maheshwari and Lauffenburger, 1998). Note that the terms chemotaxis and chemorepulsion, although not equal, are both forms of directional motility and will be used interchangeably throughout this text. When directional or non-directional motility involves extracellular matrix (ECM) proteins or cell membranes, the terms haptotaxis and haptokinesis, respectively, are employed, see Anand-Apte and Zetter, 1997). The fundamental difference between directional and random motility that indeed allows their distinction is the acquisition and persistence of cell polarity. Directional motility, which is persistence of locomotion in a particular orientation, requires that the concentration of a signaling molecule be sufficiently different along the body of a cell that such differences are distinguishable and thereby confer orientation to the cell, and additionally requires that the cell is able to recruit the appropriate cellular machinery to respond to these differences (Anand-Apte and Zetter, 1997). By contrast, cell polarization in random motility is only transient and is governed by intrinsic factors (Wilkinson, 1998). Motility requires contraction and ultimately, cells must form adhesions with a substratum in order for a contractile process to occur. Therefore it is the cell: substratum contact area, the number and strength of

adhesions both to the substratum as well as to the cytoskeleton, the structure of the cytoskeleton, and the force generated by the contractile machinery which enable contraction (Maheshwari and Lauffenburger, 1998). This implies that, front vs. rear asymmetry of adhesions and their properties (i.e. expression, function, affinity), regardless of how such asymmetry is established, is essential for a resultant net difference in traction and hence movement rather than isometric contraction (Huttenlocher et al., 1995; Lauffenburger and Horwitz, 1996).

1.2.1 Actin Polymerization

The process of actin polymerization, the current paradigm for cell migration, is complex and involves multiple biochemical pathways (Figure 1.1). Mechanisms of actin polymerization involve Rac, cell division cycle 42 (cdc42), and RhoA, which are all subtypes of Rho GTPases within the Ras superfamily. The activation of both Rac and cdc42 are elicited by a multitude of signaling cascades and effector proteins. Activated Rac and the adaptor protein Nck cause the dissociation of Scar/WAVE (WASP (Wiskott-Aldrich Syndrome Protein)-family verprolin homologous protein) from a complex composed of heat shock protein C300 (HSPC300), Abi, Sra-1, and Nck-associated protein 125 (Nap125) (Eden et al., 2002) while activated cdc42 activates WASP and N-WASP (Vicente-Manzanares et al., 2005). WAVE/Scar-HSPC300 and WASP/N-WASP both activate the actin-related protein 2/3 (ARP2/3) complex which binds to the pointed end of F-actin and causes extension of nascent filamentous (F)-actin at a 70^0 angle (Mullins et al., 1998). Activated cdc42 also activates the formin mDia2 which, when associated with monomeric, globular (G)-actin bound profilin, binds to the pointed end of F-actin and causes extension of F-actin in a linear fashion (Vicente-Manzanares et al., 2005). RhoA, is similarly governed by a variety of signaling cascades and effector proteins, and results in the activation of the formin mDia1 which, when associated with G-actin bound profilin, also binds to the pointed end of F-actin and causes extension of F-actin in a linear fashion (Kobielak et al.,

2004; Li and Higgs, 2003). The interaction of vasodilator-stimulated phosphoprotein (VASP) with vinculin is also important for actin-filament assembly (Brindle et al., 1996).

1.2.2 Actin Stabilization

The stability of F-actin is dependent not only upon polymerization, but also on disassembly. Both Rac and RhoA activate LIM kinase (LIMK) which in turn phosphorylates and inactivates cofilin leading to the disassembly of F-actin into G-actin. Ena/ VASP associated with profilin interact with Zymin and α-actinin to stabilize the barbed end of F-actin as does the capping protein gelsolin, which is under the control of phosphoinositides (Janmey and Stossel, 1987; Krause et al., 2003).

1.3 Gap Junctions

The ability of cells to communicate is of fundamental importance to their survival. Not surprisingly, many different forms of cellular communication exist. One form of cellular communication is mediated by gap junctions, membrane-spanning channels which are formed by the association of hemi channels (connexons) from adjacent cells (Figure 1.2). This association connects the cytosol of adjacent cells allowing the selective and regulated movement/exchange of amino acids, ions, small molecules and metabolites (<1kDa) between cells (Simon and Goodenough, 1998; Spray and Bennett, 1985). Recently it has been shown that siRNA is also able to pass between neighbouring cells via gap junctions, suggesting that gap junctions may play a role in the transfer of other forms of RNA such as microRNA (Valiunas et al., 2005). Cells which communicate in this manner are said to be coupled. Gap junction intercellular communication (GJIC) is essential for the viability of numerous tissues and the selective and regulated movement of gap junction permeable molecules is essential for proper vertebrate and invertebrate development and homeostasis (Reaume et al., 1995; Lo and Gilula, 1979; Ackert et al., 2001; Mercola and Levin, 2001).

Gap junctions also electrically couple adjoining cells (Bennett, 1997). Electrical communication via gap junctions is especially relevant to cardiac and neuronal activity. Cardiac gap junctions mediate the flow of current from the nodal pacemaker cells to ventricular myocytes which permits synchronous, rhythmic contraction (Herve and Sarrouilhe, 2006). In the brain, the synchronous, rhythmic spread of action potentials throughout neuronal syncytia, likely initiated by the thalamus, is defined and realized by gap junction expression and activity respectively. Normal synchronous activity is important for physiological processes such as memory and sleep (Destexhe and Sejnowski, 2003) while pathological synchrony is the hallmark of seizures (Fenner and Hass, 1989; Perez Velazquez and Carlen, 2000).

As gap junctions play a major role in vertebrate development, homeostasis, and electrical synchronizations, it is not surprising that perturbations in their expression and/or function have been implicated in many diseases (See Table 1). Such diseases include but are not limited to epilepsy/epileptogenesis (Perez Velazquez and Carlen, 2000), Charcot Marie Tooth Disease (Bergoffen et al., 1993) non-syndromic sensorineural deafness (Kelsell et al., 1997), cataracts (Shiels et al., 1998), Keratitis-ichthyosis-deafness syndrome (Richard et al., 2002), oculodentodigital dysplasia (Paznekas et al., 2003) and a variety of cancers including ovarian, liver, breast, brain (Fentiman et al., 1979; Hoffman et al., 1993; Loewenstein and Kanno, 1966; Zhu et al., 1991); (See Mesnil et al., 2005) for a review of gap junctions in cancer).

1.4 Connexins

The identification of gap junction constituent proteins, connexins (Cxs; Beyer et al., 1990), has offered much insight into the regulation and function of gap junctions. In addition to numerous species specific isoforms, 21 connexin (Cx) family members have been identified in humans thus far (Willecke et al., 2002). Two different nomenclatures exist to categorize these isoforms. The most common nomenclature uses the apparent molecular weight of the proteins on sodium dodecyl sulphate (SDS)-PAGE (polyacrylamide gel electrophoresis) mobility assays to

identify individual connexins (Beyer et al., 1990). This nomenclature will be used throughout the current study. Alternatively, individual connexins are subdivided based on structural similarity and differences into 3 types: α, β and γ or δ (Manthey et al., 1999). All members of the connexin family have 4 highly conserved alpha-helical hydrophobic domains which span the membrane and line the pore of connexons and gap junctions (Goodenough et al., 1996; Kelsell et al., 2001). Two extracellular loop domains mediate docking of adjacent connexins (when oligomerized into connexons) resulting in the formation of gap junction channels. In addition to a single cytoplasmic loop, both the amino (N) and carboxy (C) termini are cytoplasmic-facing. These cytoplasmic components are the moieties sensitive to pH and phosphorylation, important determinants for channel properties and function (Bruzzone et al., 1996). The C-terminus exhibits the highest variation in amino acid sequence and is therefore thought to be responsible for unique channel properties and function conferred by individual connexins.

The noncovalent oligomerization of connexins into hexameric membrane spanning connexons can occur in several ways (see Figure 1.3) with a vast number of possible combinations. Identical connexins can oligomerize with one another to form homomeric connexons. The docking of identical homomeric connexons form homotypic junctions while non-identical associations between homomeric connexons form heterotypic junctions. Non-identical connexins can also oligomerize with one another to form heteromeric connexons. When a heteromeric connexon associates with a homomeric connexon, a heteromeric heterotypic junction is formed while the association of two heteromeric connexons forms a heteromeric homotypic junction (Cx-Cx interactions are reviewed in (Herve et al., 2004a). The various combinations of connexon associations and the compliment of connexins that form an individual connexon alter channel properties such as selectivity and conductivity (White et al., 1995) and hence individual members of the Cx family can be studied independently of one another or in various combinations (Brink et al., 1997). The physiological consequences of differing channel

properties are particularly relevant since a given cell type can express a distinct yet variable compliment of connexins, and therefore both compartmentalization and syncytia can be realized.

For example, granulosa cells express both Cx43 and Cx37. Direct homocellular interactions between granulosa cells are mediated by Cx43 (Gittens et al., 2003; Ackert et al., 2001) while heterocellular interactions at the granulosa-oocyte interface are mediated by Cx37 (Veitch et al., 2004; Simon et al., 1997). The differential permeability properties of Cx43 and Cx37 suggest that the granulosa syncytium may be a compartment distinct from that of the oocyte and thus enable discrete and independent signaling within the developing follicle (Elfgang et al.,1995; Gittens and Kidder, 2005).

1.5 Connexin43

Connexin43 (Cx43) is the most prevalent and well studied of the isoforms within the Cx multigene family. In cancer research, Cx43 is best known for its growth suppressive effect: it is downregulated in many types of tumor tissue (see section 1.8) and its reconstitution leads to a reduced proliferation rate and tumor formation (Naus et al., 1992; King et al., 2002). These findings were previously explained by GJIC alone however, more recently channel independent effects have been observed (Omori and Yamasaki, 1998; Huang et al., 1998c; Zhang et al., 2003b; Lin et al., 2003b). Indeed cells transfected with Cx43 have a different gene expression profile than mock transfected cells that may account for tumor suppression partially or wholly (Naus et al., 2000). The variable C-terminus of Cx43 is perhaps the best candidate to explain non-junctional effects. At 151aa long (40% of the full length protein) it is the longest region of variability among connexin isoforms. The C-terminus has 14 identified sites of phosphorylation regulated by 6 kinase proteins (v-src, MAPK, PKC,p34cdc2, CK1, PKA-dependent; for reviews see Lampe and Lau, 2000; Lampe and Lau, 2004). The phosphorylation profile of Cx43 is implicated in junctional conductance and channel gating, protein turnover, and modulates

interaction with many of the associating proteins identified thus far (Herve et al., 2004b; Giepmans, 2004).

1.5.1 Cx43 Life cycle

1.5.1.1 Regulation of Cx43

Connexins can be regulated by endogenous and exogenous factors at several levels including transcription, translation, and posttranslational modifications. The promoter region of the Gja1 gene which encodes Cx43 is regulated by numerous transcription factors (i.e. c-*fos*, c-*jun*, CREB, Sp1) which bind to AP-1, AP-2, Sp1, p53, and CRE sites (Sullivan et al., 1993; Yu et al., 1994) as well as other positive and negative regulatory sites (Chen et al., 1995). Activation of such transcription factors is realized by several identified factors including: phorbol esters, protein kinase C (PKC; Geimonen et al., 1996) ciliary neurotrophic factor (CNTF) in combination with it's soluble receptor CNTFsRα (Ozog et al., 2002; Ozog et al., 2004), bone morphogenic proteins (BMPs) (BMP2 and BMP4; Chatterjee et al., 2003; Bani-Yaghoub et al., 2000), Wnt1, likely via T-cell factor (TCF)/LEF (lymphoid enhancer factor) binding elements (Ai et al., 2000; van der Heyden et al., 1998), and retinoids (through the RXR-β receptors (Batias et al., 2000).

Translation of Cx43 mRNA is facilitated by a strong internal ribosomal entry site (IRES; Schiavi et al., 1999). The posttranslational modification of Cx43 is regulated by numerous biochemical signaling pathways, including kinases and phosphatases, but importantly the life cycle of Cx43 is a major site for the regulation of Cx43 expression. The half life for Cx43 is 1.3-5h (Beardslee et al., 1998; Berthoud et al., 2004; Darrow et al., 1995; Laird et al., 1991) and therefore the expression and persistence of gap junction plaques represents a balance between stabilizing mechanisms such as phosphorylation and degradation pathways. Indeed the upregulation of Cx43 by inhibitors of both protein synthesis and the proteasome underscores the important physiological role for Cx43 in maintaining cellular homeostasis, particularly when the

viability of the cell is compromised (Musil et al., 2000). Furthermore, the disappearance of Cx26 mRNA is completely inhibited by the protein synthesis inhibitor cycloheximide (Kren et al., 1993). It is possible that in addition to Cx43 protein, Cx43 mRNA might also be stabilized by the inhibition of protein synthesis, supporting Cx43 stabilization when protein synthesis is compromised.

1.5.1.2 Cx43 Trafficking and Plaque formation

Cx43 is cotranslationally inserted into the endoplasmic reticulum (ER) membrane (White et al., 1995) and oligomerized into hexameric connexons in the trans Golgi compartment (Bennett and Zukin, 2004; Das Sarma et al., 2001; Musil and Goodenough, 1993; Saez et al., 2003). However, the exogenous overexpression of Cx43 (VanSlyke, 2005), induces premature assembly of connexons in the ER. The aggregation of connexons (10,000/μm^2; Lauf et al., 2002) in the plasma membrane forms gap junction plaques (Bruzzone et al., 1996) which are important for the opening of channels and functional GJIC (Bukauskas et al., 2000). Connexons are delivered via vesicular transport along microtubules to the plasma membrane (Lauf et al., 2002). Note that the formation of a Cx43/zona occludens-1 (ZO-1)/β-catenin complex is required to target Cx43 to the plasma membrane in rat cardiomyocytes (Wu et al., 2003). Although mictotubules directly interact with Cx43 at gap junction plaques (Giepmans et al., 2001), connexons are transported to nonjunctional plasma membrane rather than to the center of gap junction plaques (Lauf et al., 2002). One proposed mechanism for the plasma membrane localization of individual connexons to gap junction plaques involves $G_{i\alpha}$ proteins as their inhibition by pertussis toxin reduced plasma membrane levels of Cx43 specifically in low-density lipoprotein (Lampe et al., 2001).

1.6 Cx43 Associating Proteins

1.6.1 Overview

Recently, Cxs have received attention as proteins in their own right, independent of gap junction channel formation. In addition to their interaction with other Cx family members via extracellular loop domains, Cx43 associates with a variety of other proteins, the majority of which associate with the cytosolic C-terminus of Cx43. The variable homology domain of Cx43 contains 14 phosphorylation sites which are important for SH2 interactions (Lampe and Lau, 2004).

Cx43 associating proteins identified thus far include: ZO-1, Occludin, Lin-7, β-catenin, α-catenin, p120ctn, pp60c-src, pp60v-src, PKCε, PKCα, DMPK, MAPK, P38MAPK, N-Cad, NOV, F-actin, actin, drebrin, clathrin, Caveolin-1, ubiquitin, α-tubulin, β-tubulin, CIP62, and numerous connexins (see Herve et al. (2004a) and Giepmans (2004) for review of Cx associating proteins).

These associations are important not only for Cx43 trafficking, degradation, and function, but have also been implicated in cell adhesion and signaling. Given the plethora of Cx43 associating proteins, many effects attributed to Cx43 may be mediated via these proteins and/or their interaction with Cx43 independent of gap junction channel formation. While all of these associating proteins are of interest in their own right, several are of particular interest to Cx43-mediated motility.

1.6.2. Cadherins

Cadherins are a widely expressed multigene family of transmembrane proteins and are the major constituents of adherens junctions. The extracellular N-terminus consists of five tandem repeat domains which selectively mediate specific cell-cell adhesions via associations with other cadherins (Pecina-Slaus, 2003). The short, highly conserved cytosolic region associates with numerous proteins, most notably the actin cytoskeleton and actin binding

proteins including the α/β-catenins, vinculin, α-actinin, and zona occludens-1, and determines the specificity of adhesive interactions (Klingelhofer et al., 2000; Nagafuchi, 2001). The extracellular and cytoplasmic domains enable cadherins to act as both receptors and ligands. Cadherins are divided into subclasses (including the predominant E, N, and P subclasses) depending on tissue distribution (Takeichi, 1988). The function and localization of cadherins to cell-cell contacts may be a prerequisite for gap junction formation as disruptions in cadherin function can perturb gap junction formation and impair or inhibit GJIC (Frenzel and Johnson, 1996). Furthermore, Ca^{2+}-dependent regulation of GJIC involves Ca^{2+}-dependent cell adhesion molecules (i.e. E-Cadherin (Jongen et al., 1991; Wang and Rose, 1997).

1.6.2.1 N-Cadherin

N-Cadherin (N-Cad, Neural cadherin) is a 130kDa protein and it is the major adhesion protein in the CNS (Hatta et al., 1985). While the loss of E-Cadherin is associated with endothelial to mesenchymal transition (EMT; Takeichi, 1988), N-Cad expression is often associated with increased invasion (Mareel and Leroy 2003). A potential mechanism involves the interaction of N-Cad with FGFR-1 which leads to prolonged mitogen activated protein kinase (MAPK)/ERK (extracellular signal regulated protein kinase) activity and increased matrix metalloprotease-9 (MMP-9) gene transcription (Wheelock and Johnson, 2003). N-Cad has been reported to associate with other proteins including actin, catenins, and Cx43. The N-Cad/Cx43 interaction has been studied extensively in neural crest (NC) and NIH3T3 cells. Knockdown of N-cad expression by siRNA in NIH3T3 cells decreased the cell surface expression of Cx43, and similarly the knockdown of Cx43 by siRNA reduced the cell surface expression of N-Cad in these cells (Wei et al., 2005). The Cx43/N-Cad interaction also appears to be essential for the function of gap junctions containing Cx43 as N-Cad-deficient neural crest cells exhibit reduced GJIC despite retaining abundant gap junction plaques containing Cx43 (Xu et al., 2001). The association of these proteins with one another has particularly important physiological

consequences as perturbations in their expression and/or localization modulate cell motility (Wei et al., 2005; Xu et al., 2001). The specific site of the N-Cad/Cx43 interaction is not yet known (Wei et al., 2005).

1.6.3 Catenins

Catenins are a family of proteins best known for their cytoplasmic association with cadherins. This association serves to help cluster cadherins and produces a complex which is linked to the actin cytoskeleton (Aberle et al., 1996; Xu et al., 2004). Thereby, catenin-cadherin associations play an important role in cadherin-mediated cell adhesion and cytoplasmic functions of cadherins (Gumbiner and McCrea, 1993). Importantly, catenins are also involved in biochemical pathways and are able to mediate transcription.

1.6.3.1 P120 catenin

p120 catenin (p120ctn), a 120kDa protein associates with the cytoplasmic domain of N-Cad, facilitating N-Cad clustering (Wei et al., 2005) and has also been shown to colocalize with Cx43 (Xu et al., 2001). Cells in which Cx43 has been knocked down by siRNA exhibit a reduction in the cell surface expression of p120ctn and an increase cytoplasmic and nuclear p120ctn were observed (Wei et al., 2005). The cytoplasmic and nuclear localization of p120ctn is associated with increased malignancy (Bellovin et al., 2005; van Roy and McCrea, 2005). Cytoplasmic p120ctn inhibits Rho kinase and activates Rac and Cdc42 (Anastasiadis et al., 2000; Anastasiadis and Reynolds, 2001; Grosheva et al., 2001; Hou et al., 2006) and the nuclear localization of p120ctn inhibits kaiso-mediated transcriptional repression (van Roy and McCrea, 2005). Therefore, p120ctn signaling has been proposed to mediate the cross-talk between Cx43 and cell motility mechanisms (Xu et al., 2001).

1.6.4 Drebrin

Drebrin is a soluble 70 kDa protein that binds and stabilizes F-actin (Asada et al., 1994). In addition to localizing to plaques of adhering junctions, drebrin localizes to leading edges of

lamellipodia and filopodia and plays a role to the formation of protrusions, thereby contributing to cell motility (Peitsch et al., 2001). Drebrin was identified as an interaction partner of the Cx43 C-terminal domain at the plasma membrane in astrocytes and is required to maintain functional Cx43-containing gap junctions at the cell surface (Butkevich et al., 2004).

