The Role of the Gap Junction Protein Connexin43 in Glioma Migration and Invasion

by

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Abstract

Glioblastoma Multiforme (GBM), an aggressive form of adult brain tumor, is difficult to treat due to its invasive nature. A molecular change frequently observed in GBM is a decrease in the expression of the gap junction protein Connexin43 (Cx43); however, how a reduction in Cx43 expression contributes to glioma malignancy is still unclear. The first objective of this thesis was to establish an in vitro human GBM cell model to clearly delineate the role of Cx43 in the migration and invasion phenotype. Characterization of a panel of immortalized high grade human GBM cell lines showed variability in Cx43 protein expression, subcellular localization, gap junctional coupling and migration.

For the second objective of my thesis I selected the human GBM cell line U118 from the aforementioned panel and developed a 3D spheroid migration model that mimics the in vivo architecture of tumor cells to quantify migration changes. Down-regulation of Cx43 expression increased migration by reducing cell-ECM adhesion. Using live imaging my findings are the first to show that glioma cells change their migration pattern from collective to single cell when Cx43 is reduced. In addition, reducing Cx43 expression enhanced relative migration by increasing the cell speed and affecting the direction of migration. Subsequently, gap junction intercellular communication (GJIC) played a more prominent role in mediating migration than the cytoplasmic interactions of the C-terminal tail. Taken together my findings reveal an unexplored role of Cx43 in facilitating collective glioma migration.

The third objective of this thesis was to assess the role of homocellular and heterocellular gap junctions in glioma invasion using a syngeneic in vivo mouse model. A reduction in invasion was observed when we reduced Cx43 in mouse GL261 glioma cells and deleted it in host astrocytes. Interestingly, blocking the channel in GL261 did not decrease invasion. In summary,
a reduction in homocellular gap junction communication increases migration of glioma cells in vitro however when they encounter astrocytes in the brain a lack of heterocellular gap junction communication reduced invasion. This suggests that gap junctions may have opposing roles when formed between glioma cells versus when formed between glioma and astrocytes.
Chapter 2: Characterization of human glioma cell lines

All of the experiments in Chapter 2 were carried out by me with the exception of experiments presented in Figure 2.2. The experiments in Figure 2.2 were carried out by Dr. Moises Freitas Andrade. The remainder of the experiments were designed by me under the guidance and mentoring of Dr. Wun Chey Sin and my supervisor. The impetus for characterizing a panel of human glioma cell lines was to obtain a suitable model cell line to examine Cx43 mediated migration in vitro. The data obtained from human glioma cell line LN229 was published with a visiting MSc student Paul Gielen, Department of Tumor Immunology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, in the journal Neuropharmacology.


Chapter 3: Reduction in gap junction communication increases glioma migration

All of the experiments in Chapter 3 were carried out by me. The experiments were designed by me under the guidance and mentoring of Dr. Wun Chey Sin and my supervisor. The objective for this project was to use a U118 human glioma cell line and 3D spheroids to decipher the role of Cx43 in glioma migration. This chapter is in press in Oncotarget. The manuscript was written entirely by me with editing provided by Dr. Sin and my supervisor.

Chapter 4: Characterizing the role of Cx43 channel function in invasion in vivo

This chapter was adopted from the following manuscript which has been reviewed pending publication. All breeding and animal procedures were approved by The University of British Columbia Animal Care Committee (Protocol No: A09-0847 to the Naus Lab) and performed in accordance with the guidelines established by the Canadian Council on Animal Care. Dr. Sin was the first author on the manuscript. The intracranial mouse surgeries were performed by John Bechberger. I am the second author on this paper, and my exact contributions are listed below.


- My role in this project was to study the Cx43 channel in glioma cells in vivo.
- I generated the GL261 cell line expressing the Cx43 T154A mutant and its control. This mutant has been used in human glioma cell lines to study the Cx43 channel but this was the first incidence of it being used in a mouse glioma cell line.
- I carried out Western blot and immunofluorescence experiments to examine the expression and localization of T154A mutant.
- I carried out the preloading assay to quantify the GJIC for GL261 wildtype and GL261 cells expressing Cx43 T154A. Data analysis was performed by me as well.
- I modified and optimized the preloading assay to quantify heterocellular gap junctional coupling between GL261 cells and astrocytes. Data analysis was performed by me as well.
- The cell lines were injected intracranially into the mice by John Bechberger.

- I carried out immunohistochemistry experiments on fixed brain slices, and obtained images for quantification by confocal microscopy.

- I used Image J analysis to quantify invasion. I interpreted the data I produced.

- I wrote the methods for the experiments I carried out. I wrote the results for the data that was acquired by me, and contributed to the relevant portions of the Discussion. Both of these sections were modified by Dr. Sin and my supervisor.

- I was quite involved in the writing and editing process for this manuscript.

- I made the figures pertaining to my experiments for the paper, which were modified by Dr. Sin.

- Experimental data in Figure 4.1 panels B-C were obtained by me. Panels A, D-G were obtained by Dr. Wun Chey Sin.

- Experimental data in Figure 4.2 panels A-D was obtained by me and F-I was obtained by both me and Dr. Wun Chey Sin. Panel E was obtained by Dr. Wun Chey Sin.
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<thead>
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg</td>
<td>Micrograms</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>µm²</td>
<td>Micrometer squared</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Asn</td>
<td>Asparagine</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>ATP</td>
<td>Adenosine 5’-triphosphate</td>
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<tr>
<td>BCA</td>
<td>Bicinchoninic assay</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>C</td>
<td>Celsius</td>
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<tr>
<td>C-</td>
<td>Carboxy</td>
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<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>CBX</td>
<td>Carbenoxolone</td>
</tr>
<tr>
<td>CCN</td>
<td>Cyr61/connective tissue growth factor /nephroblastoma-overexpressed</td>
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<tr>
<td>Cdc42</td>
<td>Cell division control protein 42 homolog</td>
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<tr>
<td>CDKN2A</td>
<td>Cyclin dependent kinase inhibitor 2A</td>
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<td>CL</td>
<td>Cytoplasmic loop</td>
</tr>
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<td>Cl-</td>
<td>Chloride</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>CNTF</td>
<td>Ciliary neurotrophic factor</td>
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<td>c-Src</td>
<td>C-terminal Src</td>
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<td>C-terminus</td>
<td>Carboxy-terminus</td>
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<td>CTGF</td>
<td>Connective tissue growth factor</td>
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<td>Connexin</td>
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<td>Cxs</td>
<td>Connexins</td>
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<td>Da</td>
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<tr>
<td>DAPI</td>
<td>4’6-diamidino-2-phenyllindole</td>
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<td>DMEM</td>
<td>Dulbecco’s modified Eagle Medium</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
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<td>ERK</td>
<td>Extracellular signal related kinase</td>
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<td>FAK</td>
<td>Focal adhesion kinase</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>Flt1</td>
<td>Fms-related tyrosine kinase</td>
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<td>GABRA1</td>
<td>Gamma-aminobutyric acid A receptor 1</td>
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<td>GAPs</td>
<td>GTPase activated proteins</td>
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<td>GBM</td>
<td>Glioblastoma Multiforme</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
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<td>GEF</td>
<td>Guanine nucleotide exchange factors</td>
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<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>Gj</td>
<td>Gap junction genes in mouse</td>
</tr>
<tr>
<td>GJ</td>
<td>Gap junction genes in human</td>
</tr>
<tr>
<td>GJIC</td>
<td>Gap junctional intercellular communication</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
<td>GZA</td>
<td>Glycyrrhizic acid</td>
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<td>HCl</td>
<td>Hydrochloric acid</td>
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<tr>
<td>hr</td>
<td>Hour</td>
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<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
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<tr>
<td>IGEPAL</td>
<td>Octylphenoxy poly(ethyleneoxy)ethanol</td>
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<tr>
<td>INKα-ARF</td>
<td>Inhibitor of cyclin dependent kinase-Alternate reading frame protein</td>
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<td>IP₃</td>
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<td>K⁺</td>
<td>Potassium</td>
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<td>Molarity</td>
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<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<td>Mesenchymal</td>
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<td>min</td>
<td>Minute</td>
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<td>----------------------------------</td>
</tr>
<tr>
<td>ml</td>
<td>Milli liter</td>
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<td>MLCK</td>
<td>Myosin-light chain kinase</td>
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<td>Milli meter</td>
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<td>MMP</td>
<td>Matrix metalloproteins</td>
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<td>Microtubule organizing center</td>
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<td>N-</td>
<td>Amino</td>
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<td>NaCl</td>
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<td>NAD+</td>
<td>Nicotinamide adenine dinucleotide</td>
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<td>NEFL</td>
<td>Neurofilament light polypeptide</td>
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<td>NF1</td>
<td>Neurofibromin 1</td>
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<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
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<td>PECAM1</td>
<td>Platelet/endothelial cell adhesion molecule 1</td>
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<td>pH</td>
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<td>PI3K</td>
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<td>PKC</td>
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<td>Retinoblastoma</td>
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<td>Rho</td>
<td>Ras homolog gene family</td>
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<td>Radioimmunoprecipitation assay buffer</td>
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<td>ROCK</td>
<td>Rho-associated protein kinase</td>
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<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>shRNA</td>
<td>Short hairpin ribonucleic acid</td>
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<td>siRNA</td>
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<td>Sarcoma kinase</td>
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<td>SYT1</td>
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<tr>
<td>WASP</td>
<td>Wiskott-aldrich syndrome protein</td>
</tr>
<tr>
<td>WAVE</td>
<td>WASP-family verprolin-homologous protein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zona Occludens-1</td>
</tr>
</tbody>
</table>
Acknowledgements

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Dedication

I dedicate my thesis to my incredible parents (Ammi and Abbu). You instilled in me the value of hard work and education and taught me that I can do anything I put my mind to. You have demonstrated by example how to work hard and persevere to achieve one’s goal. You took on the challenge of immigrating to a new country not once but three times just so your kids can have a brighter future. You faced the hardships of being immigrants with integrity and determination. Whenever I start to doubt myself I use you as my inspiration to keep going. The sacrifices you have made so I can have a better life are countless. The words ‘thank you’ doesn’t do justice to the gratitude I feel. I could not have made it this far without the two of you…I love you!
Chapter 1: Introduction

1.1 Gap junctions

1.1.1 Discovery of gap junctions

By the middle of the 20th century, the cell was believed to be a ‘self-contained’ entity with an external surface that behaved as a continuous diffusion barrier [1]. Indeed electron micrographs of cells revealed a continuous surface membrane in various cells and thus it was believed that the cell membrane served to be a continuous diffusion barrier of hydrophilic molecules where only the smallest inorganic ions could pass via channels in the membrane [1]. However this was not found to be true for all cells, especially cells of organized tissues, where the barrier proved to be incomplete at cell to cell contact sites. In fact in 1952 the findings of a low core resistance in Purkinje fibers of the heart, which are organized in a series along the fiber axis, demonstrated that there must be an incomplete barrier [2]. In addition, in 1959 Furshpan and Potter reported electrical signals transmitted directly between cells in the nerve synapse of crayfish motor neurons [3]. This observation was contrary to the unidirectional action potential generally observed at synapses.

At the time these findings came to light the idea of channels that linked adjacent cells was met with criticism; it was postulated that the electrical transmission that was observed in the aforementioned studies could be due to having a high density of non-junctional membrane channels at the site of transmission. It was not until 1964 when Loewenstein and Kanno demonstrated that a large hydrophilic molecule called fluorescein could pass from one epithelial
cell to another [4, 5]. This molecule was significantly larger than the organic ions that transmit electrical signals in nerves and muscle fibers. The authors reported passage of molecules from 300 -1000 Da between cell junctions; molecules of 100,000 Da or greater were unable to pass through [4, 5]. Most of these molecules were unable to enter nonjunctional cells. In addition, the authors also observed the cell-cell junctions to be “rather leak-proof”, in that the dyes were unable to leak out of cells at junctions, leading them to hypothesize the intercellular channels were insulated [6]. From these findings the authors concluded that “in passing from cell to cell, the molecules take a specialized route through the cell membranes at the junction” [6]. These intercellular channels found at cell-cell junctions would later be termed gap junctions.