1.6.5 NOV (Nephroblastoma Overexpressed)

Also known as CCN3, NOV was originally identified as an aberrantly expressed gene in avian nephroblastomas induced by myeloblastosis-associated virus (Lin et al., 2003a). It is a member of the Cysteine-Rich61/Connective Tissue Growth Factor/Nephroblastoma Overexpressed (CCN) family of genes. It is upregulated in Cx43 transfected cells (McLeod et al., 2001) and has been shown previously to associate with the C-terminal tail of Cx43 (Fu et al., 2004; Gellhaus et al., 2004). Consistent with a growth suppressive role for Cx43, NOV inhibits glioma cell growth and tumorigenic potential (Gupta et al., 2001). NOV induces directed endothelial cell migration (Lin et al., 2003a), promotes migration and invasion in Ewing's sarcoma (Benini et al., 2005), and leads to an increase in glioblastoma cell migration via a platelet-derived growth factor receptor (PDGFR)-alpha-dependent mechanism (Laurent et al., 2003).

1.7 Involvement of Cx43in Motility

While the anti-proliferative effects of Cx43 are well established, its role in motility remains unclear. In skin wound healing assays, a loss of Cx43 staining at the wound margins during initial wound healing and persistence of Cx43 expression in non-healing wounds (Brandner et al., 2004) supported the finding by (Qiu et al., 2003) that targeting Cx43 expression by antisense technology enhanced the rate of wound repair. However, no correlation between Cx43 expression and cell migration or invasion was observed upon Cx43 transfection into in basaloid squamous cell carcinoma (Shima et al., 2006). Knockdown of Cx43 expression by siRNA led to enhanced invasion of MDA-MB-231 and Hs578T breast carcinoma cells (Shao et

al., 2005). Complete ablation of Cx43 by knockout methods were employed for explant outgrowth studies by Lo and coworkers. In proepicardial explants from Cx43KO mice outgrowth was enhanced (Li et al., 2002). In contrast, neural tube explants from Cx43KO mice exhibited decreased neural crest cell outgrowth (Huang et al., 1998a). Supporting these findings, increased outgrowth of neural crest cells was observed in neural tube explants of CMV43 transgenic mice (animals in which Cx43 is overexpressed by the cytomegalovirus (CMV) promoter; Huang et al., 1998a). Furthermore, Cx43 knockdown by siRNA inhibited NIH3T3 cell motility (Wei et al., 2005). This motility promoting ability of Cx43 has also been observed in several overexpression studies. For example, stable transfection of Cx43 into HeLa (Graeber and Hulser, 1998) and C6 cells (Lin et al., 2003b; Oliveira et al., 2005; Zhang et al., 2003b) increased cellular motility and invasion. Finally, examination of the human glioblastoma cell lines, GL15 and 8-MG, human glioma biopsies xenografted and maintained in nude mice, and fresh human glioma biopsies revealed that endogenous Cx43 expression was proportional to cellular motility and invasion (Oliveira et al., 2005)

1.7.1 Mechanisms of Cx43 mediated motility

Although the mechanism by which Cx43 influences motility, irrespective of affect, has not yet been determined, junction-dependent and junction-independent mechanisms have earned nominations. Junction dependent mechanisms have traditionally explained the effects of Cxs and it has been suggested that in the context of cancer invasion this might owe to the ability of cancer cells to metabolically hijack adjacent cells (Wei et al., 2005). Junction independent mechanisms, however, are equally able to explain such phenomena. The disruption of cadherin localization and adherens junction formation upon Cx43 knockdown (Wei et al., 2005) along with the finding that deletion of the Cx43 carboxy terminus attenuates motility of 3T3 A31 fibroblasts (Moorby et al., 2000) supports junction-independent mechanisms. Furthermore, using a gene array approach,

Iacobas et al. (2004) have identified 18 genes involved in motility that are differentially regulated in Cx43KO astrocytes compared to wild type.

1.8 Connexin43 in cancer

The implication of gap junctions in cancer was first described by Loewenstein and Kanno in 1966 when they noted that GJIC was decreased in liver tumor cells compared to untransformed counterparts. Cx43 has since been implied in carcinogenesis, tumor susceptibility and progression, as well as tumor suppression and differentiation.

The reduction or elimination of Cx43 in several types of cancer has been documented (see above). Reconstitution of Cx43 in cells derived from these tumors has reduced their proliferation and/or tumorgenicity and directly identified Cx43 as a tumor suppressor (Fernstrom et al., 2002; Fishman et al., 1990; Goldberg et al., 2000; Jou et al., 1993; Naus et al., 1992; Shima et al., 2006; Zhu et al., 1992).

There also exists considerable indirect evidence for the tumor suppressive role of Cx43. Compounds involved in decreasing tumorigenicity such as retinoids, carotenoids (Bertram and Vine, 2005), vitamin A (Naves et al., 2001), Diallyl disulfide (DADS; Huard et al., 2004), and CNTF (Ozog et al., 2002; Ozog et al., 2004), have led to parallel increases in Cx43. Furthermore, the expression of tumor suppressors (e.g. breast cancer metastasis suppressor 1 (BRMS1; Kapoor et al., 2004) correlates with increased levels of Cx43.

Complimentary studies have investigated the correlation between the loss of Cx43 and increased tumorigenicity. Numerous carcinogens result in decreased Cx43 (reviewed by (Trosko and Ruch, 2002) including the phorbol ester tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA; Enomoto et al., 1981; Swierenga and Yamasaki, 1992), *N,N'*-nitroso-methylurea (Benda et al., 1968), and dimethylbenzanthracene (DBMA; Kamibayashi et al., 1995). Similarly, the overexpression of oncogenes that increase tumorigenicity including Src, Neu, Ras, and Myc result in a downregulation of Cx43 protein or function (Azarnia et al., 1989; Kalimi et al., 1992;

Jou et al., 1995; Hayashi et al., 1998). Additionally, heterozygous (Cx43(+/-)) mice exhibit reduced expression of Cx43 and have an increased susceptibility to tumorigenesis upon exposure to environmental agents such as urethane (Avanzo et al., 2004).

1.9 Brain cancer

The most comprehensive study of brain cancers has been undertaken by the Central Brain Tumor Registry of the United States (CBTRUS) and is therefore the source of the statistics provided in this section (unless indicated otherwise). Like most cancers, brain cancers can be malignant or benign however benign brain cancers are not necessarily harmless –they can be life threatening if they compromise vital brain activity (by occlusion, edema, or otherwise) and lead to neurological dysfunction. Indeed prognosis is dependent on tumor location: for malignant brain tumors the five year survival rates differ markedly between tumors located in the frontal, temporal, parietal, occipital lobes of the brain compared to tumors located in the brain stem, pituitary gland, pineal gland, or cerebellum (CBTRUS). Brain cancers are rarely metastatic but can originate from within the brain *de novo* (primary), which is frequently associated with epidermal growth factor receptor (EGFR) amplification, or arise as a result of metastasis from elsewhere (secondary), which is associated with tumor protein53 (TP53) inactivation (Benjamin et al., 2003).

1.9.1 Glioma

Gliomas are the most common of malignant central nervous system (CNS) cancers accounting for greater than 78% of primary malignant CNS tumors (including tumors of the spinal cord, CBTRUS). Glioma is a category of primary brain cancer, specifically a type of neuroepithelial tumor, which arises from the neoplastic transformation of glial cells, or perhaps their stem cell precursors (Singh et al., 2003). The neoplastic transformation of oligodendrocytes (or their precursors) accounts for 8% of gliomas while the neoplastic transformation of astrocytes accounts for 69% of gliomas (CBTRUS).

Biopsied gliomas are divided into categories according to presumed cellular origin; astrocytic, oligodendroglial, mixed gliomas (oligoastrocytoma and anaplastic oligoastrocytoma), ependymal tumors, and neuroepithelial tumors of uncertain origin. These categories are further graded according to the world health organization (WHO) classification system (which is the most widely employed of such systems, see Louis et al., 2001). Astrocytomas, the most prevalent of gliomas, are then histologically tiered into four grades according to their malignancy (in increasing order of malignancy): grade I (pilocytic astrocytomas), grade II (low-grade nonpilocytic/diffuse astrocytomas), grade III (anaplastic astrocytomas), and grade IV (glioblastoma multiforme (GBM; Kleihues P, 2000).

A comprehensive review of cases reported between 1972 and 2002 indicates that the relative survival rates of patients differ markedly between tumor grades: the 10 year survival rate for pilocytic astrocytoma is 89.3% while the 2 year survival rate for glioblastoma multiforme is 8.7% (CBTRUS). Unfortunately, glioblastoma is the most common of gliomas accounting for nearly 50% of gliomas (CBTRUS).

1.9.2 Nature of gliomas

Glioblastomas form aggressive tumors which grow quickly and infiltrate relentlessly. The tumors spread through the brain along surfaces and white matter tracts and deccusation via the corpus callosum and invasion into the contralateral hemisphere is a common characteristic of these neoplasms. Furthermore, *in vitro* assays using spheroids of rat glioma (C6) cells provide evidence for the secretion of a chemorepellent by the tumor cell mass that promotes the invasion of glioma cells (Werbowetski et al., 2004). In these studies, cells responded to a gradient of chemorepellent cues and migrated out of the spheroid at an angle perpendicular to the spheroid edge. The addition of an adjacent spheroid altered the trajectory of cell motility away from both spheroids while cells readily invaded adjacent astrocyte aggregates (Werbowetski et al., 2004).

According to mathematical modeling, the diameter of glioblastoma expands at a rate of 2.5mm/month which is much more explosive than the expansion rate for lower grade astrocytomas (4.4mm/year, Swanson et al., 2003).

1.9.3 Causes

The WHO grading system is very valuable as it not only dictates prognosis and treatment strategies but it also provides a framework for research as excised tissue is examined for morphological and biochemical changes (Louis et al., 2001). While the causes of gliomas remain unknown, important advancements in both detection and treatment have been accomplished in this manner, leading to the identification, classification, and expression profiling of several important predisposing genes.

TP53 mutations, often associated with gliomas, are significantly more frequent in low-grade astrocytomas suggesting that they may contribute to the initiation of these tumors (Ohgaki and Kleihues, 2005a). The most common inherited tumor predisposition syndromes in the CNS arise due to mutations in the tumor suppressor neurofibromatosis 1/2 (NF1/2) genes which cause neurofibromatosis 1/2 and render patients with an increased susceptibility to astrocytoma (Korones et al., 2003; Gutmann and Giovannini, 2002). Alterations in papillary thyroid carcinoma (PTC) and adenomatous polyposis coli (APC) genes are the inherited source of Turcot syndrome and Gorlin syndrome respectively and are associated with medullablastoma (Gutmann and Giovannini, 2002), the most common primary brain tumor found in children (CBTRUS). Patients who have germline mutations in the tuberous sclerosis complex-2 (TSC2) gene acquire tuberous sclerosis and have an increased susceptibility to subependymal giant cell astrocytomas (Woods et al., 2002).

Of note, several studies have implicated Cx43 in glioma progression. While astrocytes normally express very high levels of Cx43, its expression is decreased in low grade tumors and it is often absent in glioblastoma (Soroceanu et al., 2001; Huang et al., 1999; Pu et al., 2004).

Environmental factors may well play a role in initiation or progression of these tumors. While exposure to vinyl chloride has been associated with these neoplasms (Moss, 1985), only therapeutic X-irradiation is unequivocally associated with an increased risk of brain tumors, including gliomas (Ohgaki and Kleihues, 2005a)

1.9.4 Treatment/management strategies

Although surgical resection of the tumor mass has provided invaluable insights for research and treatment and is important for reducing cranial pressure, the invasive nature of diffuse gliomas often precludes surgery as an effective means to cure the disease. Similarly, chemotherapy and radiotherapy are not curative as these approaches target only dividing cells. Because division and invasion are mutually exclusive events (Chicoine and Silbergeld, 1995), non-dividing cancerous cells are able to reenter mitosis at a later time. Combination therapy approaches involving resection, chemotherapy and radiotherapy can best be described as management strategies for the betterment of quality of life –although the incidence of glioblastoma is low (3.05 per 100,000, CBTRUS), so too is the median survival time of 0.4 years (Ohgaki and Kleihues, 2005b). Gene therapy is hence an attractive therapeutic approach. The transfer of the herpes simplex thymidine kinase (HSVtk) gene to neoplastic cells renders their viability sensitive to glanciclovir (GCV), an antiherpetic drug. The toxic metabolites produced upon HSVtk phosphorylation of GCV can be transferred via gap junctions to adjacent cells (Elshami et al., 1996). This process, called the 'bystander effect', leads to the killing of untransduced cells adjacent to cells expressing the HSVtk gene (Elshami et al., 1996). The first *in vivo* study involving Cx43 and the HSVtk-GCV approach measured tumor formation when mixed cultures of transduced and non-transduced C6 cells were injected subcutaneously into CB.17/SCID-beige mice (Dilber et al., 1997). When C6 cells were transfected with Cx43 and exhibited a high level of GJIC, tumors were frequently undetectable at the inoculation site, even when 75% of cells were HSVtk negative. Aggregates of cells from primary astrocytomas,

primary astrocytoma cell cultures, and glioblastoma cell lines demonstrated a varied bystander effect during HSVtk gene therapy depending on the level of Cx43 mRNA expression (Shinoura et al., 1996). Unfortunately, the HSVtk-GCV approach has not yet proven successful for gliomas due to low sensitivity of these tumor cells to viral infection and further by a limited bystander effect due to low levels of Cx43 (Rosolen et al., 1998).

1.10 C6 Cells

The C6 cell line was originally isolated from tumors in Wistar-Furth rats (*rattus norvegius*) exposed to *N,N'*-nitroso-methylurea and based primarily on histopathological features C6 is categorized as astrocytoma (Benda et al., 1968). These cells model human gliomas, particularly GBM, in that they are morphologically similar to human gliomas and exhibit diffuse invasion when injected into the brains of healthy neonatal rats (Grobben, Auer et al 1981). Although when C6 spheroids are implanted into neuronal tissue circumscribed tumors are formed (Naus et al., 1992), when implanted in suspension the cells invade individually as single cells located in micropockets (Bernstein et al. 1990, Izumoto et al. 1996, Goldberg et al. 1991, Grobben et al., 2002). C6 cells therefore provide an adequate means to study biochemical pathways, growth, invasion, and angiogenesis of glioblastomas both *in vitro* and *in vivo*. In addition, it is an ideal system to examine Cx43 as similar to human tumor tissue, Cx43 is downregulated. The persistence of Cx43 in this cell line ensures that cells express the appropriate machinery required for the proper gating, localization and function of Cx43. Furthermore, the cells also form confluent monolayers *in vitro* and are therefore amenable to wound healing assays unlike other glioma cell lines such as the murine delayed brain tumor (DBT) and human U87.

1.11 Rationale

Various genetic and biochemical changes occur during the neoplastic transformation of astrocytes. There is a downregulation of Cx43 expression throughout glioma progression and expression is often absent in glioblastoma multiforme. Several studies have indicated that Cx43 enhances motility in glioma cells, however all have taken an exogenous overexpression approach. It has been reported that exogenous Cx43 overexpression affects Cx43 trafficking. Furthermore, Cx43 transfected cells have a different gene expression profile than untransfected cells. Here we aim to investigate the role of endogenous Cx43 in glioma motility and further explore the mechanism by which such a role is carried out. We hypothesize that the Cx43 C-terminus may play a significant role for Cx43-mediated motility in C6 cells.

Figure 1.1 Actin Polymerization.

Activated Rac and the adaptor protein Nck cause the dissociation of Scar/WAVE from a complex composed of HSPC300, Abi, Sra-1, and Nap125 while activated cdc42 activates WASP and N-WASP. WAVE/Scar-HSPC300 and WASP/N-WASP both activate the ARP2/3 complex which binds to the pointed end of F-actin and causes extension of nascent filamentous (F)-actin at a 70^0 angle. Activated cdc42 also activates mDia2 which, when associated with monomeric, globular (G)-actin bound profilin, binds to the pointed end of F-actin and causes extension of F-actin in a linear fashion. RhoA results in the activation of the mDia1 which, when associated with G-actin bound profilin, also binds to the pointed end of F-actin and causes extension of F-actin in a linear fashion. The interaction of VASP with vinculin is also important for actin-filament assembly. Both Rac and RhoA activate LIMK which in turn phosphorylates and inactivates cofilin preventing the disassembly of F-actin into G-actin. Ena/VASP associated with profilin interact with Zymin and α-actinin to stabilize the barbed end of F-actin as does the capping protein gelsolin, which is under the control of phosphoinositides.

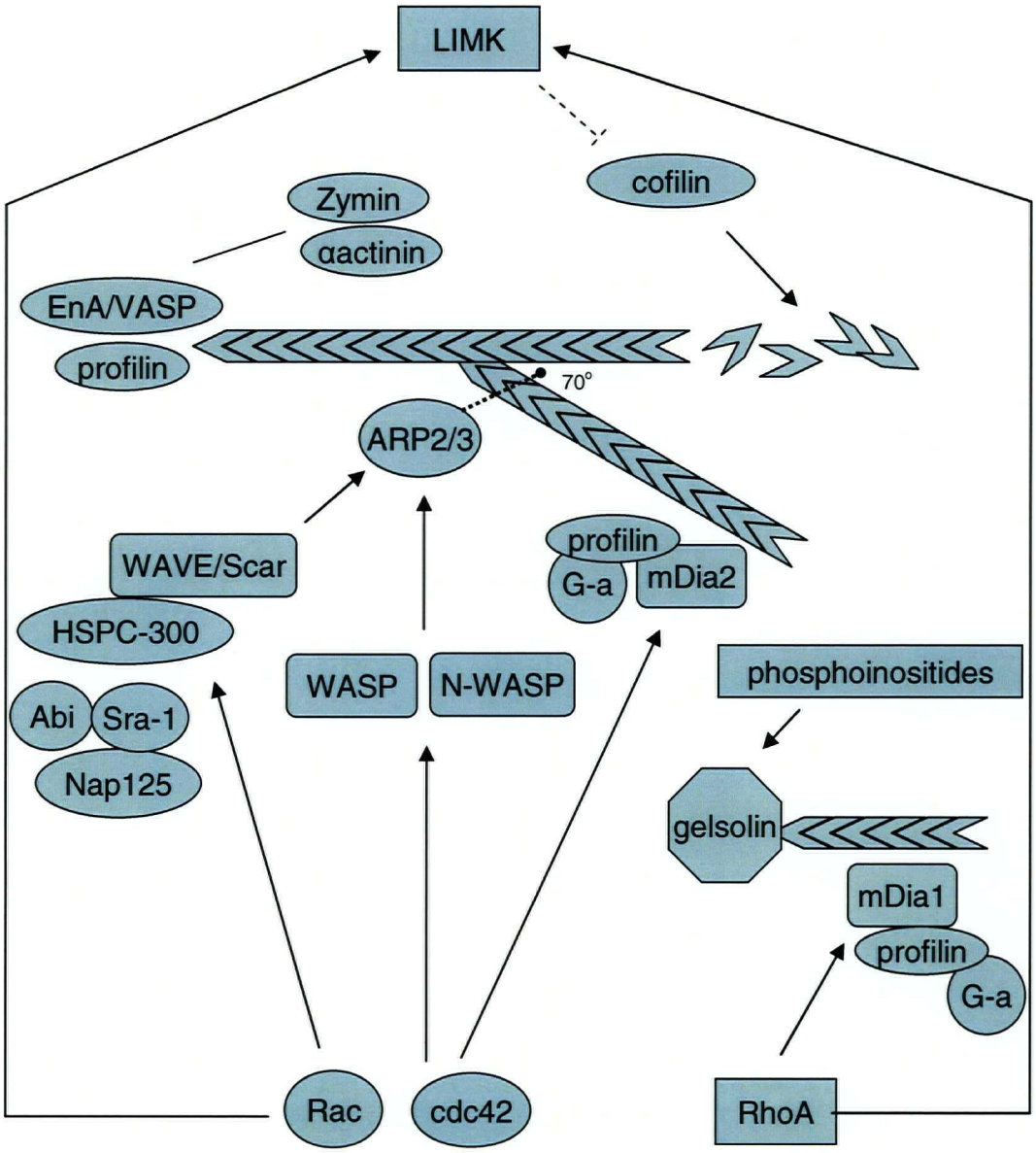

LIMK
cofilin
Zymin
αactinin
EnA/VASP
profilin
70°
ARP2/3
WAVE/Scar
HSPC-300
Abi
Sra-1
Nap125
WASP
N-WASP
profilin
G-a
mDia2
phosphoinositides
gelsolin
mDia1
profilin
G-a
Rac
cdc42
RhoA

Figure 1.2 Connexins, Connexons, and Gap Junction Formation.

A) Connexin topology. Connexins have 4 highly conserved transmembrane domains, two extracellular loop domains and one cytosolic loop domain, and both N and C termini are cytosolic facing. B) Connexon assembly. The noncovalent hexameric oligomerization of connexins forms a connexon or hemichannel. C) Gap Junction assembly. Connexons from adjacent cells associate with one another to form transmembrane channels. Clustering of these channels forms plaques which are called gap junctions. These junctions connect the cytosol of adjacent cells allowing the selective and regulated movement/exchange of amino acids, ions, small molecules and metabolites (<1kDa) between cells and also electrically couples adjoining cells.

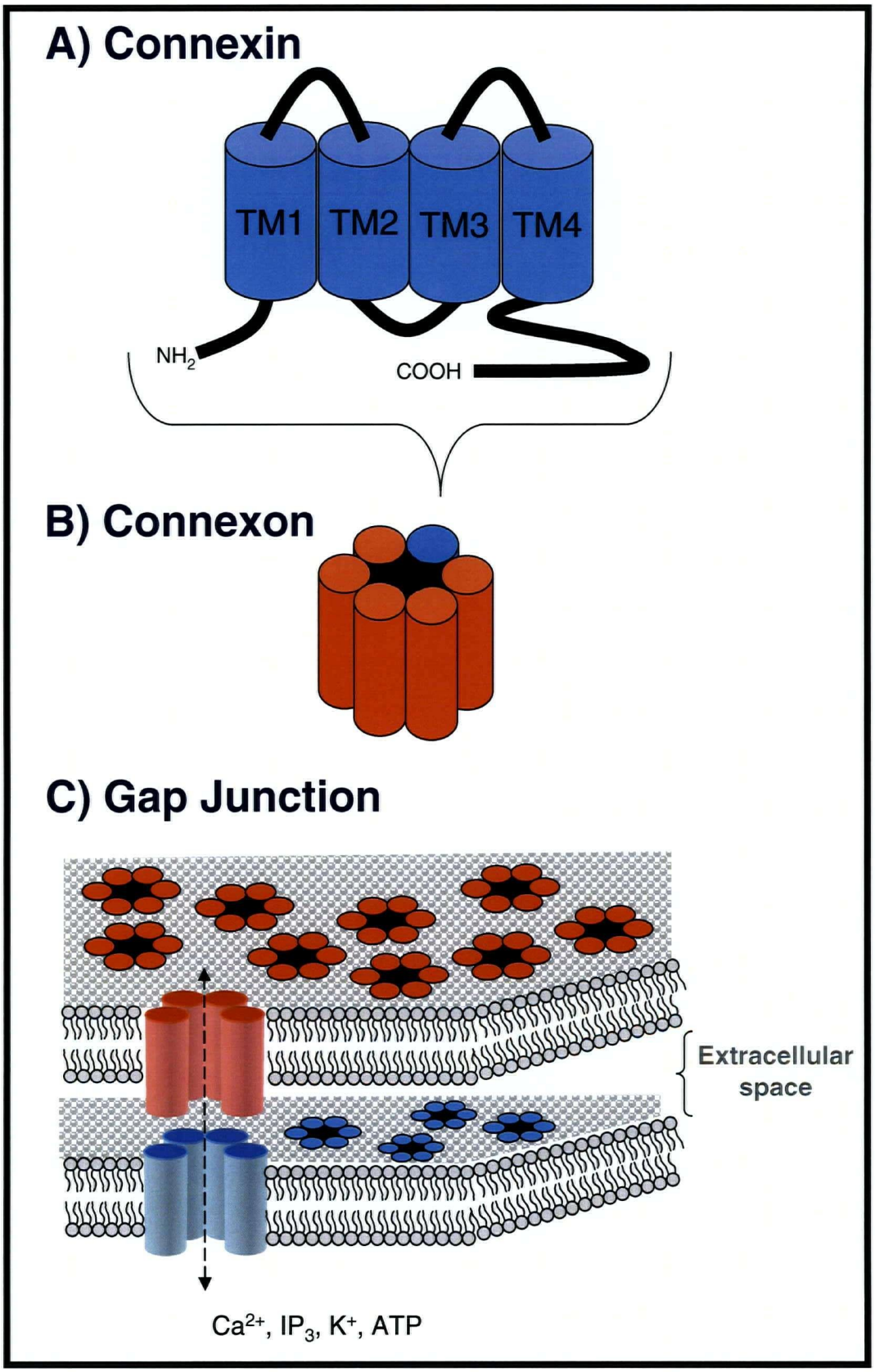
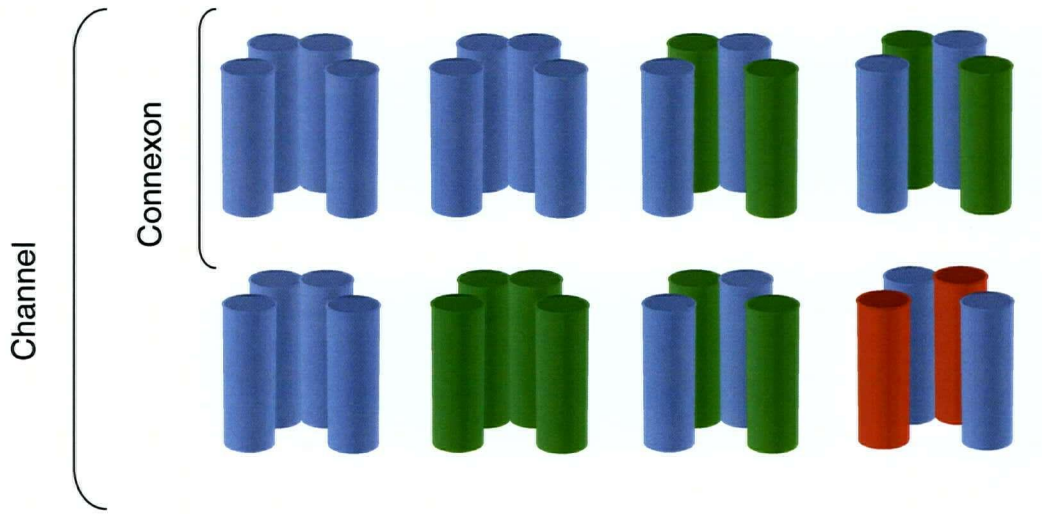

A) Connexin
TM1
TM2
TM3
TM4
NH2
COOH
B) Connexon
C) Gap Junction
Extracellular space
Ca2+, IP3, K+, ATP

Table 1. Implication of Gap Junctions in Disease

Disease State	Connexin involvement	Reference(s)
Epilepsy/epileptogenesis	Associated with alterations in GJIC	Perez Velazquez and Carlen, 2000
Charcot Marie Tooth Disease (X-linked)	Associated with mutations in GJB1 encoding Cx32	Bergoffen et al., 1993
Deafness (non-syndromic) sensorineural	Associated with mutations in GJB2 encoding Cx26	Kelsell et al., 1997
Keratitis-Ichthyosis-Deafness Syndrome	Associated with mutations in GJB2 encoding Cx26	Richard et al., 2002
Oculodentodigital Dysplasia	Caused by mutations in GJA1 encoding Cx43	Paznekas et al., 2003

Figure 1.3 Types of Gap Junction Channels. Several types of gap junction channels can be classified based on the compliment and organization of connexins which compose the connexon hemichannels. When a connexon is composed of only one type of connexin, it is called a homomeric hemichannel. The association of identical homomeric hemichannels creates homomeric homotypic gap junction channels while the association of non-identical homomeric hemichannels creases a homomeric heterotypic channel. Connexons composed of more than one type of connexin are called heteromeric hemichannels. The association of identical heteromeric hemichannels (i.e. containing the same compliment of connexins) creates heteromeric homotypic gap junction channels while the association of non-identical heteromeric connexons creates heteromeric heterotypic gap junction channels.



Connexon	Homomeric	Homomeric	Heteromeric	Heteromeric
Channel	Homotypic	Heterotypic	Homotypic	Heterotypic

CHAPTER 2 MATERIALS AND METHODS

2.1 Cell Culture

C6 rat astrocytoma cells (Benda & Lightbody 1968) were grown on non-pyrogenic, Nunclon$^{TM}\Delta$ Surface plates (NUNC) and maintained in low glucose DMEM containing L-glutamine (Sigma) supplemented with 10% Fetal Bovine Serum (FBS, Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator (37°C; 5% CO_2/95% air).

HEK293 retroviral packaging cells (generous gift from Dr. Richard C. Mulligan, Children's Hospital, Boston, MA) were maintained in low glucose DMEM containing L-glutamine (Sigma) supplemented with 10% FBS (Sigma), 1 μg/ml tetracycline, 2 μg/ml puromycin, 0.3 μg/ml G418, and 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator (37°C; 5% CO_2/95% air). See ***Transfection and infection of shRNA*** (below) for details regarding generation of infectious medium.

2.2 Gap junction blocker treatments

The non-selective gap junction blocker carbenoxolone (CBX, Sigma) was dissolved in sterile H_20 to final concentration of 150μM. The inactive analogue, glycyrrhizic acid (GZA, Sigma) was also dissolved in sterile H_20 to a final concentration of 150μM and used as a comparative control. Based on previous studies (Bani-Yaghoub et al., 1999) and preliminary tests, the lowest concentration of CBX that completely blocked the transfer of calcein (150μM) was used for experiments in this study.

2.3 Immunocytochemistry

Cells grown on coverslips were fixed in 4% paraformaldehyde for 10 min at 37°C and were subsequently washed twice at 37°C for 10 min in phosphate buffered saline (PBS). Coverslips were incubated in blocking solution (4% normal goat serum or bovine serum albumin) for 30 min at room temperature followed by incubation for 1h at room temperature in blocking solution containing primary antibody against Cx43 (Sigma, rabbit polyclonal, 1:400), N-Cad (mouse

monoclonal, BD Biosciences, 1:500), mouse monoclonal anti-p120ctn (mouse monoclonal, BD Biosciences, 1:500),. Coverslips were then washed three times for 10 min in PBS prior to incubation for 1h at room temperature in the dark in blocking solution containing secondary antibody (Molecular Probes, goat anti-rabbit IgG, 1:100). Coverslips were again washed three times for 10 min in PBS at room temperature in the dark and then rinsed in ddH_2O. Coverslips were mounted onto microscope slides with Prolong Gold® containing 4',6-Diamidino-2-phenylindole (DAPI, Molecular Probes). Note that no immunoreactivity could be detected when the Cx43 antibody was used in experiments performed on astrocytes taken from Cx43KO mice.

2.4 Fluorescence Microscopy (Image acquisition)

Fluorescent and differential interference contrast (DIC) or brightfield images were acquired with an AxioCam MRm (Zeiss) using the Axioskop2 epifluorescent microscope (Zeiss). Between cell types and treatments, the same exposure time was used and any modifications (i.e. min/max) were performed equally using AxioVision software (Zeiss).

2.5 Protein Collection

Cells were grown on 60mm dishes. Upon reaching confluency, cells were rinsed in ice cold PBS and lysed in 250µl radioimmune precipitation lysis buffer (RIPA; 0.005% sarkosyl w/v, 0.01% IGEPAL v/v, 0.001% SDS w/v, 150mMNaCl, 50mMTris, pH8.0) buffer containing phosphatase (Roche) and protease inhibitors (minicomplete, Roche) until lysis was complete. After DNA was sheared by titration with a fine gauge needle or sonicator, samples were centrifuged at 4°C for 10 min at 14,000rpm. The protein-containing fraction (supernatant) was subjected to the colorimetric BCA Protein Assay Kit (Pierce). Results were read at a wavelength of 570 nm on a Wallac1400 plate spectrophotometer (Wallac) to determine protein concentration.

2.6 SDS-PAGE

Protein samples were diluted in RIPA and denaturing sample buffer containing 0.1µl/mL β-mercaptoethanol. After boiling the samples for 3min, a volume containing 30µg was loaded onto 8% (for N-Cadherin and p120ctn) and 10% (for NOV, Cx43, and GAPDH) acrylamide-containing gels. Electrophoretic separation was conducted in a pre-mixed buffer (0.3% w/v Tris, 1.44 % glycine w/v, 1% SDS w/v) using a Mini-Protean 3 electrophoresis system (Biorad) at 100V for approximately 2 hours. Separated protein was electrically transferred (15V, 35min) onto an Immun-Blot® PVDF (polyvinylidene difluoride) membrane (Biorad) using a Transblot SD ® semidry transfer cell (Biorad).

2.7 Immunoblotting

Blots were equilibrated for approximately 5 min in Tris-Buffered Saline (TBS; 50mM Tris, 150mM NaCl, pH7.5) following semidry transfer and subsequently incubated in a solution of 5% non-fat dried milk (Carnation, Nestle) in TBS containing 0.1% Tween (TBST) for 1 hour at room temperature to block non-specific antibody binding. Blots were then incubated in a 1% milk-TBST solution containing antibodies to the protein of interest for 1-2h at room temperature or overnight at 4°C. Antibodies included: rabbit polyclonal anti-Cx43 (Sigma, 1:8,000), rabbit polyclonal anti-NOV (Fu et al. 2004; 1:800), mouse monoclonal anti-GFP (Chemicon, 1:1,000), mouse monoclonal anti-N-Cadherin (BD Biosciences, 1:2500), mouse monoclonal anti-p120Catenin (BD Biosciences, 1:1,000), and mouse monoclonal anti-GAPDH (HyTest Ltd., 1:10,000). Blots were then washed with 3 changes of TBST for 10 minutes each at room temperature and incubated in a 1%milk-TBST solution containing appropriate secondary antibody conjugated to horseradish peroxidase (goat anti-rabbit HRP or goat anti-mouse HRP, Ceaderlane, 1:10,000) for 1h at room temperature. Finally, blots were washed at room temperature with 2 changes of TBST for 10 minutes each, followed by an additional 10 min

wash with TBS and incubated for 5 minutes at room temperature in SuperSignal chemiluminescent substrate (Pierce) before exposure to X-ray film (Kodak). Densitometry analysis was carried out using AlphaImager^{TM}3400 software (AlphaInnotech) to determine relative changes in protein levels.

2.8 Preloading/Dye Coupling

Intercellular coupling between cells was evaluated according to the established preloading method described by Goldberg et al.(1995). Briefly, cells were seeded in 10% FBS-containing medium into 35-mm plates. Upon reaching confluency, donor cells were incubated in a dye solution (5 μM calcein-AM [Molecular Probes] and 10 μM DiI [Sigma-Aldrich] in an isotonic [0.3 M] glucose solution) for 20 min in an incubator. Donor cells were then rinsed several times with isotonic glucose, trypsinized, seeded onto recipient sister cultures at a ratio of 1:500, and maintained for 3.5 h in the incubator. Cells were examined by epifluorescence microscopy and gap junctional coupling was assessed by the passage of calcein from donor cells to, and among, recipient cells.

2.9 Wound healing motility assay

The following assay was modified from Lagana and Goetz et al (2006). Cells (1-2 x10^6) were grown to confluency (1-3d) on 60mm dishes in DMEM supplemented with 10%FBS and penstrep. The confluent monolayer was scraped using a cell lifter (Costar) in close proximity to a line which had been etched onto the underside of the dish using a razorblade. Cells were rinsed several times in PBS to remove loose and detached cells. Images were acquired immediately and 24h after the scrape. The images acquired at time 0 and time 24h were overlain in Adobe Photoshop. Migration distances were measured using Axiovision software (Zeiss) by drawing a line from the edge of the scrape at time 0 to the leading edge of the ten most migratory cells after 24h. (Unpaired t-tests and one way ANOVA were preformed using Instat software, see statistical analysis).

2.10 Transfection and infection of shRNA

Transfections and infections were modified from Shao et al. (2005) and Mao et al. (2000) respectively. RNAi-1 and RNAi-2 shRNA sequences in retroviral pH1.1QCXIH (GeneScript) vectors were transfected into HEK293 packaging cells using LipofectAMINE 2000 reagent and MEM. (Invitrogen, 20μg plasmid onto 100mm dishes). 6 hours following transfection 3mL of serum-free DMEM containing tetracycline was added. 24h post transfection the medium was replaced with serum-containing DMEM without tetracycline to allow for the production of the G glycoprotein of vesicular stomatitis virus (VSVG). 48-72h following transfection the medium was removed and filtered (0.45μm filter, BD Biosciences). Cell culture media of C6-H cells was replaced with filtered retroviral supernatant for infection. Following three rounds of infection cells were further cultured in selection media containing 100μg/ml hygromycin. Antibiotic-resistant cells were passaged in hygromycin-deficient medium prior to further experimentation. The entire cell population was kept rather than isolating clones.

2.11 Transwell migration invasion assays

Transwell assays were modified from (Zhang et al., 2003a; Vigetti et al., 2006; and www.fisher.co.uk/techzone/life/table-pdfs/protocol/transwell_trypsin_protocol.pdf). Medium (600μl) was added to lower compartment and $7.5x10^4$ cells (suspended in 200μl medium) were added to upper chamber. After 10-14h incubation at 37°C inserts were removed and transferred to 24 well plates in which each well contained 600μl 0.25%Trypsin-EDTA. Following a 10 min incubation at 37°C the 24 well plate was agitated gently by vortexing to detach cells from the underside of the PET (polyethylene terephthalate) membrane. 500μl of cell suspension was counted in a COULTER COUNTER ® Z1 Series Particle Counter (Coulter Electronics, Burlington, Ontario). (NOTE: For invasion experiments, 200μl 5% gelatin (in PBS) was added to upper chamber and allowed to dry (24h). Prior to seeding cells, inserts were first rehydrated by adding 200μl medium to upper chamber.) Assays were performed in duplicate. To

determine the motility index, the number of migrated cells was normalized to companion plate controls.

2.12 Statistical analysis

Quantitative data were applied to statistical analysis using InStat® software. Data were first subjected to a one-way analysis of variance (ANOVA) where applicable followed by an unpaired, two-tailed Student's t-test to determine whether significant differences existed between samples.

CHAPTER 3 THESIS RESULTS

Section 3.1 Motility of C6-H subclones

Similar to high grade human gliomas, C6 rat astrocytoma cells typically express very low levels of Cx43. Previous studies investigating the consequence of Cx43 on the motility of C6 cells have employed an exogenous overexpression approach (Lin et al., 2002; Oliveira et al., 2005; Zhang et al., 2003a). Although these studies all indicate that Cx43 overexpression leads to an increased motility and/or invasion, it has been reported that such exogenous overexpression causes premature assembly and altered trafficking of Cx43 (VanSlyke, 2005). Furthermore, we have previously shown that cells in which Cx43 is overexpressed have a very different gene profile than mock-transfected controls (Naus et al., 2000). While C6 cells typically express low levels of Cx43, we have isolated subclones by serial dilution in which Cx43 is endogenously expressed at moderate to high levels (C6-H, Figure 3.4A,B). Similarly, GJIC is also increased in these cells (Figure 3.4 C). We exploited the C6-H subclones to examine the consequence of endogenous upregulation of Cx43 in C6 cell motility. Wound healing assays (schematic, time 0h, 24h Figure 3.5A) were conducted to assess directional motility and it was found that the subclones endogenously expressing higher levels of Cx43 traveled a distance significantly greater than parental C6 cells expressing low levels of Cx43 ($P<0.001$, Figure 3.6B). Transwell chambers (schematic, Figure 3.5B) were also used to measure the random (i.e. non-directional) motility of these cells. Similar to directional motility, a significant (approximately 75%) reduction in the percentage of randomly migrating cells was observed for C6-L cells compared to C6-H subclones ($n=3$, $P<0.0001$, Figure 3.7).

Section 3.2 Knockdown of Cx43 in C6-H subclones by shRNA

The results described in the previous section prompted us to investigate whether the increased motility exhibited by the C6-H cells could be attributed directly to increased levels of

Cx43 or whether it was merely coincidental. We employed an RNAi approach to explore this possibility.