Several advances were made to understand the physical and chemical nature of gap junctions following the initial studies that identified them. Electrophysiology and dye tracer experiments furthered the understanding of how the pore formed in gap junctions. X-ray diffraction, electron microscopy and chemical studies of the mouse liver gap junction gave insight about the structure of gap junctions [1]. Thus the concept of what a junctional channel would look like in a cell and how it behaved evolved significantly between 1960-1980 (Figure 1.1).
Figure 1.1: The evolution of the gap junction channel concept (1960-1980).

A. This model of the gap junction incorporated structural attributes of the membrane channel. It demonstrates that two matching transmembrane channels from each cell connect and allow bi-directional exchange of molecules. It demonstrates that the insulation is provided by the docking of the two transmembrane channels at the intercellular junction. B. This model inferred structural information obtained from X-ray diffraction, electron microscopy and fluorescent tracer studies of the mouse liver gap junction. It shows that the individual transmembrane channels are formed by hexamers that are tightly joined. This schematic was taken from [1] with the permission of ©Elsevier.

1.1.2 Connexins

We now know that gap junctions are a common way for cells to communicate with each other; cell-cell communication mediated by gap junctions is called gap junctional intercellular communication (GJIC). There are two large families of transmembrane proteins that form gap junctions; invertebrate gap junctions are comprised of proteins called innexins and vertebrate gap
junctions are comprised of connexins (Cx) [7, 8]. Although these two families of proteins are not similar in their amino acid sequence they do exhibit structural convergence [7, 8].

1.1.2.1 Nomenclature

There are 20 members in the connexin family in the mouse genome and 21 in the human genome [9, 10]. There are two different nomenclature systems for Cx proteins, the first one is based on the molecular weight of a single subunit; i.e., connexin43 (Cx43) is a 43 kDa isoform. The other nomenclature system groups the connexin genes by sequence identity and length of the cytoplasmic loop (α, β, and γ) and numbers them in the order they were discovered. For example Gjα1 for Cx43, as this was the first Cx discovered in the α group (‘Gj’ is abbreviation for gap junction) [10]. The molecular weight nomenclature is widely used and will be in this work as well. A list of the different Cxs and their respective genes in the mouse and human genomes are presented in Table 1.1.
<table>
<thead>
<tr>
<th>Mouse</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx protein</td>
<td>Cx gene</td>
</tr>
<tr>
<td>Cx23</td>
<td>Gje1</td>
</tr>
<tr>
<td>Not found in mouse</td>
<td>Not found in mouse</td>
</tr>
<tr>
<td>Cx26</td>
<td>Gjb2</td>
</tr>
<tr>
<td>Cx29</td>
<td>Gjc3</td>
</tr>
<tr>
<td>Cx30</td>
<td>Gjb6</td>
</tr>
<tr>
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<td>Gjd3</td>
</tr>
<tr>
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<td>Gjb4</td>
</tr>
<tr>
<td>Cx31</td>
<td>Gjb3</td>
</tr>
<tr>
<td>Cx31.1</td>
<td>Gjb5</td>
</tr>
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<td>Cx32</td>
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<tr>
<td>Cx33</td>
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</tr>
<tr>
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<td>Gja8</td>
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<td>Not found in mouse</td>
</tr>
<tr>
<td>Cx57</td>
<td>Gja10</td>
</tr>
</tbody>
</table>

Table 1.1: This table presents the nomenclatures of connexin genes in mouse and human genomes and their corresponding proteins.
The gene nomenclature is based on evolutionary relationships between the connexins, while the protein names are based on the molecular weight of the protein. The information presented in this table was acquired from NIH GenBank database.

1.1.2.2 Structure

Each Cx has four hydrophobic transmembrane domains that are connected by two extracellular loops. The N- and C-termini of connexin and a cytoplasmic loop that connects the second and third transmembrane domains extend into the cytoplasm (Figure 1.2). The two extracellular loops have six conserved cysteine residues, in the first loop [C-X₆-C-X₃-C] and the second loop [C-X₅-C-X₅-C]. These cysteines form intraconnexin disulfide bonds which aid in the docking of gap junctions [11, 12]. The transmembrane domains, extracellular loops, and the N-terminus of Cx isoforms are conserved among species, whereas the carboxy-terminus (C-terminus) and cytoplasmic loop show variation in sequence and in length. Six connexins oligomerize to form a hemichannel, and when a hemichannel from one cell connects to a hemichannel in another cell a gap junction is formed (Figure 1.2) [9, 13].
Figure 1.2: Schematic of the molecular structure of gap junctions.

Monomeric Cx molecules have four transmembrane domains, two extracellular loops, N-terminal (NT), cytoplasmic loop (CL), and a C-terminal tail (CT). Six connexins oligomerize to form a connexon or hemichannel. Docking of hemichannels between adjacent cells forms gap junctions.

Given that overlapping expression patterns of various Cxs have been observed in different cells and tissues, the type of gap junction formed depends on the Cxs expressed in the given cell. The variation in gap junction configuration can occur at the monomeric Cx state and at the hexameric hemichannel state. A hemichannel can consist of the same Cx (homomeric) or a combination of two or more different Cxs (heteromeric) (Figure 1.3). Consequently, a gap
junction can be composed of the same Cxs (homotypic) or different Cxs (heterotypic). For example, a gap junction consisting of only Cx43 is a homomeric, homotypic gap junction. Therefore the 20-21 Cx isoforms in mammals allows for substantial combinatorial variety in gap junction composition [14-20].

![Figure 1.3: Different combinations of gap junctions.](image)

A. Different Cx isoforms are shown in red and yellow. B. Homomeric hemichannels/connexons consists of the same Cx isoform while heteromeric connexons consist of different isoforms. C. Examples of different types of gap junctions that can form.
1.1.3 **Physiology**

Gap junctions exhibit voltage-dependent gating irrespective of the type of gap junction formed (homotypic or heterotypic) [21]. Parameters such as voltage dependence, and chemical or pH gating have been useful in determining the open probability of gap junctions. The amount of time a channel remains open versus closed is what determines the open probability of a channel; these measurements are made under steady state conditions at different voltages and pH conditions [21]. Cx43 gap junctions show very high open probabilities in the range of 0.6-0.9 at a transjunctional voltage of 40 mV and pH 6.8, suggesting that gap junctions are open under a wide range of intracellular conditions [21, 22]. Amino acid side chains that line the pore of the Cx43 channel provide selectivity and therefore dictate what passes from cell to cell. Homotypic Cx43 gap junctions can pass molecules that are <1.2 kDa in size; this includes ions such as K\(^+\), Ca\(^{2+}\), Cl\(^-\), inositol triphosphate, glucose, nucleotides, small interfering RNA, microRNAs, amino acids and even small peptides [21, 23-26]. This becomes complicated when the gap junctions are heterotypic, consisting of more than one Cx. In a heterotypic gap junction the two different Cxs contribute different side chains to the pore and thus create a very different channel from that of a homotypic gap junction. This complexity presents a way of controlling what passes through the gap junction channel.

1.1.4 **Gating the gap junction channel**

Various gating mechanisms have been suggested to control the gap junction channel. A rise in intracellular calcium has been shown to have an inhibitory effect on gap junction
channels indirectly via the calmodulin signaling pathway [27]. In addition, a drop in intracellular pH facilitates dimerization of the adjacent Cx C-terminal tail within a hemichannel/connexon, facilitating an interaction with the cytoplasmic loops rendering the channel blocked [28, 29].

Gap junctions display a property common to other ion channels in that the conductance is sensitive to voltage. This is referred to as voltage gating and this mechanism plays an important role in modulating gap junction channels. All Cxs can sense transjunctional voltage (Vj) changes between coupled cells [30]. Unopposed hemichannels or hemichannels that comprise gap junctions have two molecularly different Vj-sensitive gating mechanisms that regulate the channel. These gating mechanisms are termed ‘fast’ and ‘slow’ [31]. The fast gating mechanism is sensitive to Vj and elicits fast gating transitions between the open state and the residual conductance state. The slow gating mechanism is sensitive to Vj and the following conditions transmembrane voltage (Vm), intracellular Ca2+ levels, and pH, chemical uncouplers and gap junction channel opening during de novo channel formation [31]. Gap junctions composed of different Cxs differ significantly in sensitivity to Vj and chemical gating. Although it is unclear how heterotypic gap junctions are regulated by Vj, studies on homotypic gap junctions have given us insight about the basic characteristics of how gap junctions may be gated [31].

1.1.5 **Hemichannels**

Cx hemichannels are hexameric structures oligomerized in the trans-Golgi network and subsequently delivered to the plasma membrane. Once at the plasma membrane, hemichannels will dock with hemichannels on adjacent cells forming gap junctions. Hemichannels are delivered at the periphery of existing gap junction plaques, and older plaques are degraded from
the center to make room for new gap junctions (Figure 1.4) [32-34]. Gap junction formation time can vary from 3-30 minutes [35]; once formed gap junctions cluster at high density and form structures called ‘gap junction plaques’ that exhibit fast turnover times (1-5 hours half-life) [36-40].
Figure 1.4: Gap junction formation.

Unattached hemichannels are delivered to the plasma membrane. At a cell-cell junction, hemichannels from adjacent cells dock to form gap junctions. Recent studies have suggested that hemichannels can communicate with the extracellular surrounding. This image was taken from [41] with the permission of © Macmillan publishers.
Up until the early 2000s hemichannels were believed to be non-functional transitional structures in the formation of gap junctions. This changed with studies proposing a new role for hemichannels, one in which the hemichannels weren’t mere intermediates but functional channels that could communicate with the extracellular environment. Since gap junctions are quite permeable, it can be assumed that opening of an undocked hemichannel would affect the integrity of the electrochemical gradient leading to cell death [42]. In fact, one of the first compelling studies for functional hemichannels showed that Cx46 transfected oocytes underwent lysis unless an osmolyte was added to the medium [43]. Subsequent studies found that cell lysis could be prevented by increasing the extracellular concentration of divalent ions such as Ca$^{2+}$ [42, 44, 45]. Hemichannels have been shown to open in response to mechanical stimuli, drops in extracellular Ca$^{2+}$, fluctuations in pH and redox potential [46, 47]. Thus it was found that functional hemichannels existed under specific conditions.

Manipulation of physiological conditions such as membrane potential, inflammation and metabolism have also been shown to influence hemichannel activity. Overexpression of Cx43 in HeLa cells resulted in hemichannel activity under conditions where membrane depolarization was above +20mV. The authors found the single channel conductance to be twice as much as the conductance of Cx43 gap junctions [48]. Some groups have observed functional hemichannels under pathological conditions such as inflammation. Treatment of cultured astrocytes with microglia conditioned media showed an increase in hemichannel activity; interestingly, treatment with the media also caused a reduction in total and surface Cx43 and the coupling between astrocytes was reduced as well [49]. An increase in hemichannel activity in conjunction with a decrease in GJIC was also observed in astrocytes following metabolic inhibition [48-50].
In general, hemichannels have twice the conductivity of their respective gap junction due to the reduction in the length of the channel by half. Functional hemichannels have been reported under specific physiological and pathological conditions [51]. However, the findings of functional Cx hemichannels are met with caution mainly due to the discovery of another protein that exhibits hemichannel-like properties, the pannexins.

1.1.6  **Pannexins**

In 2000 a new family of transmembrane channel proteins called pannexins (Panx) was discovered by Panchin and colleagues. Panxs were identified to be homologs of innexins, the invertebrate gap junction proteins [7]. Although Panxs do not share similarity with Cxs in their primary amino acid sequence [52], they do possess structural similarities to Cxs. Indeed, similar to Cxs, Panxs have four transmembrane domains, two extracellular loops, a cytoplasmic loop, N-terminus and C-terminus, however instead of six extracellular cysteine residues they only have four [53]. To date three members of the Panx family have been identified, Panx1, Panx2, and Panx3 [53]. Interestingly, Panx1 has been preserved in the teleosts as two independent ohnologs due to whole genome duplication [54].

Panx1 has been shown to form hexamers similar to Cxs, however unlike the Cxs, Panx1 undergoes N-glycosylation on its extracellular loops. Site-directed mutagenesis has shown that glycosylation at Asn-254 may influence trafficking to the plasma membrane [55]. The steric hindrance created by glycosylation of the extracellular loops prevents Panx1 hexamers from docking with each other to form gap junctions. Indeed electron micrographs show that Panx1 is dispersed all over the plasma membrane at non-junctional sites and in intracellular vesicles; a
lack of intercellular communication was also observed in cells over-expressing Panx1 [55]. The oligomerization of Panx2 is different from Panx1 in that it forms octamers rather than hexamers [56]. The N-glycosylation site for Panx2 is predicted to be Asn-86 on the first extracellular loop of the protein. Treatment with N-glycosidase enzyme clearly showed a band shift indicating that the protein is indeed glycosylated [57]; site-directed mutation analysis has not been done to confirm the site of glycosylation. The oligomerization of Panx3 has not been reported yet, however it is predicted to form hexamers given its amino acid sequence similarity to Panx1 [53]. Similar to Panx1, Panx3 has also been reported to undergo N-glycosylation; however the glycosylation occurs in the first extracellular loop of the protein. Site-directed mutagenesis suggested that the glycosylation occurs at Asn-71, and is important for localization to the plasma membrane [58].

Panxs are suggested to form channels with large pores that communicate either with the extracellular or intracellular environment. The steric hindrance caused by the N-glycosylations prevents Panx from forming gap junctions. Interestingly, upon enzymatic digestions of the N-glycans on the cell surface Panx1 proteins were able to form functional gap junctions [59]. Since under normal physiological conditions Panx1 proteins would be glycosylated, the steric hindrance would not permit formation of Panx1 gap junctions in vivo [54]. In contrast, Panx1 was also reported to form gap junctions when it was over-expressed in C6 glioma cells; this was suggested to be due to a weak passage of sulforhodamine 101 between cells [54, 60]. Although the aforementioned studies suggested a role for Panx in intercellular communication, one of the first studies on the channel function of Panx1 demonstrated that it formed non-junctional transmembrane channels in Xenopus oocytes [53, 54, 61]. Unitary conductance of Panx1
channels was reported to be 550 pS, much larger than the unitary conductance of any of the Cxs [53]. Dye uptake assays have indicated that all Panxs can form single membrane channels [57]. Panxs have been implicated in both developmental and pathological processes; with respect to this thesis I will discuss the role of Panxs in gliomagenesis.

Over-expression of Panx1 in C6 glioma cells decreases the rate of proliferation, motility and anchorage-independent growth [60]. A change in cell morphology was observed in that cells over-expressing Panx1 were flatter, suggesting that Panx1 expression could be influencing the cytoskeleton; it should be noted that this observation was not quantified [60]. In a nude mice assay a reduction in in vivo tumor growth confirmed the in vitro reduction of cell proliferation and anchorage independent growth [60]. The same phenotype was observed when Panx2 was over-expressed in C6 glioma cells [62]. Both Panx proteins suppressed oncogenic traits such as cell proliferation, motility and anchorage independent growth suggesting that Panxs exhibit tumor-suppressive properties.

1.1.7 Cx43 functions

A decrease in Cx43 expression has been observed in GBM tissues and more clarification is needed to understand the significance of this association [63, 64]. The focus of this thesis was to examine if and how Cx43 modulates glioma migration and invasion. To reveal the mechanism of Cx43 mediated glioma migration we need to understand how the protein structure of Cx43 influences its functions. The different domains of Cx43 each serve a purpose that contributes to the functioning of the gap junction in a coordinated manner. Hemichannels assist the cells in communicating with their surrounding environment during mechanical or ischemic stress; they
modulate the passage of molecules such as ATP, glutamate, and NAD$^+$ into the extracellular space, producing different physiological responses [65]. The binding of two hemichannels to form a gap junction occurs via non-covalent interaction between the extracellular loops of adjacent hemichannels [66]; hence the extracellular loop provides adhesion which can assist cell aggregation [67, 68]. Recently it has been shown that this adhesive property of gap junctions is involved in neuronal migration to the cortical plate in the rat embryonic neocortex [68]. Gap junction activity is regulated by interaction with various proteins; in particular the carboxy tail of Cx43 has been shown to interact with structural proteins such as Zona Occludens-1 (ZO-1), drebrin an actin binding protein, β-catenin and N-cadherin, growth regulators such as Cyr61/connective tissue growth factor /nephroblastoma-overexpressed-3 (CCN3) and kinases such as Sarcoma (Src) and Protein Kinase C (PKC) [66, 69]. The C-terminal tail of Cx43 is also a site of post-translational modification throughout Cx43’s life cycle. It is predominantly phosphorylated on the serine residues [70-72], while some studies have also reported phosphorylation of tyrosine residues [73, 74]. Phosphorylation of the Cx43 is necessary for proper trafficking to the plasma membrane, gating the channel and degradation [75-77]. Several phosphorylation sites that are targets for specific kinases have been identified, which gives us an insight into the signaling pathways involved in regulating gap junctions [76] (Figure 1.5).
Figure 1.5: Phosphorylation sites in Cx43.

The different colours indicate the various kinases that are proposed to phosphorylate the C-tail.

This image was taken from [76] with the permission from © Elsevier.

1.2 Connexins and carcinogenesis

Several Cxs have been implicated in the process of carcinogenesis. In fact, the loss of gap junctions has been identified in cancer cells from various tissues and animal species [1, 41, 78-80]. One of the first observations implicating Cxs in cancer was made in 1966 when a lack of electrical coupling was observed in rat hepatomas [81, 82]. Lack of electrical coupling was also observed in cells from human carcinoma of the stomach [83]. Enhanced and unregulated growth is the most recognizable phenotype of solid tumors, and therefore it was hypothesized that the
lack of GJIC in cancer facilitates deregulated growth [1]. This hypothesis was substantiated by studies that demonstrated tumor promoting agents to be blockers of gap junctions [78, 84-86]. The well coupled cells of the liver make it a suitable model system to establish the relationship between reduction in gap junctions and carcinogenesis. Both Cx26 and Cx32 have been shown to be expressed in liver cells, and microinjections with gap junction permeable dyes have been used as a tool to monitor the decrease in GJIC as carcinogenesis progresses [87]. Interestingly, in human liver cancer, a decrease in GJIC is observed as carcinogenesis increases but this was not associated with a decrease in Cx32 expression; rather it was due to the aberrant localization of Cx32 in the cytoplasm [78, 88, 89].

Decrease in GJIC has also been observed in breast cancer. Specifically, Cx26 and Cx43 have been studied extensively. Cx26 was not expressed in normal tissue but was upregulated in breast carcinomas [90]. Cx43 showed heterogeneous expression in some of the carcinomas studied. A complete absence of Cx43 expression was found in lobular and ductal carcinomas regardless of the grade, suggesting Cx43 as marker for early breast carcinogenesis [91]. In addition, lung cancer cells obtained from primary tissue as well as cultured cell lines display a decrease in Cx43 expression [92, 93]. On the contrary, studies with chemically induced lung carcinomas have shown extensive Lucifer yellow dye (gap junction permeable dye) passage in late stage carcinomas. Loss of GJIC in carcinoma cells was only observed after several months of culturing [78, 94]. This discrepancy between studies highlights that the molecular changes that take place in a chemically derived carcinoma versus a naturally occurring carcinoma might be different.

The observation of decreased GJIC does not always correlate with a decrease in Cx expression. In fact, several studies have shown that aberrant localization of Cx proteins in the
cytoplasm versus intercellular junctions may be the cause of decreased GJIC. In skin cancer
Cx43 was found at intercellular junctions as small plaques and in cytoplasmic vesicles; normal
cells did not show similar intracellular localization [87]. A significant proportion of Cx26 protein
was found in cytoplasmic vesicles in human breast carcinoma and bladder tumors [78, 90, 95].
The aforementioned studies are just a few of many that implicate Cxs and therefore gap junctions
in carcinogenesis. However, it is hard to discern whether loss of GJIC is a prerequisite or a
consequence of the carcinogenesis process.

1.2.1 Glioblastoma multiforme (GBM)

Each year around 64,000 cases of primary central nervous system tumors are reported in
the United States, of which 38% are malignant. Gliomas make up 80% of malignant brain tumors
[96, 97]. Gliomas can arise through de-differentiation of glial cells such as astrocytes,
oligodendrocytes and ependymal cells or through the transformation of precursor cells in the
central nervous system [98].

1.2.1.1 World health organization (WHO) classification system

Gliomas are divided into astrocytoma, oligodendroma, and ependymoma based on the
cellular morphology of the tumor cells; the most commonly occurring gliomas are grade IV
astrocytomas called Glioblastoma Multiforme (GBM). Astrocytomas are divided into four grades
by the World Health Organization (WHO) with tumorigenicity increasing from grade I-IV [99].
The grades are divided based on histopathogical features, morphology, and genetic alterations.
Grade I tumors are benign and can be cured by surgical resection (if possible); grade II tumors are malignant and exhibit early ‘diffuse infiltration’ into healthy parts of the brain which makes them impossible to cure by resection; grade III tumors show increased anaplasia and proliferation and therefore can be quickly fatal; grade IV tumors are the most malignant exhibiting features such as vascular proliferation and necrosis (Table 1.2), proving unmanageable with standard surgery and radio/chemotherapy (death occurs within 12 months) [100]. In addition, after resection the tumor frequently recurs which, depending on the location of the tumor, could prove difficult to surgically resect. The different types of astrocytic tumors and their grades are summarised in Table 1.2. The focus of this thesis will be to study Cx43’s role in grade IV GBM migration.

<table>
<thead>
<tr>
<th>Astrocytic Tumors</th>
<th>WHO Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subependymal giant cell astrocytoma</td>
<td>I</td>
</tr>
<tr>
<td>Pilocytic astrocytoma</td>
<td>I</td>
</tr>
<tr>
<td>Pilomyxoid astrocytoma</td>
<td>II</td>
</tr>
<tr>
<td>Diffuse astrocytoma</td>
<td>II</td>
</tr>
<tr>
<td>Pleomorphic xanthoastrocytoma</td>
<td>II</td>
</tr>
<tr>
<td>Anaplastic astrocytoma</td>
<td>III</td>
</tr>
<tr>
<td><strong>Glioblastoma</strong></td>
<td><strong>IV</strong></td>
</tr>
<tr>
<td>Giant cell glioblastoma</td>
<td>IV</td>
</tr>
<tr>
<td>Glissarcoma</td>
<td>IV</td>
</tr>
</tbody>
</table>

Table 1.2. WHO classification of astrocytomas.
This table shows the different types of astrocytomas categorized by their WHO assigned grade. The focus of this thesis is Glioblastoma (yellow). This information was obtained from [99].

1.2.1.2 Molecular classification

GBM is a brain neoplasm and as its name implies it consists of a group of tumors that are genetically and phenotypically heterogeneous [101]. GBM cells display polymorphic morphology with cells being either polygonal or spindle shape and containing nuclei that are either oval or elongated in shape. The tumor generally consists of binucleated or multinucleated cells, in addition immune cells such as lymphocytes, neutrophils, macrophage and microglia can also be present [101, 102]. In addition the GBM present complex characteristics in many ways: 1. macroscopically with obvious signs of necrosis and hemorrhage; 2. microscopically with areas of ‘pseudopalisading necrosis, pleomorphic nuclei and cells and microvascular proliferation’[98]; 3. genetically with chromosomal rearrangements leading to deletions, amplification, and point mutations of important genes [98]. Most of the genetic aberrations lead to activation of signalling pathways that are downstream of receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR). As well deletions or mutations in genes such as Inhibitor of cyclin dependent kinase –alternate reading frame protein INKα-ARF, tumor protein 53 (p53) and retinoblastoma (Rb) can obstruct regulation of the cell cycle resulting in uncontrolled proliferation [98].