(C6-H) subclones were infected with Cx43shRNA or control (empty vector or scrambled Cx43 shRNA) constructs and the generation of stably expressing cells (C6-Cx43shRNAempty, C6-Cx43shRNAscrambled, C6-Cx43shRNA-1, C6-Cx43shRNA-2) was realized by hygromycin selection as described in the materials and methods. Consistent with previous observations (Shao et al., 2005), only the Cx43shRNA-1 construct led to a reduction in Cx43 protein (Figures 3.8 and 3.9). Densitometric analysis revealed that in cells stably expressing the Cx43shRNA-1 construct expression of Cx43 was reduced to 36% of scrambled control (Figure 3.8). To avoid clonal selection artifact and maintain the heterogeneity of the C6 cell line, the entire population of hygromycin-resistant cells was collected. Importantly, the high level of Cx43 retained in the C6-Cx43shRNAscrambled cells, and the low level of Cx43 in the C6-Cx43shRNA-1 cells was uniform throughout the entire cell population as indicated by immunocytochemistry (Figure 3.9).

Section 3.3 Knockdown of Cx43 by shRNA attenuates cell motility and invasion

Wound healing assays were again conducted to examine the consequence of Cx43 downregulation on directional cell motility. Untransfected, empty, and scrambled controls as well as cells expressing the Cx43shRNA-2 construct (which did not knockdown Cx43 protein) migrated a distance significantly greater than cells in which the level of Cx43 protein had been knocked down by the expression of the Cx43shRNA-1 construct (Figure 3.10).

The motility of cells expressing scrambled Cx43shRNA or Cx43shRNA-1 was further investigated using transwell chambers to assess random (i.e. non-directional) motility. In this assay, the number of migrating cells is examined rather than the migration distance (by counting the number of cells that traverse a perforated (8μm), uncoated PET membrane, Figure 3.5B). In addition to a reduction in directional motility upon Cx43 knockdown, a 50% reduction in the

percentage of randomly migrating cells was observed for cells expressing the Cx43shRNA-1 construct compared to cells expressing Cx43shRNA scrambled control (Figure 3.11).

Notably, glioma cells do not simply migrate passively through the brain but also actively invade into the brain parenchyma. Therefore, the consequence of Cx43 knockdown on invasivity was also investigated using transwell chambers in which the perforated (8μm) PET membrane had been coated with 5% gelatin prior to seeding the cells (Figure 3.5C). The consequence of Cx43 knockdown was more dramatic on cell invasivity than on cell motility: a 75% reduction in the percentage of invading cells was observed for cells expressing the Cx43shRNA-1 construct compared to cells expressing Cx43shRNA scrambled control (Figure 3.12).

Section 3.4 Mechanisms of Cx43 Mediated motility

In addition to attenuated motility and invasivity, GJIC was also decreased in C6-Cx43shRNA-1 cells compared to C6-Cx43shRNAscrambled as indicated by restricted passage of calcein dye in preloading experiments (Fig 3.13). We reasoned that the attenuation in cell motility may be a direct result of attenuated GJIC. Therefore, if functional gap junctions are essential for Cx43-mediated motility than blocking the channels would affect cell motility. Our next aim was consequently to elucidate a potential mechanism for Cx43 mediated motility. Previous studies have identified both junction-dependent (Oliveira et al., 2005) and –independent (Moorby, 2000; Wei et al., 2005) mechanisms.

The non-selective gap junction blocker carbenoxolone (CBX) is widely employed for studying channel-dependent activity. In agreement with Bani-Yaghoub et al. (1999), application of 150μM CBX during preloading assays restricts dye passage to unlabeled recipient cultures while application of the same concentration of inactive analog, glycyrrhizic acid (GZA) does not restrict dye passage to adjacent unlabeled recipient cells (Figure 3.14, see materials and methods section for assay details).

To evaluate the contribution of functional channel activity to C6 motility we carried out directional and non-directional motility assays in the presence of CBX. Blocking gap junction channel activity with CBX did not significantly attenuate the distance cells traveled in wound healing assays compared to untreated or control (GZA) treated cultures ($P>0.05$, Figure 3.15). Similarly, the percentage of cells that traversed through 8μm pores on uncoated PET membranes in transwell motility studies was not significantly increased upon treatment with 150 μM CBX compared to untreated or GZA treated cells (n=5, Figure 3.16).

Section 3.4.1 Protein localization and gap junctional intercellular communication in C6-Cx43ΔCT244-382GFP cells

Because the results obtained in wound healing assays containing CBX indicated that there was no difference in directional motility, we considered a mechanism for Cx43 mediated motility involving the carboxy terminus of Cx43. To this end, we employed C6 cells which had previously been infected with either full length Cx43 tagged with GFP (C6-Cx43GFP) or a truncated mutant in which the entire carboxy terminus had been deleted and replaced with GFP (C6-Cx43ΔCT244-382GFP; see Figure 3.17 and (Fu et al., 2004). The truncated protein localized to the periphery of the plasma membrane and formed obvious gap junction plaques at sites of cell-cell contact (Figure 3.18A).

Immunocytochemistry revealed that C6-Cx43ΔCT244-382GFP cells formed plaques composed entirely of truncated Cx43 protein (Figure 3.18B, green punctate staining) in addition to heteromeric plaques composed of both truncated Cx43 protein and endogenous Cx43 protein (Figure 3.18B, yellow punctate staining). Because heteromeric plaques composed of both truncated Cx43 protein and endogenous Cx43 protein were abundant, there existed the possibility that the truncated Cx43 protein eliminated the ability of endogenous Cx43 to form functional channels. We therefore conducted dye preloading assays to determine whether the C6-Cx43ΔCT244-382GFP cells were communication competent. Dye passage was observed in these

cells (Figure 3.19) eliminating the confounding variable that carboxy truncation of Cx43 may have imposed.

3.4.2 Expression of full length Cx43 enhances directional motility

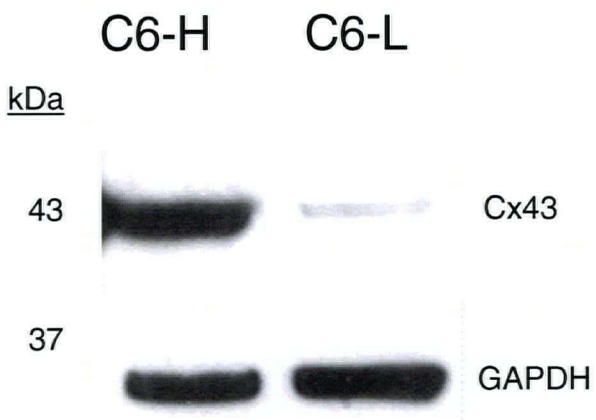
The consequence of Cx43 carboxy terminus deletion for cell motility was first examined using wound healing assays. While exogenous expression of full length Cx43-GFP demonstrated increased motility compared to control (GFP only), the communication-competent C6-Cx43ΔCT244-382GFP cells did not exhibit differences in motility compared to control cells (Figure 3.20). No differences in the non-directional motility of these cells was detected when assessed using uncoated transwell chambers in the absence of an exogenous stimulus. (Figure 3.21, n=5 $P>0.05$).

3.4.3 Cx43 associating proteins in cells expressing Cx43Δ244-382GFP

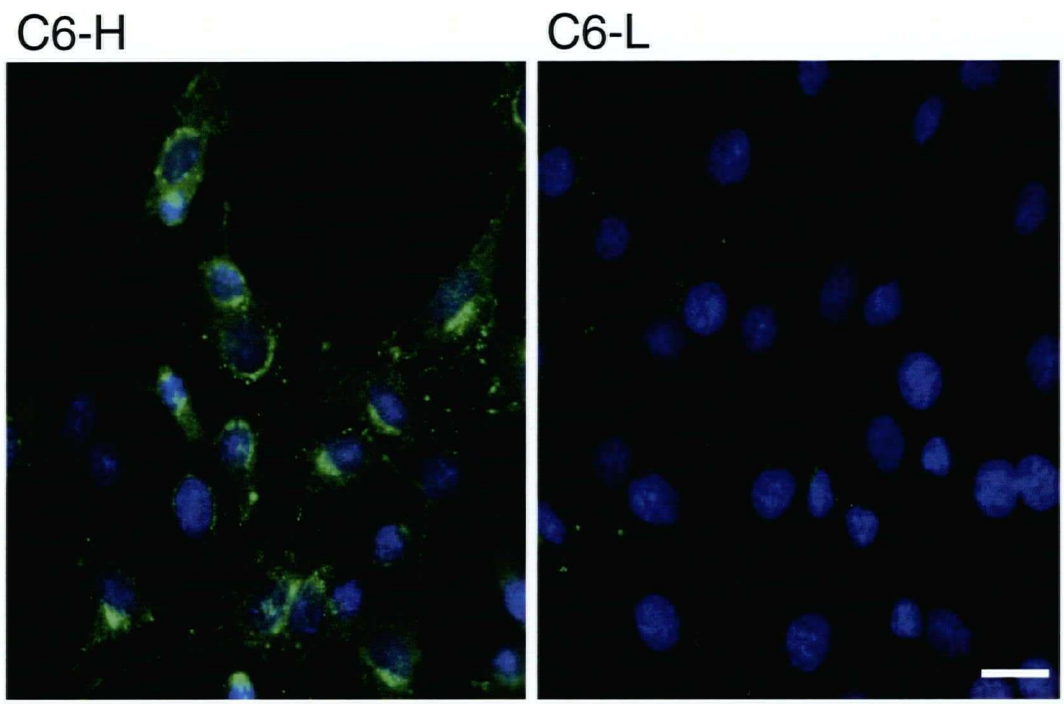
A mechanism involving the carboxy terminus of Cx43 for Cx43-mediated motility was implied in the rate of directional motility and furthermore we observed that cells expressing truncated protein appeared to migrate in collective sheets while control cells and cells expressing full length Cx43 migrated as individual cells in wound healing assays (Figure 3.22). Therefore, the expression and localization of Cx43 associating proteins with established roles in motility were examined. N-Cadherin levels were similar between C6-GFP, C6-Cx43GFP, and C6-Cx43Δ244-382GFP cells (Figure 3.23A). Furthermore, N-Cadherin localized to the membrane and at regions of cell-cell contract in both control and C6-Cx43Δ244-382GFP cells (Figure 3.23, panel B (compare upper and lower). Similarly, neither protein levels nor localization of p120ctn were obviously different between these cells (Figure 3.24, panels A and B).

Figure 3.4 Connexin43 expression in C6 subclones. C6 subclones generated by serial dilution gave rise to populations of cells in which Cx43 expression was enhanced (C6-H) compared to parental C6 cells (C6-L) as detected by A) Western immunoblotting with GAPDH serving as a loading control and B) immunocytochemistry (Bar=20μm). C) Dye preloading revealed that C6-H cells exhibit a greater degree of coupling than C6-L cells (Bar=50μm).

A



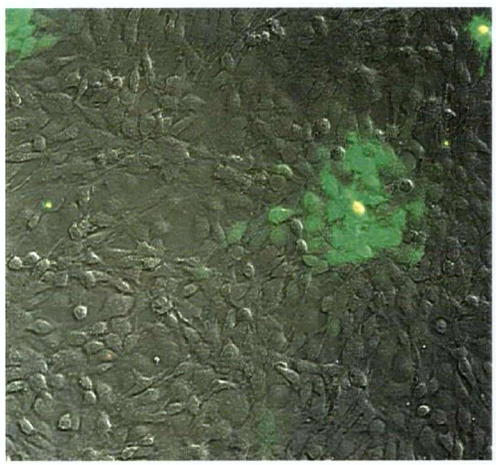
B



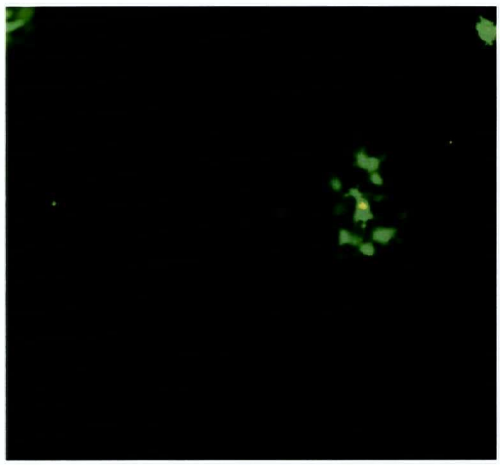
C

C6-H

DIC/FITC/Rhodamine overlay

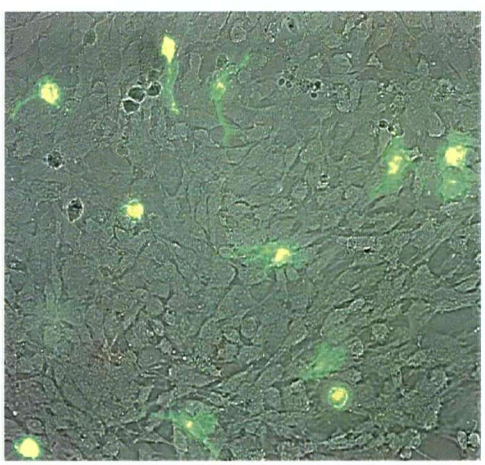


FITC/Rhodamine overlay



C6-L

DIC/FITC/Rhodamine overlay



FITC/Rhodamine overlay

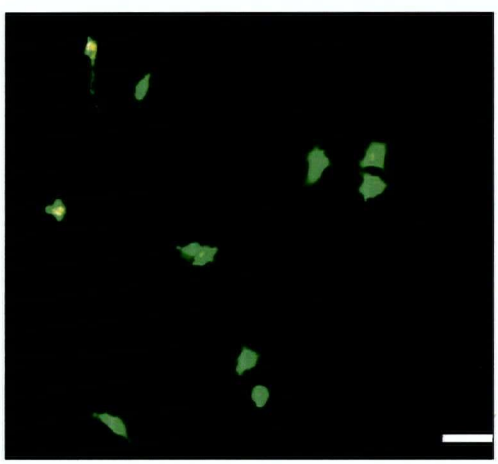


Figure 3.5 Motility and Invasion assays. A) Schematic of wound healing assays. Confluent monolayers were scraped in close proximity to a line which had been etched onto the underside of the dish using a razorblade. Images were acquired immediately (left) and 24h after the scrape (right) and were subsequently overlaid in Adobe Photoshop ® (lower) to accurately determine the site of the lesion (arrow) after cells had migrated into the void. Migration distances were measured by drawing a line from the edge of the scrape at time 0 to the leading edge of the most migratory cells after 24h. B) Schematic representation of transwell motility chambers. Cells are plated onto the upper membrane and migrate through the porous membrane to the lower chamber. C) Schematic representation of transwell invasion chambers. Transwell membranes were coated with 5% gelatin prior to experiments. Cells are plated onto the upper membrane and invade through the gelatin and porous membrane to the lower chamber.

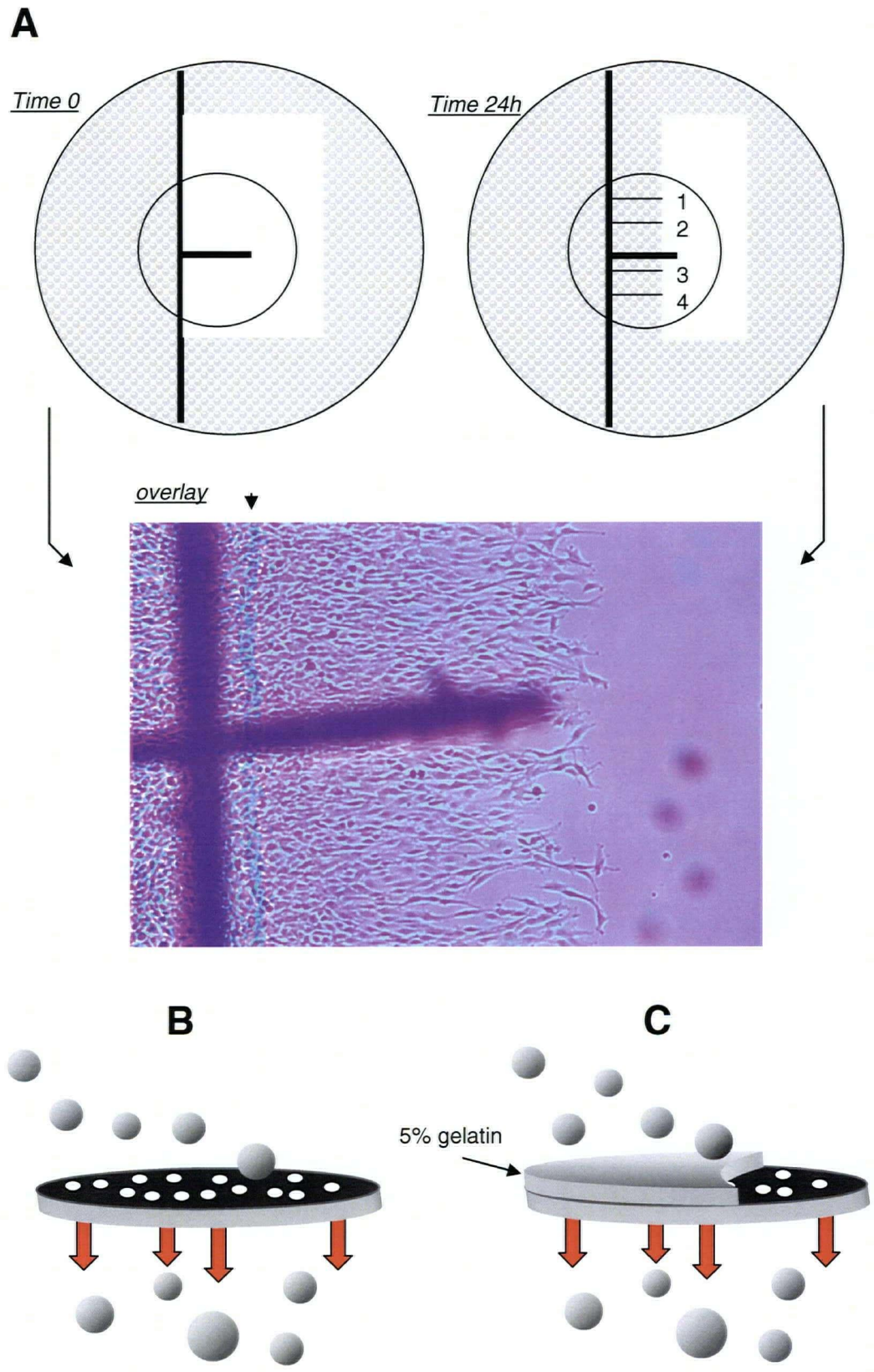
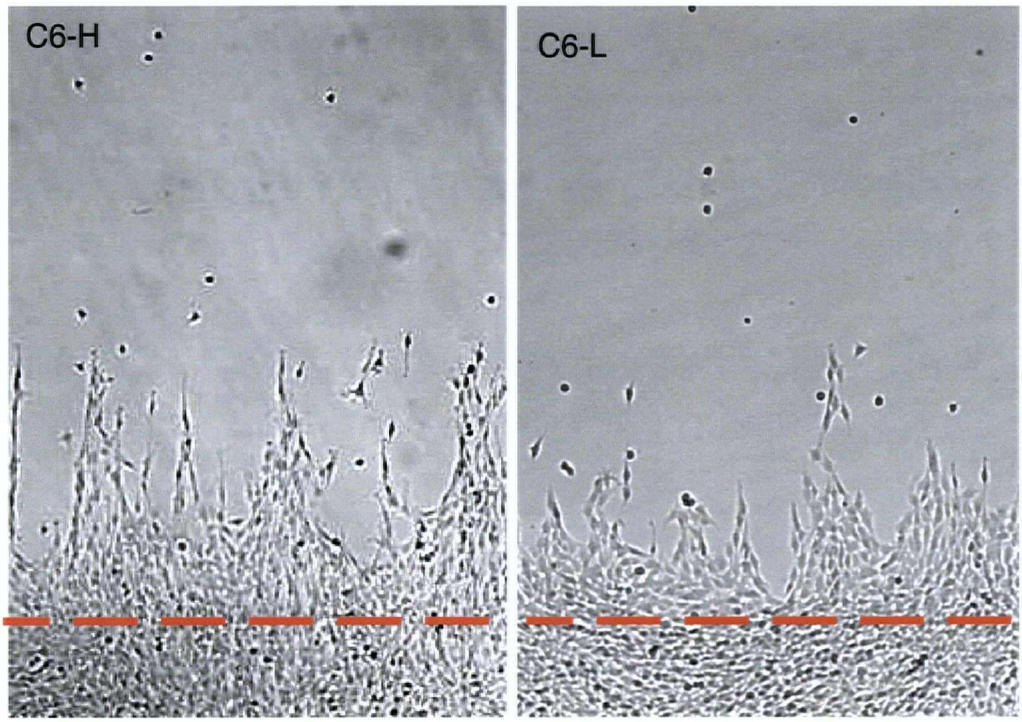

A
Time 0
Time 24h
1
2
3
4
overlay
B
C
5% gelatin

Figure 3.6 Analysis of C6 subclone motility by wound healing. A) Micrographs of subclones 24h following wounding. The red dashed line indicates the site of the lesion. B) Measurements of migration distances indicated that C6-H cells had moved a distance significantly greater than C6-L subclones 24h following wounding ($n=3$, $P<0.01$).