To add to the complexity GBMs can be subdivided into primary and secondary GBM subtypes with primary GBMs occurring at a greater frequency. Primary GBMs are generally observed in older patients while secondary GBMs generally occur in patients below 45yrs of age.
Primary GBMs come in to existence de novo with no evidence of prior lower grade pathology, whereas secondary GBMs originate and progress from lower grade astrocytomases (~70% of grade II gliomas transforming into grade III/IV within 5-10 yrs. of diagnosis) (Figure 1.6) [100].
Figure 1.6: Derivation of GBMs.

This schematic shows how the primary GBMs are derived de novo and the progressive pathway that generates secondary GBMS. It also shows the difference in genetic aberrations between the two subtypes. This image was taken from [100] with the permission of © Cold Spring Harbor Laboratory Press.

Although the derivation of the two subtypes of GBMs is different (Figure 1.6), morphologically and clinically they are indistinguishable; this is an obstacle in diagnosis and treatment of patients since histopathology is one of the main ways of classifying tumors. However with the advent of molecular approaches such as gene expression analysis by
microarray, in situ hybridization and quantitative PCR, the genetic composition of GBMs has been further distilled generating distinct molecular subclasses.

In 2006 Phillips et al [103] identified new prognostic molecular subclasses that were akin to the process of neurogenesis. These subclasses were derived from examining gene expression in high grade astrocytoma using 75 primary tissue samples and 39 RNA samples and correlating with survival time to establish prognostic value. The subclasses identified were proneural (PN), proliferative (Prolif) and mesenchymal (Mes) to represent the dominant characteristic of the gene list in each subclass; Prolif and Mes were classified as ‘poor prognosis tumor subtypes’ [103]. Upon recurrence, tumors shifted towards the Mes phenotype, indicating that recurring tumors were more aggressive than their PN predecessors. Mes subclass displayed an overexpression of angiogenesis markers such as vascular endothelial growth factor (VEGF), fms-related tyrosine kinase/vascular endothelial growth factor receptor 1 (flt1/VEGFR1), and the endothelial marker platelet/endothelial cell adhesion molecule 1 (PECAM1) [103]. As expected Prolif subclass had a higher proliferative index (determined by expression of Ki67) than PN and Mes subclasses. The more aggressive Prolif and Mes subtypes showed chromosomal aberrations such as gain in chromosome 7 and loss of chromosome 10. Subsequently, the two subtypes also lost the PTEN locus and exhibited EGFR amplification (Figure 1.7) [103]. These findings indicate that not all grade IV GBMs are equally aggressive or proliferative, which is contrary to the classification by WHO.
Figure 1.7: Summary of the GBM subtypes.

This table summarizes the characteristics of GBM subtypes: proneural, proliferative, and mesenchymal. This image was taken from [103] with the permission of © Elsevier.

<table>
<thead>
<tr>
<th></th>
<th>Proneural</th>
<th>Proliferative</th>
<th>Mesenchymal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histological grade</strong></td>
<td>WHO grade III or WHO grade IV with or without necrosis</td>
<td>WHO grade IV with necrosis</td>
<td>WHO grade IV with necrosis</td>
</tr>
<tr>
<td><strong>Cellular morphology</strong></td>
<td>Astrocytic or Oligodendrogial</td>
<td>Astrocytic</td>
<td>Astrocytic</td>
</tr>
<tr>
<td><strong>Evolution of signature</strong></td>
<td>Arises in 1° tumor, may persist or convert to Mes</td>
<td>Arises in 1° tumor, may persist or convert to Mes</td>
<td>Arises in 1° tumor or by conversion from other subtype</td>
</tr>
<tr>
<td><strong>Patient age</strong></td>
<td>Younger (~40 yrs.)</td>
<td>Older (~50 yrs.)</td>
<td>Older (~50 yrs.)</td>
</tr>
<tr>
<td><strong>Prognosis</strong></td>
<td>Longer survival</td>
<td>Short survival</td>
<td>Short survival</td>
</tr>
<tr>
<td><strong>Histological Markers</strong></td>
<td>Olig2, DLL3, BCAN</td>
<td>PCNA, TOP2A</td>
<td>CHI3L1/YKL40, CD44, VEGF</td>
</tr>
<tr>
<td><strong>Tissue similarities</strong></td>
<td>Adult and Fetal Brain</td>
<td>HSC, lymphoblast</td>
<td>Bone, cartilage, smooth musc, endothelium, dendritic cells</td>
</tr>
<tr>
<td><strong>Biological process</strong></td>
<td>Neurogenesis</td>
<td>Proliferation</td>
<td>Angiogenesis</td>
</tr>
<tr>
<td><strong>Analogous forebrain cell</strong></td>
<td>Neuroblast</td>
<td>Neural Stem Cell and/or Transit Amplifying Cell</td>
<td>Neural Stem Cell</td>
</tr>
<tr>
<td><strong>Chromosome gain/loss</strong></td>
<td>None</td>
<td>Gain of 7 &amp; Loss of 10 or 10q</td>
<td>Gain of 7 &amp; Loss of 10</td>
</tr>
<tr>
<td><strong>PTEN locus</strong></td>
<td>PTEN intact</td>
<td>PTEN loss</td>
<td>PTEN loss</td>
</tr>
<tr>
<td><strong>EGFR locus</strong></td>
<td>EGFR normal</td>
<td>EGFR amplified or normal</td>
<td>EGFR amplified or normal</td>
</tr>
<tr>
<td><strong>Signaling</strong></td>
<td>Notch activation</td>
<td>Akt activation</td>
<td>Akt activation</td>
</tr>
</tbody>
</table>

The aforementioned study also gave insight into the origins of GBMs by performing gene expression analysis of key neural stem cell markers and markers for committed neuronal lineage. The poor prognosis subclasses Prolif and Mes had higher expression of the neural stem cell
markers than the PN subclass. The authors suggest that GBMs may be derived from cells at several stages of differentiation from stem cell to glia or neuron; the phenotypic differences arise from the variation in signalling pathways rather than the cell of origin (Figure 1.8) [103-105].

**Figure 1.8:** Models showing parallel between GBM subtypes and stages in neurogenesis.

This model proposes a link between GBM subtypes and stages in neurogenesis based on differences in expression of neural stem cell markers versus neuroblast markers [103]. The Mes subtype is maintained in a more undifferentiated state such as neural stem cells. The Prolif subtype is similar to the transit amplifying cell and the PN subtype has a phenotype akin to the neuroblast stage of neurogenesis [103]. This image was taken from [103] with the permission of © Elsevier.
The recent establishment of The Cancer Genome Atlas (TCGA) Network has advanced the goal of generating a comprehensive catalog of genomic aberrations in GBMs. Verhaak et al. [106] were able to obtain 206 GBM patient samples to further characterize the molecular subtypes present in GBM. They identified two new subtypes, Classical and Neural, and gained new insights on the existing Mes and PN subtypes [106].

The Classical subtype had amplification in chromosome 7 paired with chromosome 10 loss. A high-level of EGFR amplification was seen in 97% of the Classical subtype. Deletion of p16INK4A and p14ARF occurred in conjunction with the EGFR amplification affecting the RB pathway [106]. The Neural subtype was classified based on high expression of neuronal markers such as neurofilament light polypeptide (NEFL), gamma-aminobutyric acid A receptor 1 (GABRA1), synaptotagmin (SYT1) and solute carrier family 12 (potassium/chloride transporter), member 5 (SLC12A5) [106]. PN had PDGFRA amplification and point mutations in the IDH1 gene. TP53 mutations and loss of heterozygosity was also observed in this subtype. The Mes subtype was characterized by deletion of the neurofibromin 1 (NF1) gene [106].

The identification of the different molecular subtypes of tumors that were all diagnosed as GBM highlights the heterogeneity of this disease. It gives insight into why the standard treatment for GBM (surgical resection + chemo/radiotherapy) does not cure the patient of the disease. Instead the patient’s tumor should be categorized in to a molecular subtype by carrying out gene expression analysis and whole exome sequencing. Once the subtype is identified a course of treatment that is effective for treating that subtype should be prescribed. In additions, establishing a prognostic correlation with the subtypes will aid in prescribing therapies more specific to the individual patients.
1.2.2 **Cx43 and glioma**

While the sequencing analysis mentioned earlier did not identify Cx43 as a marker for any of the subtypes, an association between decreased Cx43 expression and GBM has been demonstrated by many studies including our own (see section 4.3). In 1999 Huang et al used immunohistochemistry on 18 human glioma paraffin embedded tissues to demonstrate relatively high levels of Cx43 protein in normal brain and in grade I and II glioma [63]. In contrast, grade III had very low levels of Cx43 while grade IV (GBM) displayed undetectable levels of Cx43 [63]. A subsequent study by Soroceanu et al examined Cx43 expression levels in 11 human glioma samples also demonstrated an inverse relationship between Cx43 protein expression and glioma grade [64]. While the aforementioned studies examined Cx43 protein expression, Pu et al used Northern blot analysis to examine Cx43 mRNA levels in 44 freshly resected human glioma samples [107]. When compared to the lower grades and normal brain tissue grade IV GBMs had the lowest levels of Cx43 mRNA [107]. We examined the DNA copy number of Cx43 in 372 grade III/grade IV glioma from The Cancer Genome Atlas (TCGA) Network and found that in 11.3% of the tumors the Cx43 gene was deleted [108]. Interestingly, when we assessed the mRNA profile of the tumors (n = 424) we observed 27.3% of the tumors displayed a 2-fold down regulation of Cx43 and 20.3% of the tumors showed a 2-fold up regulation [108]. Recently, our lab performed immunohistochemistry (Appendix 4) on normal and glioma tissue microarray slides to quantify Cx43 protein expression in 474 primary human brain tumor tissue samples. We observed an overall reduction of Cx43 protein in the tumor core of grade III and grade IV gliomas (Appendix 5 A and B). Over the years multiple studies have shown an overall
decrease in Cx43 mRNA and protein expression in high grade human glioma tissues suggesting that decreasing Cx43 expression may contribute to the aggressive behavior of gliomas.

1.2.2.1 The effect of Cx43 expression on cell growth

As early as the 1960s the deficiency in GJIC in cancer was hypothesized to regulate cell growth, and in fact this is exactly what was shown in the initial studies with rodent glioma cell-based models (1990s). The rat C6 glioma cell line, derived following treatment with a chemical mutagen nitrosourea, displayed astrocytic characteristics such as expression of glial fibrillary acidic protein and glutamine synthetase [109-111]. C6 cells also expressed very low levels of endogenous Cx43 protein which was reminiscent of high grade glioma. Successful overexpression of Cx43 in this cell line showed an increase in GJIC and decrease in cell growth. In fact, a negative correlation between Cx43 expression and cell growth was established using C6 clones of varying Cx43 protein expression [112]. Intracerebral implantation of C6 cells overexpressing Cx43 in rats formed smaller tumors than non-transfected C6 cells, further confirming the relationship between Cx43 and cell growth [113]. Overexpression of Cx43 in high grade human glioma cell lines U251 and T98G also reduced their growth rate; this was attributed to the extended G1 phase in cells overexpressing Cx43 [114]. In addition overexpressing Cx43 also altered the cell morphology to a flatter shape indicating that the cytoskeleton of the cell was affected [114].

The aforementioned studies in addition to studies in other cancers established Cx43’s influence on cell growth; however the exact mechanism is not very clear. The first insight on how Cx43 expression may be regulating growth was from co-culturing experiments, where non-
transfected C6 cells co-cultured with C6 cells overexpressing Cx43 (C6-Cx43) showed a reduction in cell growth. These cells were able to form functional gap junctions with C6-Cx43 and became highly coupled, implying that passage of molecules between gap junctions could be regulating growth. However, treatment of C6 cells with conditioned media from C6-Cx43 also elicited a reduction in growth rate [115]. This suggested that overexpression of Cx43 influenced secretion of molecules that could influence cell growth in a paracrine manner. This report was the first to show that changes in Cx43 expression can affect cell growth in both gap junction dependent and independent manners.

Several studies have shown that introduction of exogenous Cx43 influences the expression of proteins that are involved in the cell cycle, corroborating the observed delay in the G1 phase. Proteins that are responsible for advancing the cell cycle such as the cyclins were found to be decreased in cells overexpressing Cx43, and consequently proteins that slow down cell cycle were expressed at higher levels, i.e. p21 and p27 [116-121]. Treatment with growth factors such as transforming growth factor beta (TGFβ1) and ciliary neurotrophic factor (CNTF) have been shown to increase GJIC and reduce cell growth [122, 123]. It is still unclear exactly what could be passing through the gap junctions to influence cell growth but one can hypothesize that permeability of molecules such as second messengers and metabolites (Ca^{2+}, ATP, glutamate, glucose) could negatively regulate cell growth.

Gap junction independent function of Cx43 has also been implicated in Cx43-mediated growth changes. Specifically the C-terminal tail of Cx43, which has been shown to regulate the channel function and interact with various kinases and cytoskeletal proteins, has also been suggested to regulate cell growth [124]. The C-terminal tail has key phosphorylation sites that are substrates for kinases such as Src, mitogen activated protein kinase (MAPK) and PKC [76].
Specifically, Herrero-Gonzales et al observed an inverse correlation between Cx43 expression and cell growth as determined by over-expressing Cx43 in the C6 rat glioma cell line [125], this coincided with previous findings by Zhu et al [112]. The authors found that expression of Cx43 reduced cell growth by preventing the cell cycle progression from G0/G1 to S phase by reducing phosphorylation of retinoblastoma (Rb) [125]. In addition, the authors also observed a reduction in C-terminal Src (c-Src) oncogenic activity in C6-Cx43 cells; c-Src can bind the Cx43 C-terminal tail at amino acid residues Tyr247 and Tyr265 and when these sites were mutated the oncogenic activity of c-Src was restored and the proliferation phenotype reversed [125]. These findings demonstrated that Cx43 decreased cell growth by arresting the cell cycle via reduction of Rb. In addition the study suggested that c-Src interaction with the C-terminal tail of Cx43 also modulated cell growth.

Another protein that has been implicated in modulating cell growth via it’s interaction with the C-terminal tail is the CCN3 (connective tissue growth factor [CTGF], Cyr61/Cef-10, nephroblastoma overexpressed [NOV]) protein. In fact, C6-Cx43 glioma cells have increased levels of two CCNs: CCN1 and CCN3 [126]. Cyr61 has been shown to influence adhesion and migration, whereas CCN3 is known to regulate cell growth [126, 127]. CCN3 was later shown to interact with the C-terminal tail of Cx43 and it was suggested that this interaction prevents CCN3 from localizing to the nucleus where it may activate expression of genes that stimulate growth [121, 128]. These studies suggest that Cx43 may be regulating the cell growth by using its C-terminal tail as a docking domain for various proteins that promote cell proliferation when not interacting with Cx43.
1.2.2.2 The effect of Cx43 expression on glioma migration and invasion

Although the initial studies of Cx43 in glioma cells were focused on establishing a correlation between cell growth and Cx43 expression, one also gave insight about the cytoskeletal changes that were induced by manipulating Cx43 expression. Transfection of Cx43 in human glioma cell lines U251 and T98G changed their morphology from fibroblast-like to flatter and larger [114]. In addition other studies showed an association between Cx43 and the scaffolding protein ZO-1 which interacts with tight junctions, and the cytoskeleton demonstrated that Cx43 expression could indeed be involved in altering the cytoskeleton, which could subsequently influence glioma migration and invasion [129-131].

Lin et al. [67] demonstrated that C6-Cx43 glioma cells were able to form functional gap junction channels with astrocytes in the rat brain. In fact, C6-Cx43 cells were able to invade much further into the brain parenchyma than the control cells. The authors also demonstrated that the C6-Cx43 cells were able to form larger aggregates than the control cells indicating a Cx43-mediated increase in cell-cell adhesion [67]. The authors used chimeric mutant Cx40*43C3 to examine the channel function of Cx43, the chimera protein was mainly Cx40 in sequence except for the C-terminal tail which was from Cx43 [132]. Since the amino acid sequence that constituted the channel was that of Cx40 rather than Cx43 it was a non-specific way of examining the Cx43 gap junction channel. They found that chimeric mutant Cx40*43C3, which can make adhesive gap junction plaques but lacks the channel function, did not invade the brain parenchyma as well as C6-Cx43, demonstrating the importance of establishing GJIC between glioma and host cells in invading the brain. Interestingly a higher proportion of Cx40*43C3 and control cells migrated along the blood vessels when compared to C6-Cx43 thus
indicating that glioma cells with reduced GJIC may need guidance from blood vessels to invade. Although this was an elegant study demonstrating for the first time the role of GJIC in glioma invasion, their approach encompassed both homocellular coupling (between glioma cells) and heterocellular coupling (glioma and astrocytes), and their approach to examine the channel function was non-specific.

In 2005 Oliveira and colleagues [133] conducted a study that distinguished the roles of homocellular and heterocellular gap junctions in glioma invasion. The authors used three different approaches: 1. culturing human glioma cell lines (GL15 and 8MG) as spheroids on ECM to test for homocellular GJIC; 2. co-culturing GL15 and 8MG spheroids on astrocytes to test for heterocellular GJIC; 3. implanting the GL15 and 8MG spheroids in brain slices to test for heterocellular GJIC [133]. The authors also used carbenoxolone (CBX), a commonly used pharmacological blocker of gap junctions, to inhibit GJIC in all aforementioned assays. Inhibiting homocellular gap junctions between glioma cells increased migration, whereas inhibiting heterocellular gap junctions between glioma and host cells decreased migration [133]. In addition, they showed that C6-Cx43-GFP cells were more invasive than control cells in vivo. These findings highlight opposing roles for homocellular and heterocellular gap junctions in glioma invasion in vivo [133].

The aforementioned studies implicated the channel function of Cx43 in facilitating glioma migration and invasion; however the C-terminal tail of Cx43 could not be ignored due to its role in channel regulation and cytoskeletal interactions. Bates et al. [134] used C6 glioma cell clones with varying Cx43 expression, C6-H (high Cx43) and C6-L (low Cx43), to show that C6-H cells are more motile in a wound healing assay. This was confirmed by reducing endogenous Cx43 in C6-H cells using a shRNA construct; cells with reduced Cx43 exhibited a decrease in
motility (wound healing assay) and invasion (transwell coated with gelatin) [134]. Treatment of C6-H cells with CBX did not alter migration. Expression of Cx43 in C6-L cells increased migration while expression of Cx43 mutant (Cx43ΔCT), that had amino acids 244-382 deleted truncating most of the C-terminal tail, did not, indicating a role for the C-terminal in mediating migration [134]. It should be noted that C6-H cells produced low levels of GJIC (approximately 3-4 coupled cells) when compared to human glioma cell line U118 (Chapter 2), so it’s possible that inhibiting such a low level of coupling is not enough to generate migration changes. In addition, overexpression of Cx43ΔCT did not enhance cell coupling as well as full-length Cx43 and so the channel function was also affected in this mutant [134].

In the human glioma cell line LN18, overexpression of full-length Cx43 and a C-terminal truncation mutant (TrCx43; amino acids 242-382) increased migration (wound healing assay), indicating that the C-terminal tail was not necessary to increase migration in this cell model [135]. It should also be pointed out that the cells expressing TrCx43 mutant were coupled poorly and hence had compromised gap junction channel function. Interestingly, the expression of just the C-terminal tail alone (243Cx43) also increased migration. In addition LN18-Cx43 and LN18-243Cx43 produced lamellipodia like structure while migrating whereas LN18-control and LN18-TrCx43 produced bleb like structures, indicating differences in the organization of the actin cytoskeleton [135]. Specifically, lamellipodia are actin-rich structures that are formed by polymerization of filamentous actin, whereas blebs are protrusive structures formed due to intracellular pressure generated by actomyosin contractility and consist of cortical actin [136-138]. Recently Bergert and colleagues found the transition between lamellipodia and blebs in Walker carcinoma cell lines occurs when there is a shift in balance between the actin polymerization forces and actomyosin contractility [138]. A shift in signaling from Rac1/Arp2/3
actin polymerization to Rho/ROCK driven actomyosin contractility was able to permit cells to transition from lamellipodia to blebs [138]. Interestingly, real-time imaging showed that the transition between the two types of protrusions occurred on a time scale of seconds without gross change in cell shape or cell polarity [138]. Hence it is possible that the observed difference in protrusions between cells expressing Cx43 and TrCx43 could be due to changes in small GTPases signaling. Additional experiments examining Rac1 and Rho activity in LN18 expressing Cx43 and TrCx43 mutant have to be done to conclusively determine if the observed phenotype is due changes in actin cytoskeleton and actomyosin contractility. In addition cell-ECM adhesion can also influence the types of cell protrusions formed during migration [139-141], therefore one cannot rule out the possibility that there might be changes in cell-ECM adhesion in LN18 cells over expressing Cx43 and TrCx43 that could be influencing the type of protrusion formed. These results indicate that the C-terminal tail of Cx43 may influence the type of protrusions formed by migrating cells however it is still unclear how that affect is attained. In addition, it should be noted that 243Cx43 mutant does not localize to the plasma membrane or intracellular membrane due to a lack of a transmembrane domain; it is predominantly cytoplasmic and therefore differs in localization with full-length Cx43 and TrCx43 [142].

A reduction of endogenous Cx43 in human U251 glioma cells by two shRNA constructs increased migration in a wound healing assay, again demonstrating that reducing homocellular GJIC is pro-migratory [143]. The authors also show a concomitant decrease in cell-ECM adhesion to fibronectin and vitronectin. An invasion assay using transwells coated with matrigel showed a decrease in invasion for U251-shRNA cells compared to control cells; no difference was observed between control and U251-shRNA cells when astrocytes were seeded in the bottom well [143]. This indicates that Cx43-mediated glioma migration is affected by the type of
matrix, 2D vs. 3D. Interestingly, U251-shRNA and control cells in the presence of astrocytes were more invasive than U251-shRNA and control cells in the absence of astrocytes. This highlights a second mechanism for glioma invasion, one that depends on the chemokines produced by the host cells rather than Cx43 expression [143]. U251-shRNA cells showed decreased invasion in mouse brain slices when compared to control cells [143]. These findings indicate that homocellular coupling versus heterocellular coupling mediate migration in opposing manners. The findings from the aforementioned studies have been summarized in table 1.3 below.
<table>
<thead>
<tr>
<th>Author</th>
<th>Cells</th>
<th>Methods</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>McDonough et al. 1999 [144]</td>
<td>Dog glioma</td>
<td>Clones of varying Cx43 expression were used. Spheroid migration assay on collagen.</td>
<td>High Cx43 reduced migration</td>
</tr>
<tr>
<td>Author</td>
<td>Cells</td>
<td>Methods</td>
<td>Findings</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-----------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Zhang et al. 2003 [146]</td>
<td>C6 rat glioma cell line.</td>
<td>Over expression of Cx43. Motility assays: (1) Matrigel invasion assay (2) co-culture with astrocytes (3) use of MMP inhibitor.</td>
<td>Over expression of Cx43 caused increase in motility after 48hrs. Cells over expressing Cx43 were 3X more invasive. Over expression of Cx43 produced high levels of MMP2 and MMP9. Blocking MMP decreased invasion.</td>
</tr>
<tr>
<td>Oliveira et al. 2005 [133]</td>
<td>Human glioma cell lines GL15 (high Cx43) &amp; 8MG (Low Cx43) &amp; human glioma in nude mice.</td>
<td>In vitro collagen migration assay showed increase in migration with CBX blocking. GL15 (high Cx43) was more invasive than 8MG (Low Cx43) on brain slice; CBX decreased migration. Glioma with high Cx43 invaded more in vivo.</td>
<td>Over expression of Cx43 and CBX as gap junction blocker. Motility assays: (1) spheroid migration assay on Collagen IV (2) co-culture w/astrocytes (3) implantation into organotypic brain slices cultures.</td>
</tr>
<tr>
<td>Author</td>
<td>Cells</td>
<td>Methods</td>
<td>Findings</td>
</tr>
<tr>
<td>------------------</td>
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<td>-------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Bates et al. 2007 [134]</td>
<td>C6 rat glioma cell line.</td>
<td>Overexpression and knockdown of Cx43. Motility assays: (1) wound healing (2) transwell with 5% gelatin.</td>
<td>C6 clone with high endogenous Cx43 showed high motility. Knockdown of Cx43 showed low motility. Over expression of C-tail truncation showed low motility.</td>
</tr>
<tr>
<td>Crespin et al. 2010 [135]</td>
<td>LN18 human glioma cell line.</td>
<td>Over expression of Cx43, trCx43, and C-tail. Motility assays: (1) wound healing (2) time lapse.</td>
<td>Over expression of Cx43 showed high motility. Knockdown of Cx43 showed low motility. C-tail truncation slows down motility.</td>
</tr>
<tr>
<td>Olk et al. 2010 [147]</td>
<td>Mouse astrocytes.</td>
<td>Knockdown of Cx43. Transwell motility +/- laminin. 0.2% BSA in top chamber and 10% FCS in bottom chamber.</td>
<td>Increased motility. Increased protrusion length and protrusions/cell. No change in cell length and roundness.</td>
</tr>
<tr>
<td>Author</td>
<td>Cells</td>
<td>Methods</td>
<td>Findings</td>
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</tbody>
</table>
| Strale et al 2011 [143] | U251 human glioma. | Knockdown of Cx43. Motility Assays: (1) wound healing (2) transwell with matrigel (3) transwell with Cx43 +/+ & Cx43 +/- astrocytes in the bottom well (4) invasion on Cx43 +/- | (1) Knockdown of Cx43 closed wound faster than mock. (2) Knockdown of Cx43 decreased invasion thru Matrigel (3) Control and Cx43 knockdown glioma cells showed increased invasion in the presence of Cx43 +/- & Cx43 +/- astrocytes. (4) Cx43 knockdown glioma cells showed invasion decreased on Cx43 +/- brain slices not Cx43 +/-.
|                      |                     |                                                                         |                                                                                                                                         |