A


C6-H
C6-L

B

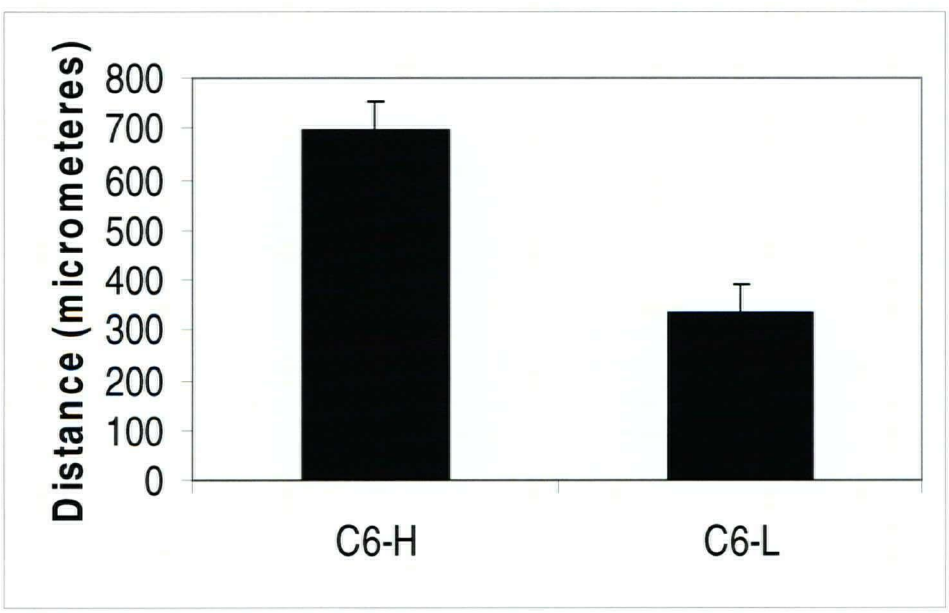

Distance (micrometeres)
800
700
600
500
400
300
200
100
0
C6-H
C6-L

Figure 3.7 Analysis of C6 subclone motility by transwell. The percentage of C6-H cells that migrated to the underside of uncoated PET membranes was significantly greater than that of C6-L subclones after14h ($n=3$, $P<0.0001$).

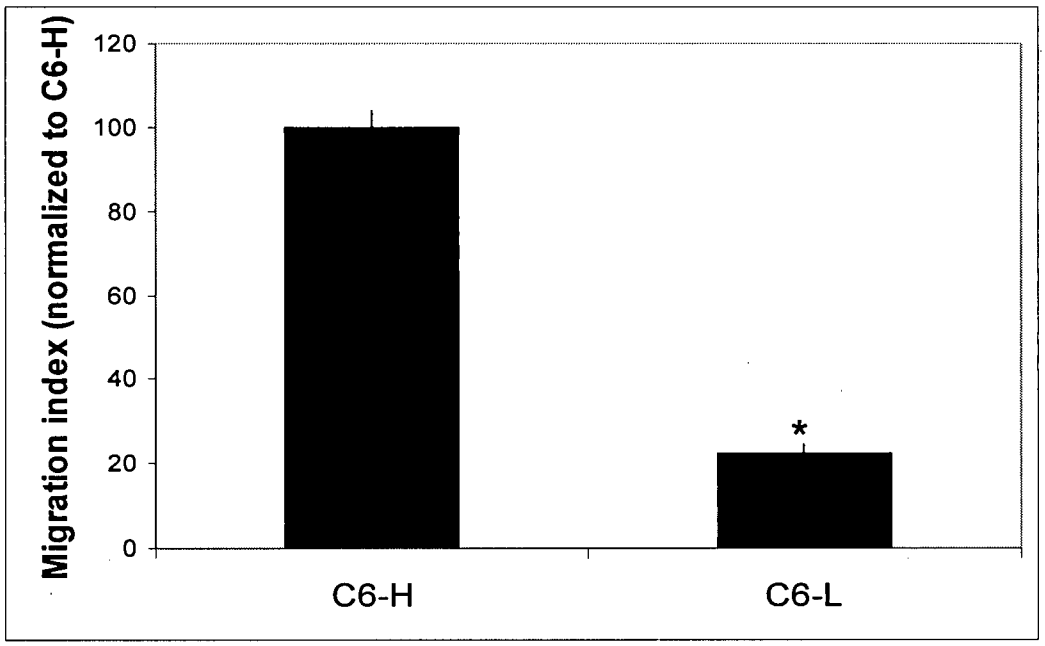
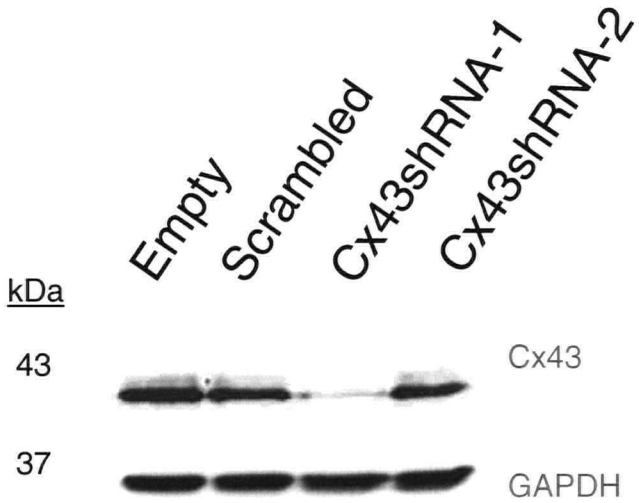

Migration index (normalized to C6-H)
120
100
80
60
40
20
0
*
C6-H
C6-L

Figure 3.8 Efficiency of endogenous Cx43 knockdown in C6 cells.

A) Cx43 protein expression after infection with Cx43shRNA-1 (but not Cx43shRNA-2) was reduced compared to both empty and scrambled controls as detected by Cx43 immunoreactivity of whole cell lysates subjected to western blot analysis. B) Densitometric analysis indicated that the intensity of Cx43 expression in C6-Cx43shRNA-1 cells was reduced by approximately 3 fold (36%) compared to cells expressing scrambledCx43shRNA following knockdown (n=2).

A


Empty
Scrambled
Cx43shRNA-1
Cx43shRNA-2
kDa
43
37
Cx43
GAPDH

B

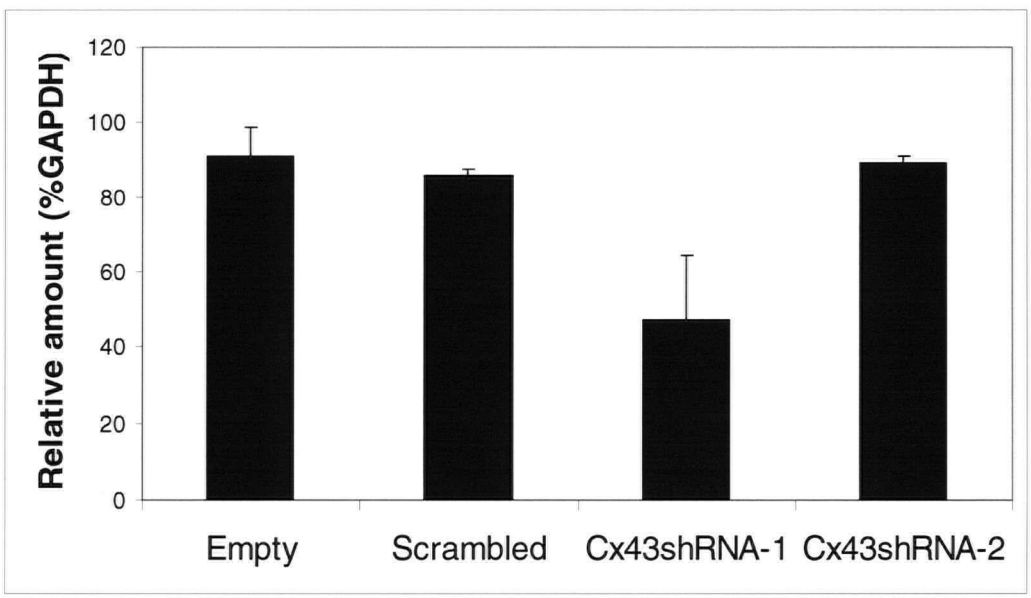
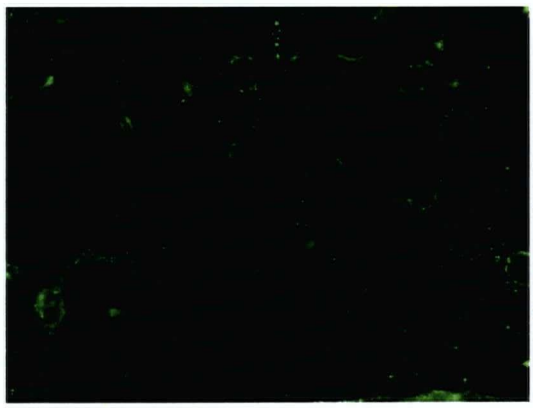

Relative amount (%GAPDH)
120
100
80
60
40
20
0
Empty
Scrambled
Cx43shRNA-1
Cx43shRNA-2

Figure 3.9 Knockdown of endogenous Cx43 in C6 cells

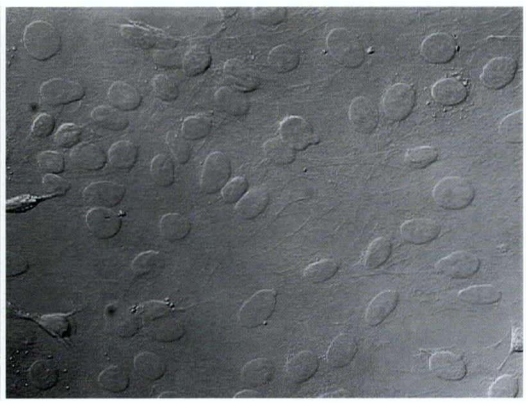
Consistent with Western immunoblotting, immunocytochemistry assays indicate that scrambled control cells express higher levels of Cx43 than Cx43shRNA cells. Note that exposure times for both scrambled and Cx43shRNA cells labeled with antibodies against Cx43 were the same.

Scrambled

FITC



DIC



Cx43 shRNA

FITC

DIC

Figure 3.10 Knockdown of Cx43 by shRNA decreases directional motility.

In wound healing assays cells stably expressing Cx43shRNA-1 moved a distance significantly less than empty or scrambled control 24h following wounding ($n=9$, $P<0.05$). Note: No significant differences were observed between empty, scrambled, Cx43shRNA-2, and untransfected parental controls ($P>0.05$)

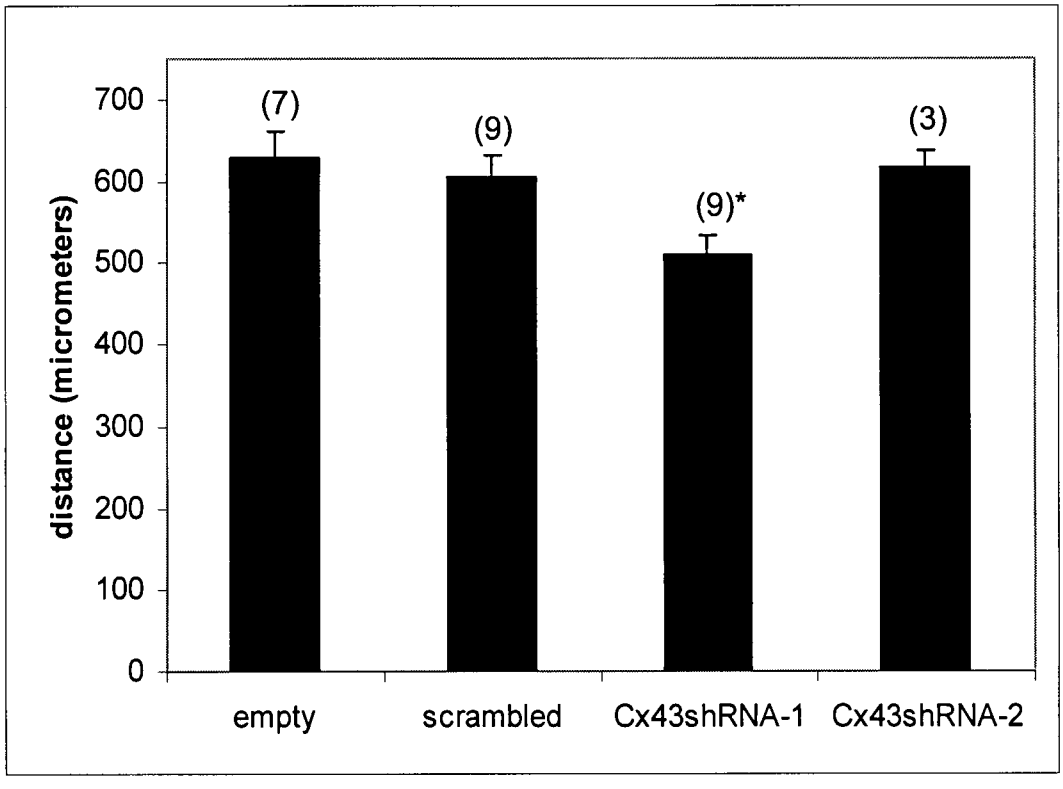

distance (micrometers)
700
600
500
400
300
200
100
0
(7)
(9)
(9)*
(3)
empty
scrambled
Cx43shRNA-1
Cx43shRNA-2

Figure 3.11 Knockdown of Cx43 by shRNA decreases non-directional motility. In transwell motility assays fewer Cx43shRNA-1 infected cells migrated through uncoated transwell membranes (8 μm pore size) than scrambled control after 10h (n=3, representative image shown)

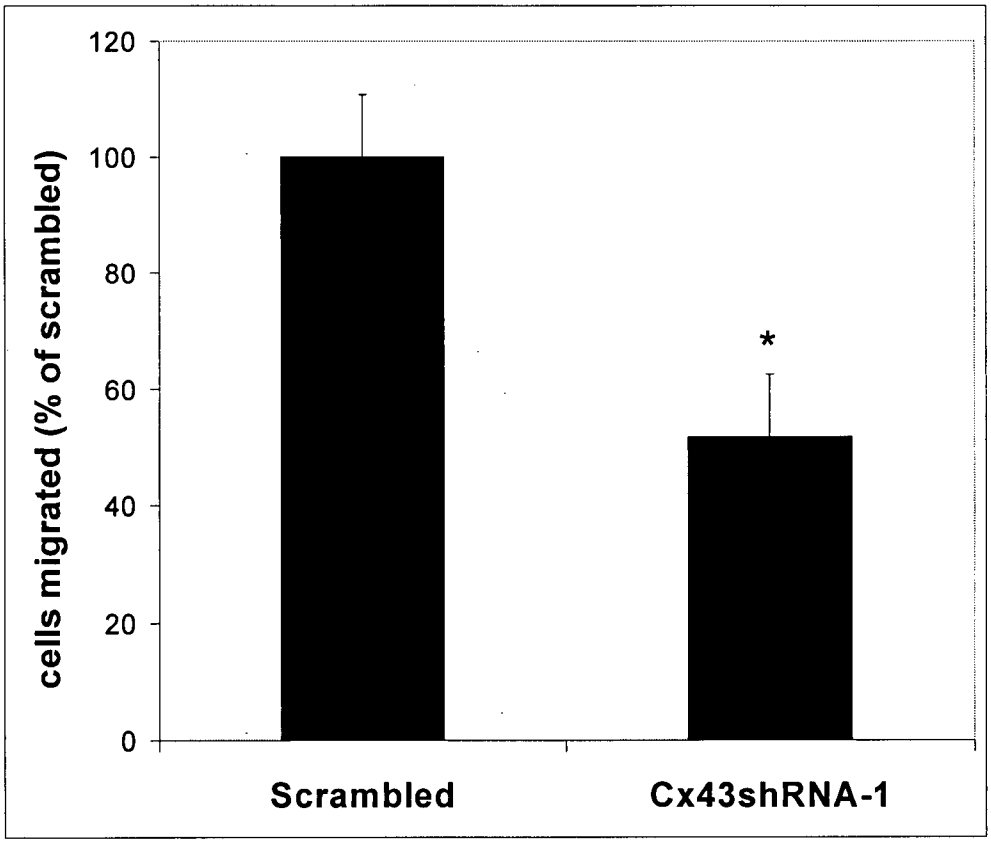

120
100
80
60
40
20
0
cells migrated (% of scrambled)
*
Scrambled
Cx43shRNA-1

Figure 3.12 Knockdown of Cx43 by shRNA decreases invasion. In transwell invasion assays fewer Cx43shRNA infected cells invaded through gelatin coated transwell membranes (8 μm pore size) than scrambled control after 10h (n=3, representative image shown)

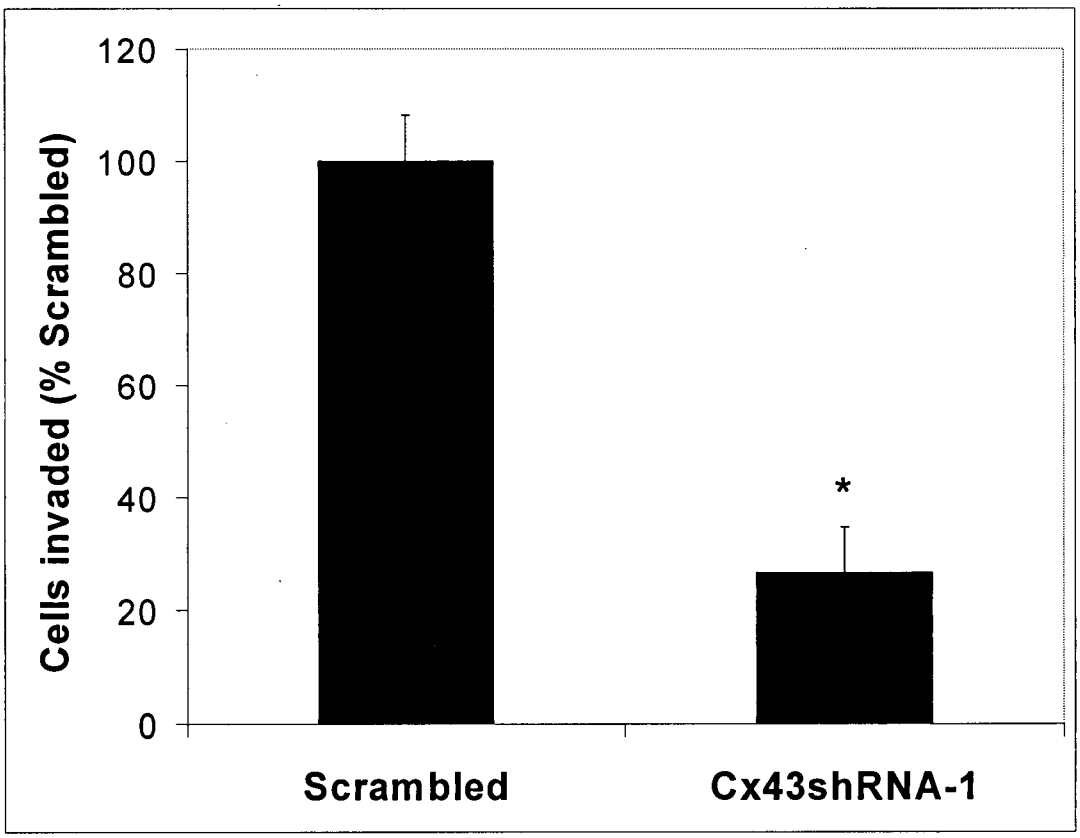
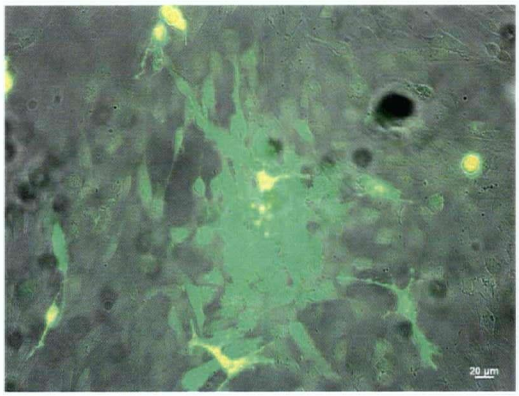

120
100
80
60
40
20
0
Cells invaded (% Scrambled)
*
Scrambled
Cx43shRNA-1

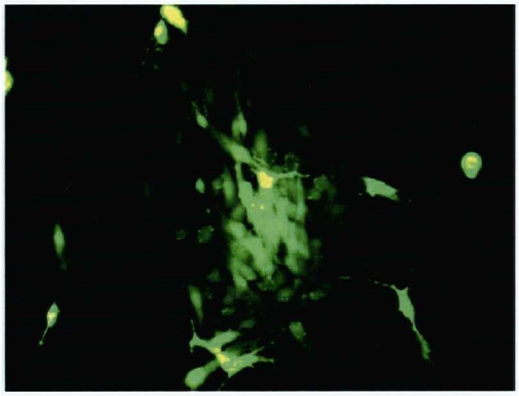
Figure 3.13 Knockdown of Cx43 by shRNA reduces gap junction intercellular communication. Gap junction intercellular communication was assessed in confluent cultures by the preloading method (see text for details). Dye transfer was restricted in cells expressing Cx43shRNA-1 compared to cells expressing scrambled control.