**Table 1.3: The effect of Cx43 expression on glioma motility.**

This table highlights the main findings of studies that were carried out over the last 15 years to examine how Cx43 affects glioma migration.
1.3 **Cell migration**

1.3.1 **Single cell migration**

The process of cell migration is important in development, maintenance of tissues and progression of diseases. Cell migration is a highly coordinated multi-step process that begins with a cell sensing its environment for pro-migratory signals, polarizing in the direction of the signal by forming actin-rich protrusions, establishing cell-ECM adhesions at the front to provide traction and disassembling adhesions at the rear to move forward (Figure 1.9) [148].

1.3.1.1 **Cell polarization**

The cell creates polarity in response to an external cue by having a different distribution of proteins in the front of the cell versus the rear. Activation of the Rho family of small GTPases such as Rho, Ras-related C3 botulinum toxin substrate 1 (Rac) and Cell division control protein 42 homolog (Cdc42) is a key event in establishing polarity [148]. Small GTPases are activated by guanine nucleotide exchange factors (GEFs) that convert the inactive GDP-bound protein to the active GTP-bound protein [149]. Conversely, they are inactivated by GTPase activated proteins (GAPs) that catalyze the hydrolysis of bound GTP [149]. Localized activation of these small GTPases is critical to establishing polarity. For example, Cdc42 is activated in the front of the cell where it plays a role in lamellipodia formation [148, 150]. In addition, Cdc42 mediates the movement of the microtubule organizing center (MTOC) and Golgi apparatus to the front of the cell facing the leading edge [148]. This re-orientation is mediated by Cdc42 interaction with
the Par6-Par3-aPKC complex and facilitates microtubule extension into the leading edge and delivery of vesicles from the Golgi to the leading edge providing proteins and membranes necessary for protrusion formation and forward motion [148, 151-153] (Figure 1.9A). Obstruction of Cdc42 in astrocytes compromises polarity and instead of directed migration the cell exhibits random migration [154].

In addition to the re-orientation of the microtubule cytoskeleton, for a cell to maintain polarity it has to relay the signal from the external gradient generated by a chemoattractant and amplify it intracellularly. The amplification of the external signal is orchestrated by phosphoinositide 3-kinase (PI3K), phosphatase and tensin homolog (PTEN) and phosphoinositide molecules phosphatidylinositol trisphosphate PtdIns (3,4,5)P$_3$ (PIP$_3$) and phosphatidylinositol diphosphate PtdIns(3,4)P$_2$ (PIP$_2$) [148]. At the leading edge of the migrating cell the activation receptor tyrosine kinases leads to the production of PIP$_3$ by PI3K, subsequently polarity proteins such as Par3 are also recruited to the leading edge. Par3 proteins work in conjunction with ‘PIP$_3$-activated exchange factors’ to employ the small GTPases Cdc42 and Rac at the lamellipodia [148, 153]. Changes in PI3K and PTEN signalling reduces the cell’s ability to move in a directional manner towards a gradient [148] (Figure 1.9A).

1.3.1.2 Protrusion and adhesion formation

The small GTPases Cdc42 and Rac are instrumental in the formation of protrusions at the leading edge. As mentioned earlier phosphoinositide molecules PIP$_3$ and PIP$_2$ are involved in recruiting the small GTPases to the plasma membrane where the leading edge has formed [148]. Rac and Cdc42 regulate actin polymerization through their interactions with proteins called
Wiskott-aldrich syndrome protein/WASP-family verprolin-homologous protein (WASP/WAVE) that in turn activate the actin-related protein 2/3 (Arp2/3) family of proteins. ARP2/3 complex binds to the sides of actin filaments and causes formation of side actin branches of the existing filament. This leads to a formation of a dendritic actin network and the formation of the protrusive structure called lamellipodia [148, 150, 155].

The dendritic network of actin is stabilized by proteins such as cortactin, filamin A and α-actinin [148, 150] (Figure 1.9B). The polymerization of actin filaments is mediated by multiple actin binding proteins, of which profilin and cofilin have been studied extensively. Actin filaments in the leading edge polymerize at the front and de-polymerize at the back thus moving forward while maintain the same length; this process is called ‘treadmilling’ [150]. Cofilin binds to the ADP-actin filaments and induces de-polymerization at the pointed end of the actin filament thus increasing the levels of monomeric actin [150]. Profilin binds to monomeric actin and exchanges the ADP to ATP; the profilin-actin complex exists at the barbed end of the actin filaments and hence increase the rate of treadmilling [150]. Capping proteins prevent elongation of actin filaments by obstructing polymerization at barbed ends close to the plasma membrane, thus increasing the concentration of monomeric actin at the pointed end; the monomeric actin is then funnelled to the barbed end uncapped actin filaments [148, 150, 155].

For a cell to move forward it needs to stabilize the protrusions formed at the leading edge; this is accomplished by adhering to the extracellular matrix (ECM) via heterodimeric transmembrane receptors called integrins. In addition to binding to the ECM, integrins are also connected to the actin cytoskeleton through adapter proteins such as talin, vinculin, and α-actinin [148, 156]. Binding of integrins to the ECM induces conformational changes in the heterodimers and leads to integrin clustering. Integrin clustering facilitates organization of the signalling
networks that are important in cell migration such as activation of small GTPases, phosphorylation of protein kinase and synthesis of phospholipids [148]. The adhesion sites formed by integrin clusters are more broadly called focal adhesions, however subclasses have been assigned depending on their size and stability [156]. The protrusions in the leading edge have nascent adhesions and focal complexes; these structures are small and very dynamic and facilitate actin polymerization through the small GTPases. These nascent adhesions can either turnover to form new adhesions or mature into more stable adhesions such as focal adhesions. A distinguishing factor between different types of adhesions are the protein constituents of the adhesion, for example nascent adhesions near the leading edge have Paxillin and are very dynamic, whereas α-actinin is present in more mature focal adhesions [148, 156-158].

By linking the cell’s cytoskeleton to the ECM focal adhesions provide traction which aid the cell in moving forward. In addition, focal adhesions also act as ‘mechano-sensors’ that relay the condition of the ECM to the cell which results in changes in the cytoskeleton [148]. For a cell to migrate it needs to be able to form cell-ECM adhesions for traction and detach from the ECM to move forward. This is dependent on the density of integrin receptors on the cell, the density of integrin receptors bound to ECM and the strength with which the receptors bind to ECM [148, 156].

1.3.1.3 Rear retraction

The retraction of the cell’s trailing edge is facilitated by disassembly of cell-ECM adhesions and contractile forces mediated through myosin II signalling. Disassembly of the cell adhesions involves weakening or breaking the interaction between integrins and ECM or
integrins and the actin cytoskeleton [156]. When the integrin and actin cytoskeleton interaction is broken the components associated with the cytoskeleton move towards the cell body [156]. Rho/Rho-associated protein kinase (ROCK) signalling has been implicated in the disassembly of cell adhesion and thus retraction of the trailing edge. In addition, focal adhesion kinase (FAK) and Src signalling have also been implicated in adhesion disassembly at the rear by regulating the myosin-light chain kinase (MLCK) and extracellular signal related kinase (ERK) [156, 157] (Figure 1.9C).
Figure 1.9: Steps in cell migration.

A. Polarity is established by the presence of activated Cdc42 and Rac, Par and aPKC proteins in the front of the cells. The microtubule organizing center (MTOC) and the Golgi apparatus is localized in the front of the cell as well. PTEN and myosin II restrict protrusions to the cell front. B. Protrusions are made at the front of the cell or the leading edge by increased actin polymerization. Rac and Cdc42 target WASP/WAVE proteins that are responsible for mediating the formation of actin branches. Protrusions are stabilized by the formation of cell-ECM adhesions. This process is mediated by integrin activation and clustering which recruits proteins
that connect integrins to the actin cytoskeleton. C. At the rear of the cell adhesions are disassembled so the cell can move forward. This image was taken from [148], with permission from © American Association for the Advancement of Science.

1.3.2 **Collective cell migration**

Collective cell migration is a mode of cell motility that involves a group of cells moving together while remaining physically connected. This mode of migration is observed during biological processes such as development, wound healing and cancer invasion. For cells to move collectively they must abide by the following criteria: 1. cells remain physically and functionally linked by maintaining cell-cell junctions between them; 2. cells must exhibit polarity as a cohort and form a ‘supra-cellular’ structure. In this structure the actin cytoskeleton organization in all cells must cooperatively produce traction and protrusion forces to propel forward; 3. modification of the tissue or ECM in the migration path [159].

The form of collective migration cells take depends on their environment. Collective migration across a flat tissue surface occurs as a 2D sheet, where the cells at the front use lamellapodia and pseudopodia [159]. This kind of cell movement has been observed in forming a single layered epithelium. Sheet-like migration has been observed in the *in vitro* wound healing assay (Figure 1.10 A). Another form of collective migration is multi-cellular 3D strand motility, which occurs in processes such as vascular sprouting in blood vessels and invasion of multicellular strands in cancer (Figure 1.10 B). The spheroid migration assay is a good tool to recapitulate the multicellular strand migration of cancer cells [159].
Figure 1.10: Types of collective migration.

A. A sheet of epidermal cells moving across a 2D ECM substrate. Cells are connected through adherens junctions and to the ECM via integrins. Lamellapodia are formed in the leader cells at the front. B. This represents collective migration of cancer cells through their environment. Proteases such as MMPs play an important role in degrading and remodeling the ECM to be more permissive for migration. The tip cells at the front form actin-rich pseudopods or lamellapodia to direct migration. This image was adapted from [159], with permission from © Macmillan Publishers Limited.

As mentioned before, collective cell migration cannot happen without cell-cell adhesion. This cell-cell adhesion is mediated by adherens junction proteins called cadherins. Cadherins connect cells by their extracellular domains, and interact with the actin cytoskeleton on the intracellular side thus mechanically linking cells. This is most commonly seen in migrating epithelial sheets of cells [160]. Cadherin based cell adhesions are rapidly remodeled and thus aid in cells changing positions within the sheet [159, 161, 162]. Loss of the epithelial cadherin (E-
cadherin) weakens the adhesions between cells and facilitates transition from collective to single cell migration; this process is termed epithelial-mesenchymal transition (EMT) [163-165]. Tight junction proteins ZO-1, claudin 1, claudin 4 and occludin are also expressed in migrating epithelial sheets [166, 167].