Scrambled Cx43shRNA

DIC/FITC/Rhodamine overlay

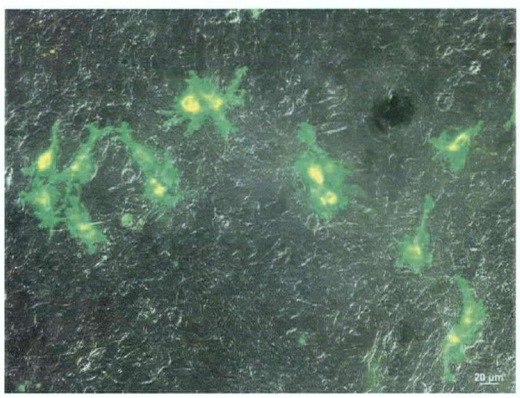


FITC/Rhodamine overlay



Cx43shRNA-1

DIC/FITC/Rhodamine overlay



FITC/Rhodamine overlay

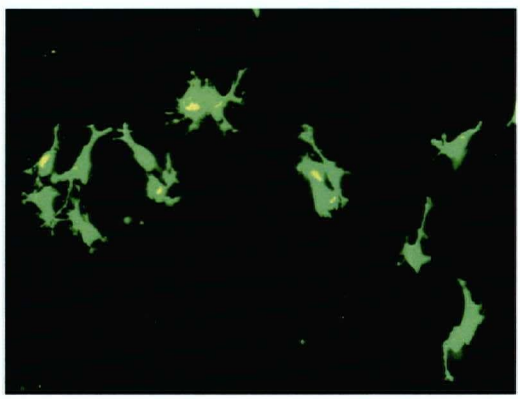
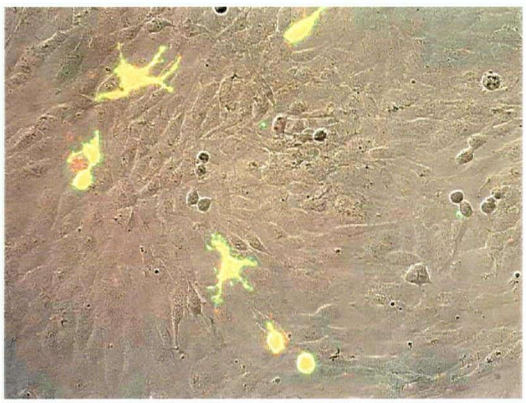


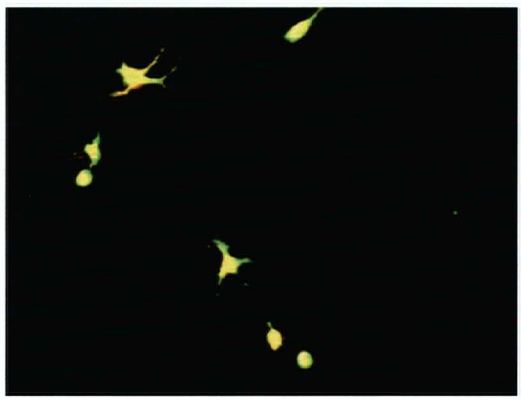
Figure 3.14 Efficacy of the gap junction blocker carbenoxolone. Preloading studies indicate that the passage of calcein dye was prevented in cultures incubated with 150μM carbenoxolone (top). The same concentration of the inactive analogue glyccccherrizzhicctic acid did not impede dye passage (bottom). Bar =50μm.

CBX- Gap Junction Blocker

DIC/FITC/Rhodamine overlay

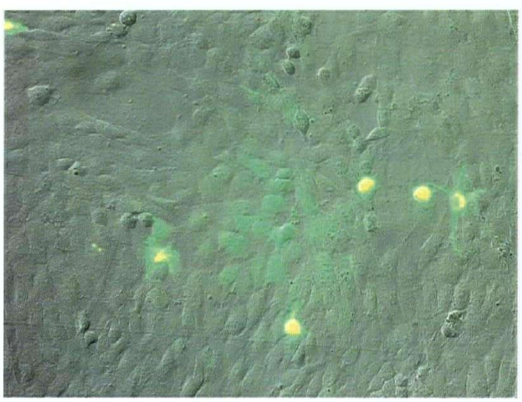


FITC/Rhodamine overlay



GZA- Inactive Analog

DIC/FITC/Rhodamine overlay



FITC/Rhodamine overlay

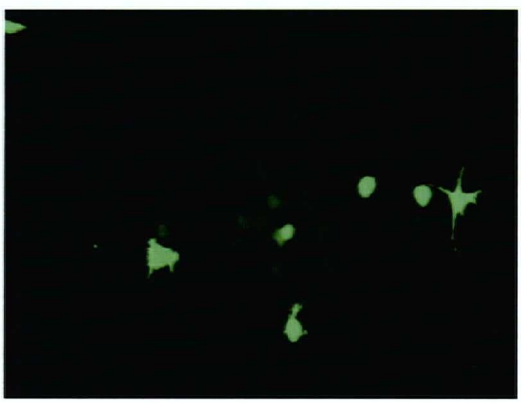


Figure 3.15 Blocking gap junction intercellular communication did not alter directional motility. Wound healing assays were conducted on C6-H cells in the presence of 150 μM CBX. The migration distance 24h after wounding was not altered compared to untreated or 150 μM GZA controls (n=6, P>0.05).

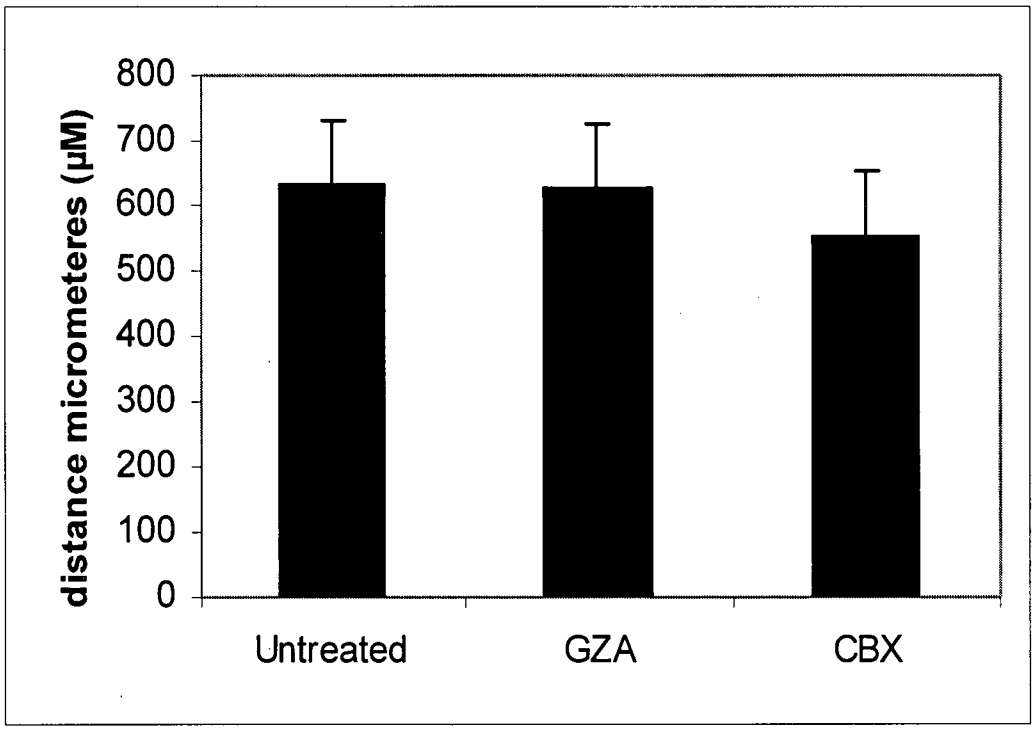

distance micrometeres (µM)
800
700
600
500
400
300
200
100
0
Untreated
GZA
CBX

Figure 3.16 Blocking gap junction intercellular communication did not alter non-directional motility. Transwell motility assays were conducted on C6-H cells in the presence of 150 μM CBX. There was no significant difference in the migration of cells treated with gap junction blocker compared to untreated or 150 μM GZA controls after incubation for 14h (n=3, $P>0.05$).

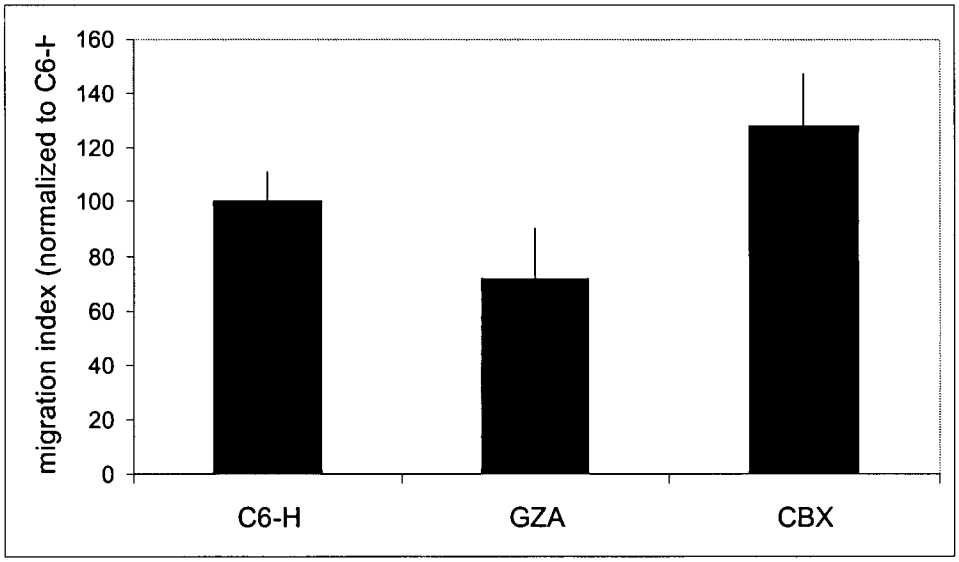

migration index (normalized to C6-H
160
140
120
100
80
60
40
20
0
C6-H
GZA
CBX

Figure 3.17 Levels of exogenous Cx43 are similar between cells expressing truncated and full length forms of Cx43. Whole cell lysates from C6 cells were run on polyacrylamide gels and probed for A) GFP to indicate exogenous GFP tagged Cx43 or B) Cx43 to indicate endogenous Cx43. GAPDH served as a loading control.

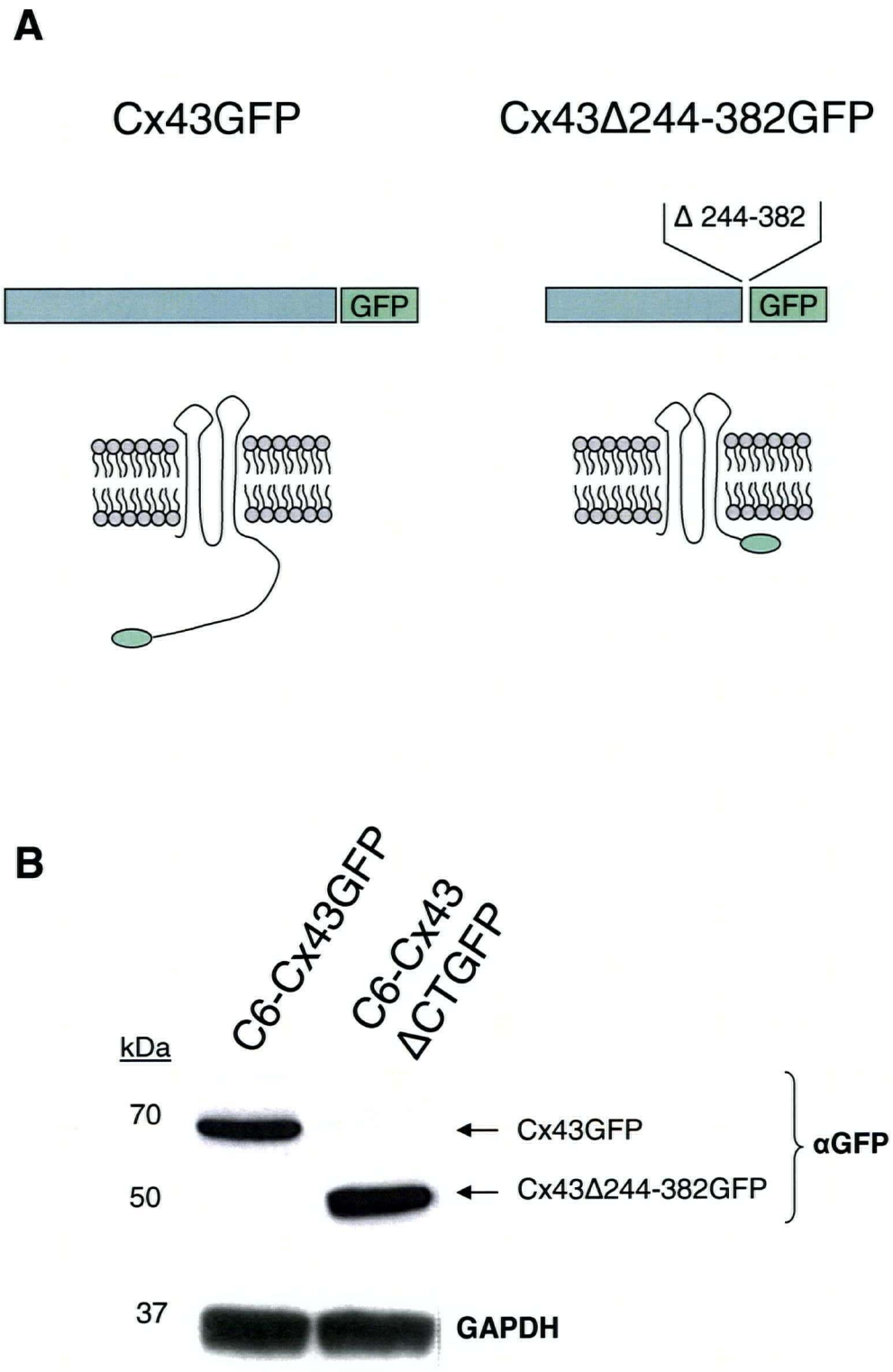
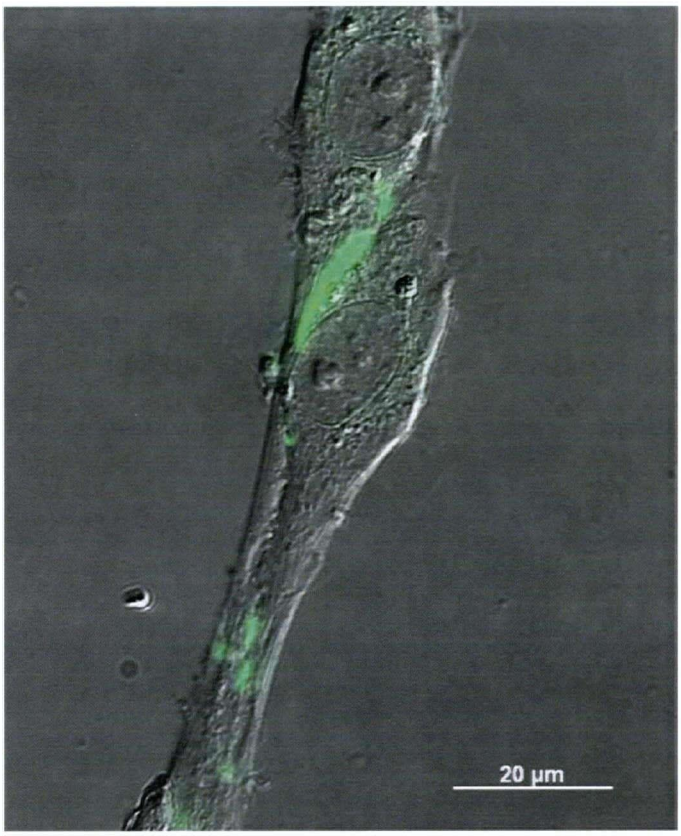

A
Cx43GFP
Cx43Δ244-382GFP
Δ 244-382
GFP
GFP
B
C6-Cx43GFP
C6-Cx43 ΔCTGFP
kDa
70
50
37
Cx43GFP
Cx43Δ244-382GFP
αGFP
GAPDH

Figure 3.18 Localization of truncated Cx43 in C6 cells. A) In live cultures, gap junction plaques composed of truncated Cx43 tagged with GFP were apparent at the plasma membranes between adjacent cells. B) In fixed cultures, endogenous Cx43 was detected using a mouse polyclonal antibody directed against the carboxy terminus of Cx43 (Red). Truncated Cx43 (GFP, green) localized to the outer membrane and formed homomeric plaques (green) or heteromeric plaques with endogenous Cx43 (yellow).

A



B

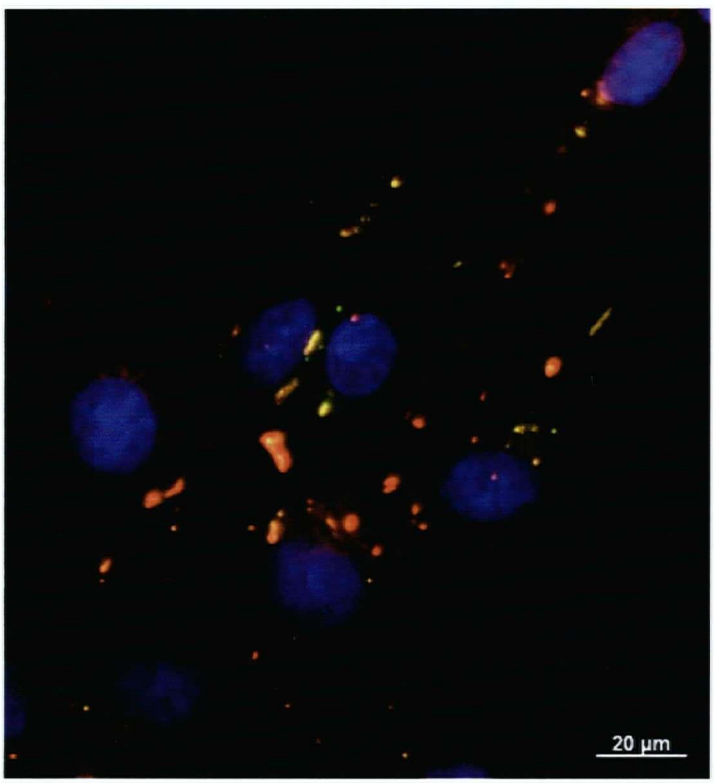


Figure 3.19 C6 cells expressing truncated Cx43 are communication competent. Dye preloading indicated that C6-Cx43Δ244-382GFP cells exhibit gap junction intercellular communication.