The gap junction protein Cx43 has also been implicated in aiding cell-cell adhesion in collective migration in different cell lines. A study by Defranco et al was the first to suggest a role for Cx43 in collective sheet-like migration by expressing Cx43-GFP proteins in human adrenal cortical cells [168]. Real-time imaging of the wound healing assay showed that adrenal cortical cells expressing Cx43-GFP move in a sheet-like manner and maintain gap junctions between cells while they migrate [168]. Analysis of GJIC showed that migrating sheets of cells have extensive coupling between them regardless of whether the cells are at the leading edge of the wound or in the center [168]. In addition the authors demonstrated the internalization of gap junctions when cells detached from one another [168]. It should be noted that the authors did not show if there were changes in migration pattern between control cells and cells over expressing Cx43-GFP, nor did they examine changes in migration rate. Brugge et al demonstrated a change in the migration pattern of immortalized non-tumorigenic mammary epithelial cells upon siRNA reduction of Cx43 [169]. Real-time imaging of the wound healing assay showed that reducing Cx43 accelerated the migration of the mammary epithelial cells in conjunction with minimal cell-cell adhesion, irregular migration and compromised front-rear polarity [169]. Consequently, the adherent sheet-like organization of the wound was lost upon closure. Interestingly, an increase in E-cadherin and a decrease in N-cadherin expression was also observed suggesting EMT [169]. These studies suggest that cells that express Cx43 and migrate collectively form gap
junctions between them, and that reducing Cx43 reduces cell-cell adhesion which facilitates detachment and migration as single cells.

The cooperative migration of cells in a cohort is predicated on establishing polarity that distinguishes the front of the supra-cellular structure from the rear. In the front the leader cells exhibit a shape that is more mesenchymal whereas the follower cells are more tightly packed and structured [159, 170]. The leader cells sense the environment for guidance cues that create changes in the cytoskeletal dynamics. The asymmetric stiffening of cortical actomyosin mediated by Rho GTPases and myosin II and the directional remodeling of the ECM by metalloproteases facilitates polarity in collective migration [159, 170]. Cells in the front work together to generate protrusive forces and cells at the rear cooperatively modulate retraction dynamics; this produces a supra-cellular cortical actin network [171-173]. Thus the supra-cellular organization of cells is crucial for a cohort of cells to move collectively.

1.4 Motivations, objectives, and highlights

*In vivo* GBMs have both heterocellular (glioma and host cells) and homocellular (glioma and glioma) gap junctions. The main objective of this thesis was to distill the contributions of the two types of gap junctions in glioma migration and invasion. Firstly, I discerned the role of homocellular gap junctions between glioma cells using an *in vitro* human glioma cell model. Secondly, studies determined whether heterocellular communication between glioma and host cells *in vivo* was important for glioma invasion by performing intracranial implantation in C57Bl mice with syngeneic mouse Gl261 glioma cells. The following sections state the specific objectives and findings of chapters 2-4.
1.4.1 Chapter 2 – Selection of a suitable human glioma cell line

Much of the work to understand Cx43’s role in the progression of glioma has been done using the C6 rat glioma cell line. The C6 glioma cell line was derived from treating adult rats with a chemical mutagen and thus this glioma cell line is not as similar to human GBM as immortalized human glioma cell lines (cultured from resected tumours from patients). Specifically, the C6 rat glioma cell line has wildtype p53 and PTEN genes, two genes that are commonly mutated or deleted in GBM. Studies using C6 and human glioma cell lines have demonstrated that Cx43 expression influences growth and migration of cells, although the exact mechanism is unclear.

Many of the studies that have used human glioma cell lines for discerning the role of Cx43 in gliomagenesis failed to characterize the cell lines for Cx43 protein expression, subcellular localization, GJIC, and migration. I believed that this information was pertinent to selecting the most suitable cell line and subsequently deriving accurate conclusions from my experiments. Thus the purpose of this chapter was to screen a panel of grade IV human glioma cell lines for the aforementioned characteristics.

From my characterization I found that the protein expression of Cx43 was variable among a panel of grade IV human glioma cell lines, a finding which is in conflict with the general assumption that Cx43 protein expression is reduced in grade IV gliomas. Interestingly, I also observed a predominant perinuclear localization of Cx43 in two of the cell lines which affected GJIC. This is an indication that the reduced GJIC observed in GBMs could also be due to improper localization of Cx43 and not just a decrease in protein expression. In addition, I
observed no correlation between endogenous Cx43 expression and migration levels. The findings from this chapter were used to select the model cell line for Chapter 3.

1.4.2 Chapter 3 – Cx43 channel function influences migration

The objective of this chapter was to determine if and how Cx43 mediates migration in glioma using a human glioma cell line. Unlike previous studies that have attempted to answer this question I took the stringent loss of function and rescue approach to answer this question. Based on my screening from chapter 2 I found the U118 human glioma cell line to be a suitable model for this approach since it had Cx43 that localized properly to the plasma membrane to form functional gap junctions. In addition, U118 also had mutations in TP53 and PTEN genes which are frequently found in GBM.

Unlike the previous findings that implicated the C-terminal tail, I found that reducing Cx43 channel function increased migration of glioma cells. This is the first study that used a specific mutant to block the Cx43 channel function in glioma cells and confirm the findings with a pharmacological blocker of gap junctions. Interestingly, I also observed a change in the migratory pattern of cells with reduced Cx43 in that they moved in a more detached manner. Although other studies with mammary epithelial and neural crest cells have reported the same phenotype, ours is the first study implicating Cx43 in the specific migration patterns of glioma cells.
1.4.3 Chapter 4 - Characterizing the role of Cx43 channel function in invasion \textit{in vivo}

The previous chapter used an \textit{in vitro} human glioma cell line to show homocellular gap junctions between glioma cells influence migration, however \textit{in vivo} the presence of heterocellular gap junctions complicates matters. Thus the objective of this chapter was to examine the role of homocellular and heterocellular gap junctions in glioma invasion using an \textit{in vivo} model. We observed a reduction in invasion when we reduced Cx43 in both glioma cells and host astrocytes. This is the first study in which a specific mutant (T154A) was used to block Cx43 channel function in mouse glioma GL261 cells; GL261 cells were subsequently intracranially implanted into syngeneic mice with Cx43 present or deleted from astrocytes. This approach blocked both homocellular and heterocellular GJIC mediated by Cx43. Blocking the channel function increased the distance of invasion but had no effect on the number of cells that distributed in to the brain.
Chapter 2: Characterization of human glioma cell lines for selection of in vitro model

2.1 Introduction

Gap junctions have been implicated in the process of carcinogenesis since the 1960’s [174], in fact a reduction in Cxs and GJIC has been observed in many different types of cancers [78]. A reduction of Cx43 expression was observed in human GBM tissues as well [63], however much of the ground-work exploring Cx43’s role in gliomagenesis was done using the rat C6 glioma cell line as a model system. The C6 rat glioma cell line was generated by using the mutagen N-nitrosomethylurea in adult rats, and the resulting tumor cells were selected based on their expression of S100 protein [109]. The C6 cell line has been classified as an astrocytoma based on their histo-pathological features [175]. Since increasing tumorigenicity has been associated with low levels of gap junction communication, the very low levels of endogenous Cx43 in the C6 cell line [176] together with its aggressive growth in vivo [113] made it a reasonable model to use.

Over-expression of Cx43 in C6 cells displayed an inverse relationship to cell proliferation [112, 113]. C6 cells overexpressing Cx43 also formed smaller tumors in vivo compared to control cells [113]. We have further shown that reducing Cx43 in C6 cell lines decreases migration and that the C-terminal may play a role [134]. Taken together, these studies demonstrated that Cx43 expression plays a role in gliomagenesis, but this finding needed to be confirmed in a model system more akin to human GBM.

Huang et al. published a study using human glioma cell lines U251 and T98G to study Cx43’s role in gliomagenesis [114]. These human glioma cell lines were selected based on the
low endogenous Cx43 expression. Over-expressing Cx43 in U251 and T98G reduced the proliferation rate [114]. A study with another human glioma cell line, LN18, showed that over-expression of Cx43 increased anchorage independent growth and motility [135]. We sought to establish an in vitro model system using a human glioma cell line to more specifically address the role of Cx43 in glioma migration. Previous studies used Cx43 protein expression as the only criteria for choosing a human glioma cell line as a model system; we extended this by characterizing a panel of high grade human glioma cell lines that had been cultured from GBM patients; Cx43 protein expression and subcellular localization, GJIC and cell migration were examined to choose an ideal in vitro model system. We discovered variability in Cx43 protein expression and subcellular localization between the cell lines. Our migration data showed no correlation between endogenous Cx43 expression and migration levels, indicating that Cx43-mediated migration changes observed in previous studies appear to be cell type specific.

The Cx43 protein consists of four transmembrane domains that make up the channel, two extracellular loops that are necessary for gap junction docking, and a C-terminal tail that is a nexus for protein-protein interaction and Cx43 regulation. The chimeric mutant Cx40*43C3 (Cx40 C-terminal domain was swapped for Cx43 C-terminal domain) blocked the channel function and disrupted glioma invasion in vivo by disrupting GJIC between glioma and host cells [67, 132]. Blocking the channel function in human glioma cell lines GL15 and 8MG using the gap junction blocker carbenoxolone (CBX) gives rise to increased migration in vitro [133]. Bates et al. implicated the C-terminal tail in facilitating glioma migration [134]. Since many of the known functions of Cx43 have been implicated in motility we used a series of mutants that specifically obstructed one function of the protein at a time to determine the function mediating glioma cell migration.
Previous studies have identified specific amino acid residues that are critical for various functions of Cx43. For example, the threonine residue in the third transmembrane domain at amino acid position 154 has been reported to be critical for channel function. Changing threonine to alanine (T154A) obstructed the channel and blocked GJIC in a dominant negative manner [177]. The two extracellular loops of Cx43 are important for the ability of Cx43 hemichannels to dock and form functional gap junctions between cells. This region of the protein contains six cysteine residues that are conserved in all vertebrate gap junctions; point mutations of these cysteine residues result in proteins that lack the ability to adhere to Cx43 proteins on adjacent cells [11, 178-180]. The C-terminal tail of Cx43 exhibits considerable variability in protein sequence between species, and has been reported to be an important site for protein-protein interactions in multiple cell types. Specifically, the C-terminal tail has been shown to interact with cytoskeletal proteins involved in cell motility such as actin, the actin binding protein debris, microtubules, and the tight junction protein ZO-1 [147, 181].

Blocking the channel function with CBX in the human glioma cell line GL15 increased migration [133]. Interestingly, point mutation of just one of these cysteine residues, at amino acid position 61 to serine (C61S), is sufficient to inhibit the protein from docking and forming gap junctions; C6 rat glioma cells expressing C61S mutant showed decreased invasion in vivo (see table 1.3) [67]. The C-terminal tail of Cx43 has been shown to modulate migration in the C6 rat glioma cell line [134]. In addition both the C-terminal tail and the extracellular loops have both been implicated in mediating neuronal migration [68, 182].

Since the channel, extracellular loops and C-terminal tail domains have all been implicated in cell motility, we used the appropriate mutants to examine which domains play a role in glioma cell migration. We characterized the following mutants: T154A, C61S and a C-
terminal tail truncation (TrCx43) by over-expressing the constructs in the human glioma cell line LN229 (Figure 2.1). The low levels of endogenous Cx43 in LN229 made it a suitable cell line for an overexpression study. We characterized the mutant proteins for expression, subcellular localization and GJIC. Surprisingly, we found no changes in proliferation or cell motility upon overexpression of wildtype or mutant Cx43. Nonetheless, we used our characterization of these human glioma cell lines to choose a suitable cell line to study glioma migration in a spheroid model by using loss-of-function and rescue experiments (Chapter 3).
This schematic illustrates where the Cx43 protein has been mutated. The C61S mutant is a point mutation of cysteine to serine in the first extracellular loop that obstructs the docking function of Cx43. The T154A mutant is a point mutation of threonine to alanine in the third transmembrane domain, rendering the channel blocked. The TrCx43 mutant is a deletion mutant in which the C-terminal tail was deleted at amino acid position 242. PTMs = Posttranslational modifications.