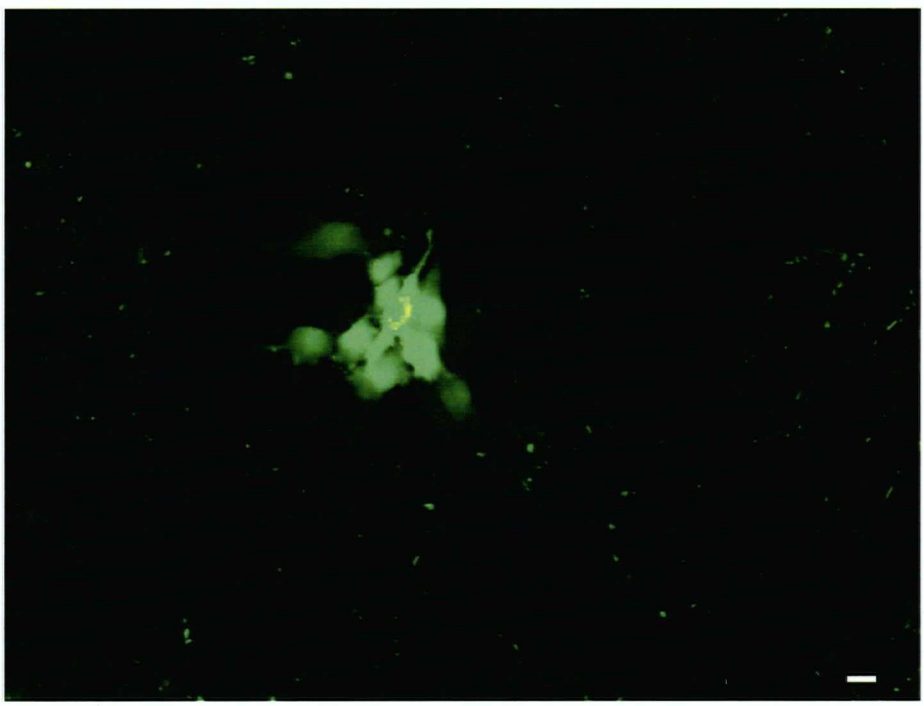


Figure 3.20 Enhanced directional motility requires full lenghth Cx43. Exogenous expression of full length Cx43 significantly enhanced directional motility compared to control ($p<0.05$). Expression of Cx43Δ244-382GFP, however, did not exhibit altered motility compared to control cells ($P>0.05$).

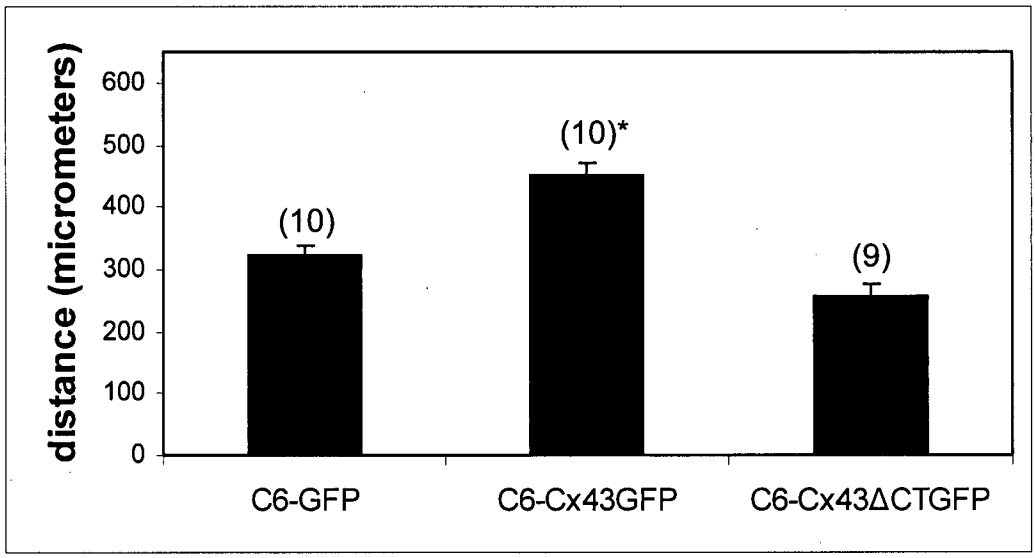

distance (micrometers)
600
500
400
300
200
100
0
(10)
(10)*
(9)
C6-GFP
C6-Cx43GFP
C6-Cx43ΔCTGFP

Figure 3.21 Truncation of the Cx43 carboxy terminus does not alter non-directional motility. Exogenous expression of full length Cx43GFP slightly but not significantly enhanced non-directional cell motility compared to control. Similarly, the expression of Cx43Δ244-382GFP did not result in altered motility compared to control cells ($P>0.05$, $n=5$).

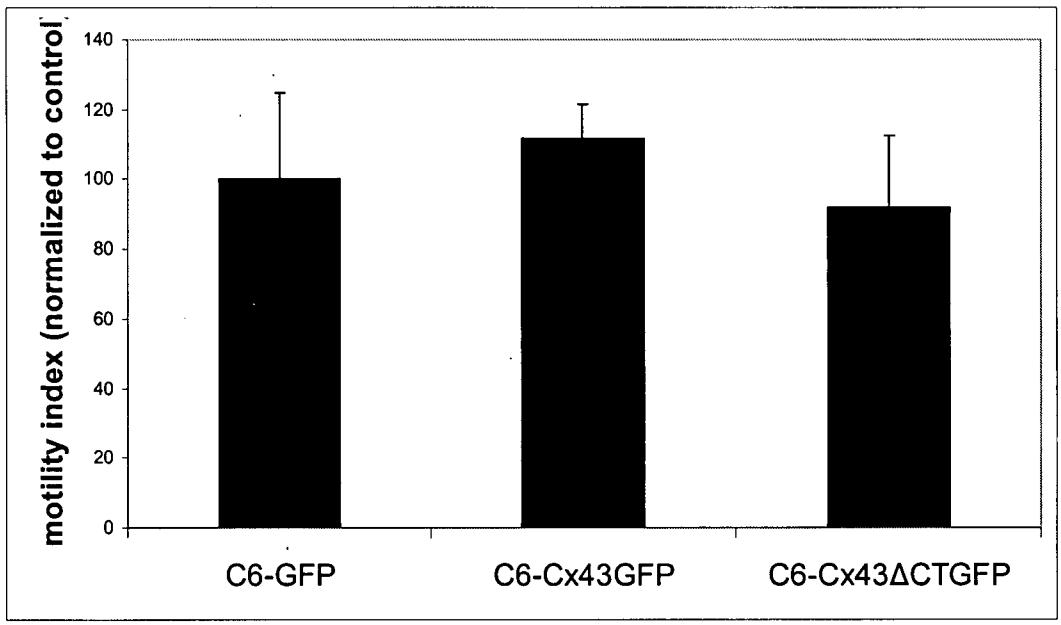
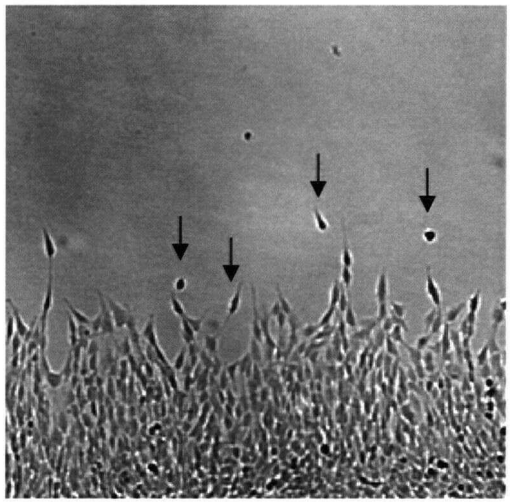
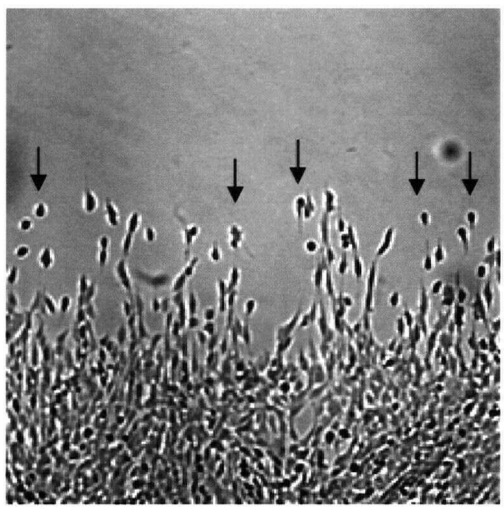

motility index (normalized to control)
140
120
100
80
60
40
20
0
C6-GFP
C6-Cx43GFP
C6-Cx43ΔCTGFP

Figure 3.22 Expression of Cx43Δ244-382GFP results in an altered motility phenotype. Micrographs of cells imaged 24h after wounding indicate that while control cells (A) and cells expressing full length Cx43 (B) migrated away from the site of the lesion as individual cells (arrows), cells expressing truncated Cx43 (C) migrated away from the wound site as a collective sheet.

C6-GFP



C6-Cx43GFP



C6-Cx43ΔCTGFP

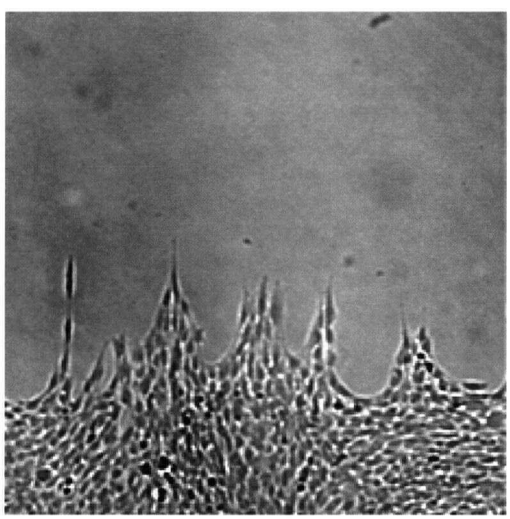
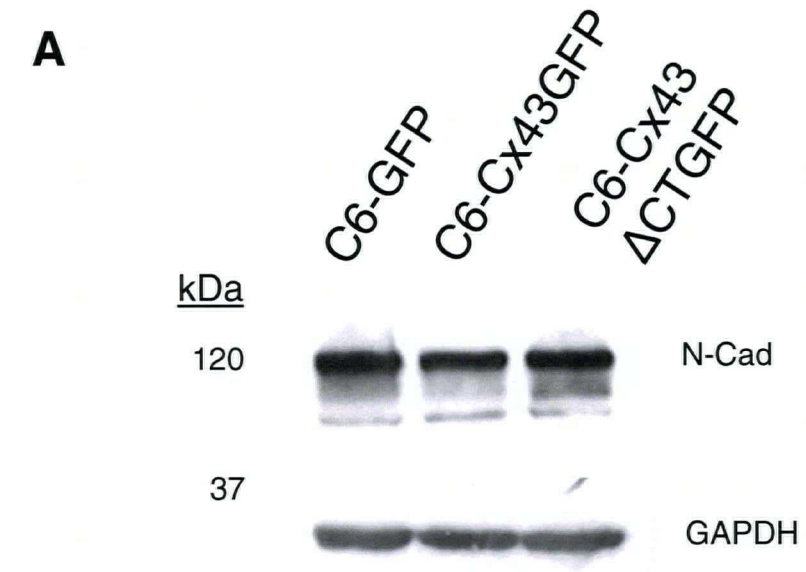


Figure 3.23 Expression and localization of N-Cadherin is not altered in C6 cells expressing Cx43ΔCT-GFP. A) The abundance of N-Cad protein is not altered in between C6-Cx43Δ244-382GFP and control cells. B) N-Cadherin is localized to the periphery of the plasma membrane at sites of cell-cell contact in both control (upper) and C6-Cx43Δ244-382GFP expressing (lower) cells (arrows).


A
C6-GFP
C6-Cx43GFP
C6-Cx43 ΔCTGFP
kDa
120
N-Cad
37
GAPDH

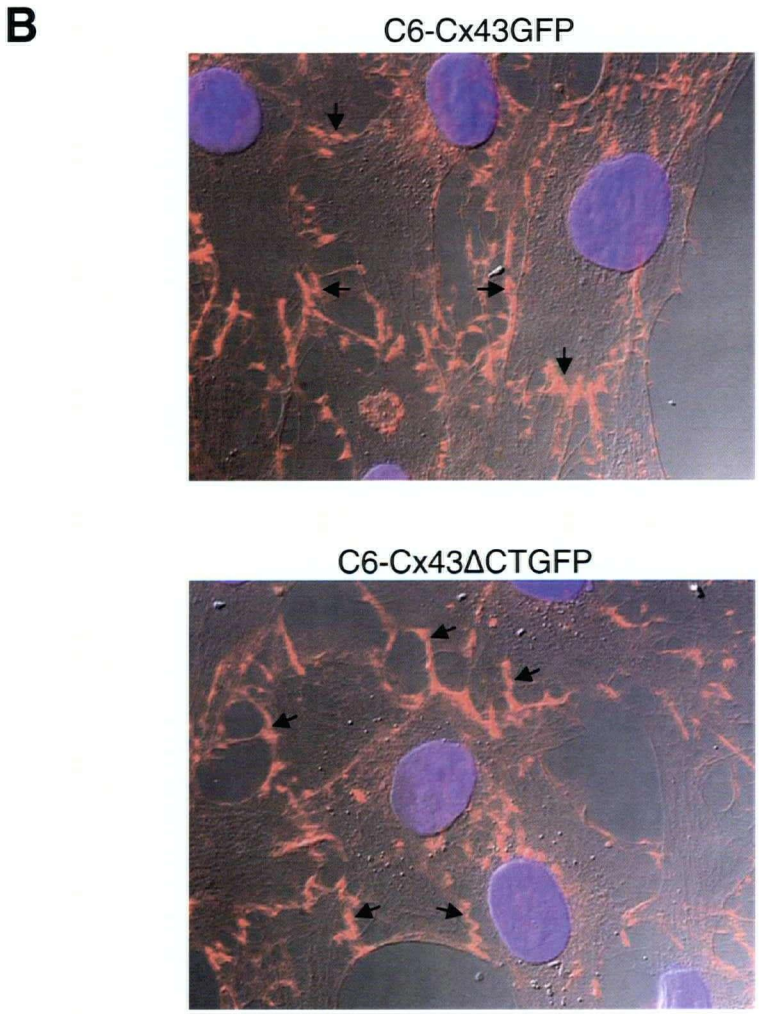
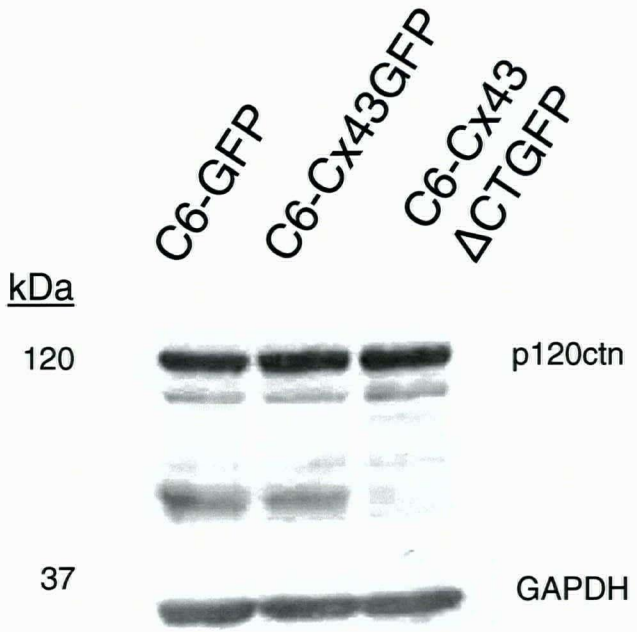
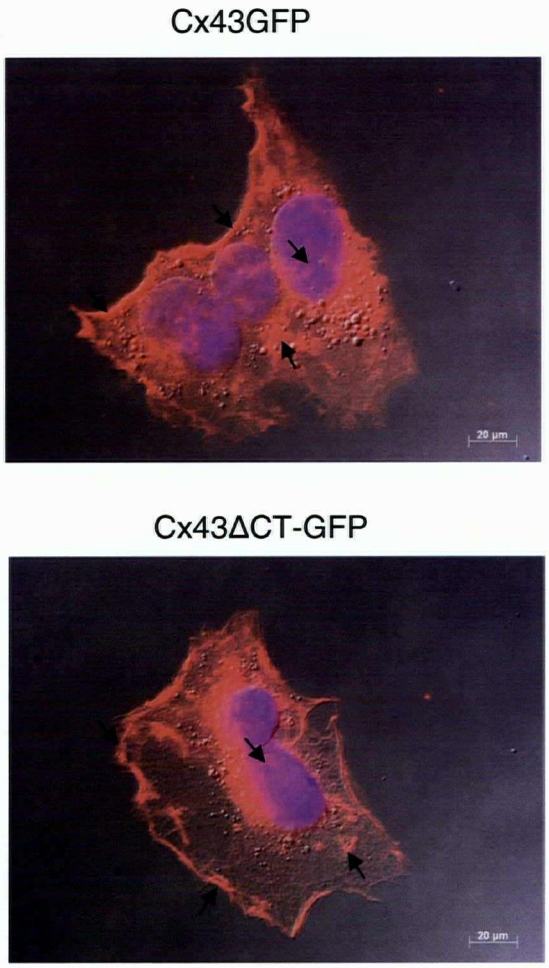

B
C6-Cx43GFP
C6-Cx43ΔCTGFP

Figure 3.24 Expression and localization of p120ctn is not altered in C6 cells expressing Cx43ΔCT-GFP. A) The abundance of p120ctn protein is not altered between C6-Cx43Δ244-382GFP and control cells. B) p120ctn is localized in the cytoplasm, nucleus, and to the periphery of the plasma membrane at sites of cell-cell contact in both control (upper) and C6-Cx43Δ244-382GFP expressing (lower) cells (arrows).

A


C6-GFP
C6-Cx43GFP
C6-Cx43 ΔCTGFP
kDa
120
p120ctn
37
GAPDH

B


Cx43GFP
20 µm
Cx43ΔCT-GFP
20 µm

CHAPTER 4 DISCUSSION

4.1 SUMMARY

The rationale for studying Cx43 in the context of glioma migration is two fold. First, it has been demonstrated that Cx43 expression is reduced in high grade glioma (Pu et al., 2004), (Soroceanu et al., 2001; Huang et al., 1999). Second, GJIC may be responsible for the observation that the invasion of malignant glioma into brain parenchyma concomitantly induces the phenotypic transformation of astrocytes, as evidenced by reactive gliosis in astrocytes surrounding the invasive tumor cells (Knott et al., 1998; Le et al., 2003). It is clear that the brain tissue plays a critical role, whether complaisant or resistant, for glioma invasion. Since Cx43 is the most prevalent Cx in astrocytes, gap junction channels composed of Cx43 likely constitute a major contribution to tumor-astrocyte interactions. Indeed direct intercellular communication between C6 glioma and astrocytes *in vitro*, as well as astrocytes *in vivo*, was increased when C6 cells were engineered to overexpress Cx43 (Zhang et al 1999, 2003, Lin et al 2002).

To date, only 3 studies have directly examined the role of Cx43 in glioma motility and/or invasion (Lin et al., 2003b; Oliveira et al., 2005; Zhang et al., 2003a). These studies have employed an exogenous overexpression approach which impose confounding variables such as defects in Cx43 assembly and trafficking (VanSlyke, 2005). To circumvent these variables, we took a novel approach and compared the motility of C6 subclones expressing high levels of endogenous Cx43 to parental C6 subclones expressing low levels of Cx43. To ensure that additional differences between these subclones (i.e. other than Cx43 expression) did not account for differences observed in functional motility assays, Cx43 expression, as determined by western blot and immunocytochemistry, was suppressed using siRNA. The results of these experiments indicated that Cx43 enhanced motility and invasion in C6 cells which is in agreement with other studies (Lin et al., 2002; Oliveira et al., 2005; Zhang et al., 2003a) that investigated the role of Cx43-mediated motility and invasion in these cells.

In addition to a reduction of Cx43 protein upon shRNA knockdown, the preloading method of dye transfer (Goldberg et al., 1995) indicated that gap junction intercellular coupling is impaired in cells expressing Cx43shRNA-1 compared to scrambled control. It was hence reasoned that the decreased cell motility observed could be a consequence of decreased GJIC or Cx43 expression independent of GJIC. To clarify these confounding factors, the gap junction blocker carbenoxolone was added to the culture medium during wound healing and transwell motility assays. No significant differences in directional or random motility could be detected between CBX, inactive control GZA, or untreated cells. While CBX is a non-specific blocker of gap junctions (Blomstrand et al., 2004), persistence of a function (i.e. motility) in the presence of this blocker is quite good evidence that GJIC does not contribute significantly to motility in C6 cells (Bennett and Zukin, 2004). Therefore, it is possible that Cx43-mediated motility may be attributed to actions of Cx43 that do not involve GJIC.

The Cx43 C-terminus is thought to mediate the interaction with the majority of Cx43-interacting proteins (Giepmans, 2004; Herve et al., 2004a). To consider the possibility that protein interactions with the carboxy terminus of Cx43 mediate the role of Cx43 in cell motility, a comparison of both directional and random motility was undertaken in communication competent C6 cells exogenously expressing full length or truncated Cx43. These cells were generated by the retroviral infection of construncts into C6 cells expressing low levels of Cx43 (however not the C6-L cells described earlier) and were not subjected to subsequent selection (see Fu et al., 2004). Random motility, evaluated by transwell chambers, was not significantly different between these cells. In wound healing assays cells expressing full length Cx43 were significantly more motile than both C6-GFP control and C6-Cx43ΔCT244-382GFP cells, while there was no significant difference in the wound healing ability of cells expressing truncated Cx43 compared to control. This indicates that the Cx43 C-terminus likely plays an important role in the ability of Cx43 to enhance motility by a mechanism that does not impair full length Cx43.

The collective sheet-like migration exhibited by C6-Cx43ΔCT244-382GFP cells suggested that the loss of the Cx43 C-terminus may correlate with increased adhesion. In an effort to elucidate differences between C6-Cx43GFP and C6-Cx43ΔCT244-382GFP cells with respect to adhesion, N-Cad and p120ctn, Cx43 associating proteins with known roles in motility and adhesion, were assessed for protein expression and localization by western blot and immunocytochemistry respectively. We were unable to detect any changes in total protein expression of either N-Cad or p120ctn between C6-Cx43GFP and C6-Cx43ΔCT244-382GFP cells. Similarly, in both cell types these proteins localized to the cell membrane, nucleus, and cytoplasmic component.

4.2 Cx43-MEDIATED INVASION IN HOMOCELLULAR POPULATIONS OF C6 CELLS

Several studies have attempted to elucidate the role of Cx43 in glioma motility and/or invasion. Zhang et al. (2003) observed increased chemokinesis of C6 cells overexpressing Cx43 (C6-Cx43) compared to mock transfected control cells (C6-mock) in radial dish assays (Zhang et al., 2003a). Using transwell chambers to evaluate chemokinesis, we noted a decrease in cell motility upon Cx43 knockdown with shRNA. Together, these studies suggest that Cx43 expression correlates positively with chemokinesis. Zhang et al (2003) further compared the invasivity of C6-Cx43 versus C6-mock cells in the presence or absence of astrocytes. In the absence of astrocytes, the rate of invasion was similar between C6-Cx43 and C6-mock cells while C6-Cx43 cells were much more invasive in the co-culture experiments. Conversely, in the present study we found that Cx43 knockdown dramatically attenuated invasion in homocellular populations of C6 cells, suggesting that the modulation of Cx43 levels alone are able to alter invasivity independent of heterocellular interactions. Although both studies employed the *in vitro* transwell invasion system, there are two notable differences between the assays.

First, Zhang et al. (2003) employed C6 cells overexpressing Cx43 while we used C6 cells in which high endogenous levels of Cx43 were knocked down using shRNA. Both of these

approaches may introduce confounding variables as hemichannels are assembled prematurely in the ER of C6 cells overexpressing Cx43 (VanSlyke, 2005) and shRNA may have off target effects. Additionally, the C6 subclones employed in these studies may not have been derived from the same parental cells. Given the heterogeneity of the C6 line, it is possible that differences unique to the cells employed in the respective studies gave rise to these differences.

Secondly, despite having demonstrated that a consequence of Cx43 overexpression was an increase in the level of gelatinase activity (Zhang et al 2003), Zhang et al (2003) used Matrigel as an invasive substrate. Matrigel, which is artificially reconstituted basement membrane, contains numerous and undefined growth factors and signaling molecules which confound *in vitro* analysis (Vukicevic et al., 1992). We employed gelatin as an invasive substrate which is devoid of such undefined growth factors and signaling molecules and is the ECM component that is preferentially degraded by the gelatinases. The former study therefore examines chemotactic properties while the latter examines chemokinetic properties. Notably, neither of these invasion experiments was conducted in the presence of gap junction blockers; therefore, the contribution of GJIC was not assessed.

Together these studies suggest that while the co-culture of C6 glioma cells and astrocytes may indeed increase the invasivity of tumor cells exogenously overexpressing Cx43, co-culture may not be essential for C6 invasion since knockdown of Cx43 expression reduces the invasivity of homocellular populations of C6 cells. These studies additionally caution that because the invasive substrate is an important determinant of cellular invasion, equivalent substrates must be employed when comparing results between studies to avoid confounding factors. Furthermore, both studies support a motility and invasion enhancing role for Cx43 in C6 cells.

The contribution of Cx43 in heterocellular interactions between C6 glioma and astrocytes was investigated *in vivo* by Lin et al. (2002). C6-Cx43 cells implanted into the striatum of Wistar rats exhibited widespread passage of Lucifer yellow to host astrocytes. In contrast, C6-mock and

C6 cells expressing chimeric Cx40/Cx43 (Cx40*43C3) proteins created by splicing the C-terminus of Cx43 into Cx40 (Haubrich et al., 1996), failed to establish Lucifer yellow coupling with surrounding host cells. Coincident with decreased GJIC, both C6-mock and C6-Cx40*43C3 cells exhibited adluminal invasion while C6-Cx43 cells aggressively infiltrated the brain parenchyma. The use of the communication-incompetent Cx40*43C3 protein in these studies suggests a requirement for GJIC in glioma invasion.

4.3 Cx43 AND GJIC IN GLIOMA CHEMOKINESIS AND CHEMOTAXIS

The contribution of GJIC in heterocellular glioma motility was evaluated using established human glioblastoma cell lines in addition to C6 cells in several *in vitro* assays (Oliveira, 2005). In an *ex vivo* brain slice assay heterocellular GJIC between brain slices and C6 cells could be manipulated throughout chemokinesis using pharmacological blockers. Blocking GJIC by CBX in the cell type that exhibited the most extensive heterocellular coupling (GL15 cells) decreased the migration distance as well as the number of migrating cells compared to GZA treated controls (Oliveira, 2005). By contrast, in the current study blocking Cx43 channels with CBX did not alter chemotaxis or chemokinesis in homocellular populations of C6 cells. Wound healing assays are a particularly relevant *in vitro* method to study the motility of glioma cells as lesions are inflicted, as they are in surgical resection. The lesion provides uniform directionality to motile cells. The imposed insult when lesioning confluent monolayers of cells provides a chemorepulsive stimulus, directing cells away from the site of injury (Sato and Rifkin, 1988). Although it is difficult to detect a chemorepulsive or chemoattractive gradient directly, it has been shown that cells hundreds of microns from the wound edge extend lamellipodia beneath cells in front of them toward the lesion. (Farooqui and Fenteany, 2005). Importantly, the authors found that this effect persisted in the presence of gap junction blockers. Furthermore, increased production of motility enhancing ECM proteins such as tenascin and thrombospondin occurs upon injury to the epithelium (Majesky, 1994). These stimuli down-regulate the assembly and

activity of focal adhesions (Zagzag et al., 2002) and may well override motility-altering effects caused by blocking GJIC. This may explain why no significant differences in wound healing ability were detected in CBX treated cultures compared to GZA treated or untreated control. The role of GJIC in chemotaxis might then be better studied using the transwell assay. However, in the transwell assay it is not clear whether cells indeed form gap junctions with adjacent cells in the absence of gap junction uncouplers and therefore blocker treatment may not have functional consequences on GJIC-mediated motility. By implanting a mixed population of C6 and C6-Cx43GFP cells into rat brain, Peschanski et al (2005) found that C6-Cx43GFP cells were preferentially located at the periphery of the tumor mass while C6 cells expressing low levels of Cx43 were present only in the tumor mass. This finding is consistent with the previous studies mentioned that examined the role of Cx43 in glioma motility, as well as by the current study in which C6-Cx43 cells exhibited increased chemotaxis compared to control (C6-GFP) cells.

In summary, the studies that have directly examined the role of Cx43 in glioma motility and/or invasion demonstrate a positive correlation between these cellular processes and Cx43 expression, although the contribution of GJIC has not yet been conclusively elucidated.

4.4 INVOLVEMENT OF GJIC

Although few studies conducted thus far have directly examined the role of Cx43 in glioma motility, a number of studies have directly investigated the role of Cx43 in other cell types. Many of these studies indicate a positive correlation between Cx43 and motility and/or invasion. The role of GJIC in transendothelial migration (diapedesis) of mammary epithelial tumors was investigated using a Cx- and GJIC-deficient, non-metastatic cell line (HBL100) in conjunction with Cx43-expressing human microvascular endothelial cells (HMVECs) derived from the lung (Pollmann et al., 2005). HBL100 cells engineered to express wild-type Cx43 exhibited enhanced diapedesis while cells expressing a non-functional (i.e. GJIC incompetent) chimeric mutant of Cx43 were similar to control (Pollmann et al., 2005). GJIC was tested

directly by blocking both homocellular and heterocellular GJIC with CBX in co-cultures and resulted in reduced diapedesis of Cx43 expressing HBL100 tumor cells (Pollmann et al., 2005). As these results indicate a positive correlation between Cx43 and invasion, they support the results found in studies investigating Cx43 in glioma invasion, including the results obtained in the present study, and further suggest the involvement of heterologous GJIC in cellular invasion.

The regulation of heterocellular GJIC may be more important than a straightforward communication-competent versus communication-incompetent scenario. The deletion of the Cx43 gene, *Gja1*, in mice results in perinatal lethality due to conotruncal heart malformations and pulmonary outflow obstruction (Reaume et al., 1995). These regions of the heart are populated by migratory cardiac neural crest cells emanating from the dorsolateral margins of the neural tube. In an *ex vivo* culture system, explants of the neural tube from transgenic mice (Cx43KO or CMV43, a transgenic mouse line in which Cx43 is overexpressed) revealed that changes in the level of GJIC correlated positively with parallel changes in the rate of neural crest migration (Huang et al., 1998a). Furthermore, blocking GJIC with oleamide significantly reduced outgrowth (Huang et al., 1998a). *In vivo* the hearts of CMV43 mice revealed an increased abundance of neural crest cells in the outflow septum while the hearts of Cx43KO mice exhibited an obvious thinning of the conotruncal myocardium and the presence of fewer neural crest cells (Huang et al., 1998a). Importantly, the hearts of CMV43 mice also suffered from malformations of the conus region and outflow tract obstructions (Ewart et al., 1997; Huang et al., 1998b) despite exhibiting high levels of GJIC (Huang et al., 1998a). These studies suggest that while the migration of neural crest cells indeed requires GJIC, the precise regulation of GJIC may be of critical importance for conotruncal heart development. Such precise regulation is intimately related to the Cx43 C-terminus. Specifically, *Xenopus laevis* oocytes expressing C-terminal truncated Cx43 were resistant to GJIC uncoupling agents such as insulin and insulin-like growth factor (IGF; Homma et al., 1998) and v-src (Zhou et al., 1999). In murine

neuroblastoma (N2a) cells, single channel analysis indicated that truncation of the Cx43 C-terminus (Cx43M257 mutant) domain did not significantly modify the magnitude of the main unitary conductance of Cx43 channels, while the mean open time of Cx43M257 channels was considerably prolonged compared to full length channels (2450±200 ms compared to 126±20 ms respectively; Moreno et al., 2002). It is therefore important to consider that GJIC may account for differences when assessing cellular functions in cells expressing mutated and/or truncated Cx43 C-termini. In our study, the motility of C6 cells exogenously expressing Cx43 in which the C-terminus had been deleted (C6-Cx43Δ244-382GFP) was not significantly altered compared to control cells, however the exogenous expression of full length Cx43 significantly enhanced directional motility compared to control and C6-Cx43Δ244-382GFP cells which suggests that the Cx43 C-terminus plays an important role in enhancing directional motility in homocellular cell populations *in vitro*. As the C6-Cx43Δ244-382GFP cells exhibited dye coupling, it is possible that the observed disturbances in differences in directional motility may be attributed to GJIC. The use of blockers throughout the motility experiments using C6-Cx43Δ244-382GFP cells is essential to rule out the possibility that the Cx43 C-terminus is involved in glioma invasion independently of gap junction channel formation.

4.5 INVOLVEMENT OF THE Cx43 C-TERMINUS IN CHEMOKINESIS

Moorby et al. (2000) recognized that low-molecular weight molecules permeable to gap junctions may account for the reduction in motility they observed in mouse 3T3 A31 fibroblasts expressing a communication-competent, truncated Cx43 mutant (Cx43-256M, described in (Moorby and Gherardi, 1999) compared to wild type 3T3 A31 cells which normally express abundant Cx43 and have a high basal level of GJIC (Moorby, 2000). The expression of Cx43-256M prevented PDGF-induced inhibition of GJIC (Moorby and Gherardi, 1999) and also prevented enhanced chemokinesis in response to PDGF (Moorby, 2000). Control cells, on the other hand, exhibited a significant increase in chemokinesis upon PDGF stimulation and

resultant impairment of GJIC. Conversely we did not see changes in chemokinesis upon blocking GJIC with CBX. While CBX is a non-specific gap junction inhibitor, its inactive analog, GZA, offers a control in addition to the untreated condition. In contrast PDGF has an overwhelming number of effects in addition to blocking GJIC which may confound the interpretation of abolishing GJIC. Chemokinesis was not altered, however, in cells expressing Cx43-256M compared to wild-type 3T3 A31 cells which is consistent with our finding that C6 cells expressing Cx43Δ244-382GFP did not exhibit altered chemokinesis compared to C6-Cx43GFP cells. The C-terminus truncation of Cx43 alters GJIC yet neither our study nor Moorby et al. (2000) were able to detect alterations in chemokinesis as a result of this truncation. Furthermore, as our results, which directly examine the consequence of blocking Cx43 channels, did not indicate a change in chemokinesis, it is likely that GJIC is not involved in regulating chemokinesis of C6 cells.

While the results of Lin et al. suggest that the Cx43 C-terminus in the absence of GJIC does not enable parenchymal invasion, the chimeric Cx40*43C3 protein employed in their studies may not accurately mimic the function of endogenous Cx43 protein sufficiently for the proper deployment of downstream Cx43 signaling cascades which may be independent of Cx43 gap junction formation. Although it is true that the majority of Cx43 interacting proteins for which Cx43 binding sites are known to associate with the C-terminus of Cx43, the specific sites of interaction for many associating proteins remains to be determined. Similarly, the interaction sites of other Cx43-associating proteins are not known. Furthermore, the functions of the Cx43 C-terminus may depend on interactions with the Cx43 intracellular loop domain (Seki et al., 2004). The use of the non-conducting Cx43 mutant which possesses an intact C-terminus (Lin et al., 2003b) would likely better elucidate the contribution of GJIC to Cx43-mediated mechanisms of glioma invasion in these studies.

4.6 THE INVOLVEMENT OF THE Cx43 C-TERMINUS IN CHEMOTAXIS

In response to a lesion stimulus in wound healing assays, Moorby et al. (2000) observed decreased chemotaxis in cells expressing truncated Cx43. Similarly, we found a significant difference in chemotaxis in cells expressing full length Cx43 compared to cells expressing truncated Cx43. However, we did not observe a significant difference in chemotaxis between C6GFP and C6-Cx43Δ244-382GFP cells. Only Moorby et al. (2000) and the current study have directly examined the consequence of Cx43 C-terminus truncation on motility, albeit using different constructs in different cells (Cx43-256M and Cx43Δ244-382GFP in 3T3 A31 and C6 cells, respectively). This present work does not aim to uncover the molecular mechanisms by which chemotaxis and chemokinesis are distinct from one another, but rather recognizes that the parameters employed in this study to investigate motility are not equal in that they measure different aspects of cell motility which have been classified as chemotaxic or chemokinetic. However, because neither study was able to detect significant differences in the random motility of cells expressing full length Cx43 or truncated Cx43, it suggests that cells expressing Cx43 which lacks the C-terminus may not express or may not be able to recruit the necessary cellular machinery required for directional motility imposed by lesion stimulus. Indeed C6-Cx43Δ244-382GFP cells migrated away from the lesion as a collective sheet unlike C6-Cx43GFP cells which did so as detached, individual cells. Although we found no differences in the total expression or sub-cellular localization of either N-Cad or p120ctn, analyzing these parameters under lesioned conditions may yield different results. Additional support for the hypothesis that the Cx43 C-terminus is involved in chemotactic response comes from the comparison of C6-Cx43shRNAscrambled and C6-Cx43shRNA-1 cells in the present study. Significant differences existed between these cells for both chemotaxis and chemokinesis. As differences in random motility were greater than differences in directional motility, it suggests that the response to the lesion stimulus may be similar in these cells, perhaps owing to the intact Cx43 C-terminus in

both cell types. Indeed Cx43 has been shown to regulate polarized cell movement essential for directional migration in cardiac neural crest cells (Xu et al., 2006). Whether this proposed scenario would be mediated via a gap junction dependent or independent mechanism can not be ascertained from the experiments conducted in our study or those of Moorby et al. (2002).

4.7 THE ROLE OF Cx43 IN MOTILITY COULD BE CELL-TYPE SPECIFIC

Although the majority of studies investigating the role of Cx43 in cell motility and/or invasion have found that Cx43 correlates positively with these processes, several studies have found an inverse correlation. Using MD-831 breast carcinoma cells in transwell migration chambers, Shao et al. (2005) observed reduced chemokinesis upon Cx43 knockdown by shRNA. Qiu et al (2003) found increased rate of wound closure upon Cx43 knockdown by siRNA while Brandner et al. (2004) noted persistence of Cx43 at the margins of non-healing wounds and a loss of Cx43 staining at wound margins during initial stages of wound healing (Brandner et al., 2004). Indeed wound closure in Cx43KO mice was realized more quickly compared to wild-type controls (Kretz et al., 2003). These studies compliment one another and together indicate that the role of Cx43 in cell motility may be cell-type specific.

4.8 CONCLUSIONS AND PHYSIOLOGICAL RELEVANCE

In conclusion, the majority of studies indicate that Cx43 expression correlates positively with chemotaxis. *In vitro* assays indicate a significant role for the Cx43 C-terminus in this process; however the mechanism by which it might be achieved has not been investigated. The use of gap junction blockers has not yet provided a clear indication of whether GJIC is involved in Cx43-mediated chemotaxis. No consensus has been reached for the role or mechanism of Cx43 with respect to chemokinesis, although the study by Shao et al. (2005) is the only study to date that has indicated altered chemokinesis as a result of Cx43 expression. While heterocellular coupling may facilitate glioma invasion, decreased Cx43 expression attenuates invasion in homocellular populations and therefore heterocellular coupling may not be a requirement for

Cx43-mediated invasion. This finding is particularly relevant as it implies that cells in the tumor core, a homocellular environment, may exhibit increased exodus concomitant with increased expression of Cx43. As numerous growth factors and cytokines to which cells in the tumor core may be exposed have been reported to upregulate Cx43 (Ozog et al., 2002), such an implication offers to reconcile the apparent paradox that while Cx43 expression is downregulated in high grade gliomas which are notoriously invasive, reconstitution of Cx43 to levels typical of normal astrocytes enhances motility.

4.9 FUTURE DIRECTIONS

4.9.1 Non-conducting Cx43 mutants could be used to evaluate the role of GJIC in glioma motility and/or invasion. These experiments could be performed using transwell chambers in the presence of a chemotaxic stimulus to differentiate between chemokinesis and chemotaxis, or alternatively in *ex vivo* assays (below).

4.9.2 The tumor environment may well contribute to tumor phenotype. The use of *ex vivo* brain slice invasion assays from $Cx43^{-/-}$ mice could be employed to evaluate the role of Cx43, and possibly GJIC, in the stromal environment during glioma invasion.

4.9.3 C6-Astrocyte co-culture experiments could be undertaken by plating both C6 cells and astrocytes in the same culture dish. A wound inflicted such that the migrating cells collide with one another would allow analysis of whether the Cx43 levels in the C6 cells change upon forming heterocellular contacts. (Note that C6 cells can be differentiated from astrocytes as C6 cells stain positively for vimentin while astrocytes do not).

4.9.4 The use of Cx43 C-terminal mutants to explore the interaction of Cx43 with Cx43 associating proteins may indicate which Cx43-associating proteins are involved in Cx43-mediated motility.

4.9.5 Protein analysis (ICC, Western, Protein Array) of cells at the wounded edge compared to cells in the confluent sub-marginal marginal zone.

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