This image was obtained from Protter - visualize proteoforms, Omasits et al., Bioinformatics. 2013 Nov 2.
2.2 Materials and methods

2.2.1 Cell lines and culturing

Human glioma cell lines LN18, T98G, LN229, U118, U138, U251, U87, A172, Hs683, and Sw1088 were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) high glucose media with 10% fetal bovine serum (FBS), with the exception of LN229 which was cultured with 5% FBS. LN229 cells expressing control (empty vector), Cx43, TrCx43, C61S and T154A constructs were cultured in DMEM high glucose with 5% FBS. All constructs were based on human Cx43 sequence.

Astrocytes were cultured from postnatal (P0) cortices as per [183]. Culture media (DMEM supplemented with 10% FBS, 10 units/ml penicillin, and 10 μg/ml streptomycin) was replaced 3 days after plating and every second day thereafter [183].

2.2.2 Western blotting

Cells were cultured to 90% confluence in 100mm dishes. The cells were washed with cold phosphate buffered saline (PBS) (for a 1L solution 0.13 g CaCl₂ * H₂O, 0.2 g KCl, KH₂PO₄, 0.1 g MgCl₂ * 6H₂O, 8.0 g NaCl, 2.16 g Na₂HPO₄ * 7 H₂O) and lysed in 500 μL radioimmune precipitation lysis buffer (RIPA) (150mM NaCl, 50mM Tris-HCl pH 8.0, 0.5% Sarkosyl, 1% IGEPAL, 0.1% SDS) containing phosphatase inhibitor cocktail 2(100X, Sigma, St. Louis, MO) and protease inhibitors (7X, Roche, Indianapolis, IN) [134]. DNA was sheared by
sonication. Protein was quantified by using the colorimetric BCA Protein Assay Kit (Pierce) and 30 µg of protein was loaded on to 10% acrylamide gels. Gel electrophoresis was carried out at 100 V 1.5 hours. The antibodies used were anti-Cx43 (C6219, rabbit, Sigma, St. Louis, MO 1:4000; 05-763, mouse, Upstate cell signalling solutions, Lake Placid, NY, 1:50), and anti-GAPDH (5G4; mouse, HyTest Ltd., Turku, Finland; 1:10,000). Enhanced chemiluminescence (Pierce) was used to visualize the protein bands. ImageJ software (National Institute of Health) was used to perform densitometry on the x-ray films to quantify intensity of protein bands.

2.2.3 Immunofluorescence

Astrocytes were plated on glass coverslips coated with poly-L-ornithine (0.01% solution, Sigma-Aldrich) or culture dishes. Human glioma cells were grown on glass cover slips in DMEM high glucose media with 10% FBS until sub-confluence, washed with PBS without Mg\(^2+\) and Ca\(^2+\) and fixed in 4% paraformaldehyde in PBS buffer for 10 minutes at room temperature. The cells were rinsed with PBS twice and incubated in blocking solution (2% BSA + 0.3% Triton X-100) for 30 minutes. Samples were incubated with primary antibodies in working solution (1% BSA + 0.3% Triton X-100) for 1 hour at room temperature. The antibody used was anti-Cx43 (C6219, rabbit, Sigma, 1:400; AP1541b, mouse, Abgent, San Diego, CA, 1:50). Coverslips were washed three times (10 minutes each) with PBS. Coverslips were then incubated with appropriate secondary antibody after which they were washed and mounted on microscope slides with Prolong Gold containing DAPI (4’,6-diamidino-2-phenylindole) (Invitrogen, Carlsbad, CA). The cells were visualized using a Zeiss epifluorescence microscope.
2.2.4 **Assessment of GJIC by preloading assay**

The preloading assay was conducted according to [184]. All cell lines were grown until confluent. Donor cells were loaded with preloading solution (1 µl of Calcein AM (C3100MP, Invitrogen) and DiI (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate, Molecular Probes, Grand Island, NY) to 1 ml of 0.3 M glucose), and incubated for 20 minutes at 37°C. The loading solution was removed and the cells were washed three times with 0.3 M glucose. Cells were trypsinized until they were completely detached. Donor cells were seeded on to the recipient cells and incubated for 2 hours at 37°C. Epifluorescence microscopy was used to image the coupled cells. The donor cells appeared yellow due to the presence of Calcein AM (green) and DiI (red). Passage of Calcein to the recipient cells labelled them green. The number of recipient cells coupled to a donor cell was counted to determine the extent of GJIC. One way ANOVA (Sigma plot) followed by Dunn’s Method to do pairwise multiple comparisons was used to calculate significance. P-value<0.05 was considered significant.

2.2.5 **Site-directed mutagenesis & retroviral infection of mutants in LN229 cells**

Cx43 and Cx43-TrCx43 cDNAs were inserted into pMSCVpuro vectors (Clontech Laboratories, Mountain View, CA) as per [135]. The point mutation for Cx43-T154A and Cx43-C61S cDNA was generated by using the Qiagen site directed mutagenesis kit; pMSCVpuro-Cx43 vectors were used as template. The primer sequence for Cx43-T154A was GGCTTGCTGAGAGCCTACATCATCACGCATCC (mutation underlined). The primer sequence for Cx43-C61S was CTCAACAACTGGCTCGAAAAACGTCTGCTATGAC. Plasmids
pMSCVpuro-Cx43, pMSCVpuro-Cx43-TrCx43, pMSCVpuro-Cx43-T154A, and the empty vector pMSCVpuro were transfected into the HEK293 packaging cell line (obtained from ATCC) using Lipofectamine 2000 (Invitrogen), and the viral titres were collected [135] and used to infect LN229 cells. Puromycin (2 µg/ml) was used to select the cells expressing the constructs. All constructs were validated by sequencing (NAPS Unit, Michael Smith Laboratory, and University of British Columbia).

2.2.6 Growth curve for LN229 cells expressing wildtype and mutant Cx43

Thirty thousand cells were seeded in triplicates into a 12 well plate with DMEM high glucose with 5% FBS. Proliferation was quantified by the number of cells per well was counted on days 1, 3, 5, 6, 7, 8 and 10 using Z1 Coulter Particle Counter (Beckman Coulter) with IsoFlow Sheath Fluid (Coulter Corp., Miami, FL) as a diluent [60]. One way ANOVA (Sigma plot) followed by Tukey’s test was performed to do pairwise multiple comparisons to calculate significance. P-value<0.05 was considered significant. No significant was found in the data.

2.2.7 Wound healing

Cells (1x10^6) were seeded in 6 well plates in DMEM high glucose with 5% FBS; at confluence (day 2) the monolayer was scratched with a P1000 pipet tip. The same field in the wound was imaged at t = 0 hours and at t = 8 hours with the Zeiss Axiovision microscope. The distance migrated was calculated by subtracting the width at t = 0 hr from the width at t = 8hrs. [134]. The images were analyzed with Axiovision software (Zeiss). One way ANOVA (Sigma
plot) followed by Tukey’s test was performed to do pairwise multiple comparisons to calculate significance. P-value<0.05 was considered significant.

2.3 Results

2.3.1 Assessment of Cx43 protein expression, localization, and GJIC in a panel of human glioma cell lines

As mentioned earlier Cx43 is the major connexin in the glial cells called astrocytes; de-differentiation of astrocytes has been implicated in the formation of gliomas. Mouse astrocytes express Cx43 protein that localizes at cell-cell contacts forming function gap junctions as demonstrated by the preloading assay (Figure 2.2 A-C). We characterized a panel of 10 human glioma cell lines for protein expression of Cx43. We chose cell lines that were classified as high grade glioma, based on the WHO classification. These cell lines also carried some of the well know mutations implicated in GBM such as P53 and PTEN deletion (Table 2.1). Western blot analysis showed varying levels of Cx43 expression in the cell lines (Figure 2.3 A). LN229 and T98G had the lowest levels of Cx43; while U118 and Hs683 had the highest levels of Cx43 (Figure 2.3 B). Hs683 and Sw1088 glioma cell lines were removed from further characterization because they exhibited changes in Cx43 expression after being cultured for 5 passages (data not shown). From the remaining analysis, we chose to focus on cell lines that are commonly used as in vitro models for studying glioma; thus we removed A172 from our panel for further characterization.
Figure 2.2: Qualitative analysis of Cx43 protein expression, subcellular localization, and GJIC in mouse (P0) astrocytes.

A. Western blot analysis was performed to detect Cx43 protein expression in mouse astrocytes. Anti-Cx43 (Sigma) antibody that targets the C-terminal tail of Cx43 was used to detect Cx43 protein. Gamma tubulin was used as a loading control. B. Immunofluorescence was carried out on mouse astrocytes using anti-Cx43 (Sigma) antibody. Cx43 (green) can be observed in plaques at cell-cell contacts (arrow) and around the nucleus (blue) (arrow). Scale bar is 20 µm. C. Preloading assay on mouse astrocytes shows GJIC between the cells as exhibited by the passage of Calcein dye (green) from the donor cell (yellow because they have both DiI (red) and Calcein AM (green)). Scale bar is 50 µm. The experiments in this figure were carried out by Dr. Moises Freitas Andrade.
Figure 2.3: Cx43 protein expression in high grade glioma cell lines is variable.

A. Western blot analysis was performed to detect Cx43 protein expression in 10 different human glioma cell lines. Anti-Cx43 (Sigma) antibody that targets the C-terminal tail of Cx43 was used to detect Cx43 protein. B. Densitometry analysis with ImageJ software reveals differing levels of Cx43 protein expression between the human glioma cell lines. GAPDH was used as loading control and the relative Cx43 protein expression was determined by normalizing to GAPDH. The experiment was repeated 3 times.
We next examined the subcellular localization of Cx43 by immunofluorescence. We discovered that in most of the cell lines Cx43 localized in intracellular vesicles and at cell-cell junctions (Figure 2.4). However in cell lines LN18 and T98G we observed a prominent population of Cx43 localizing to the perinuclear region of the cell and very low levels of Cx43 at cell-cell contacts (Figure 2.4).

We determined if Cx43 protein localizing at cell-cell junctions was making functional gap junction channels by employing a preloading assay. In this assay, the donor cell is labeled with DiI and Calcein-AM; DiI labels the cell membrane (red) while Calcein-AM is taken up by the cells. Once the AM part of the molecule is cleaved Calcein becomes a gap junction permeable dye (green) that passes through to recipient cells [184]. Our results show U118, U138, and U87 cell lines with higher Cx43 expression, had the highest level of GJIC demonstrated by the number of coupled cells. Indeed, LN18, LN229 and T98G, cell lines with the lowest Cx43 expression, had the least amount of GJIC (Figure 2.5 A and B).
Figure 2.4: Subcellular localization of Cx43 in high grade glioma cell lines.

LN18 and T98G cell lines show a prominent population of Cx43 (green) localizing to the perinuclear region (nucleus is blue). The perinuclear region is the cytoplasmic region surrounding the nucleus. Cx43 protein in LN229, U87, U118 and U138 cell lines localized in the perinuclear region and at cell-cell junctions (arrow). Scale bar is 10 µm.
Figure 2.5: GJIC in human glioma cell lines.

A. Preloading assay was used to examine GJIC in human glioma cell lines LN18, T98G, U118, U138 and U87. Donor cells (yellow because they have both dyes; see arrows) were labeled with DiI (red) and Calcein AM (green). Passage of Calcein to recipient cells through gap junctions labelled them green. B. U118 cell line had a significantly higher number of coupled cells than LN18 and T98G. The experiments were repeated 3 times. One way ANOVA (Sigma plot)
followed by Dunn’s Method to do pairwise multiple comparisons was used to calculate significance. **p<0.001. The scale bar is 50 µm.

We further characterized the cell lines U118, U138, LN18, T98G and LN229 for migration by carrying out a wound healing assay. U87 cell lines were not conducive to this assay as they cannot form a confluent monolayer under the conditions used (data not shown), a requirement for wound healing assay. All 5 high grade glioma cell lines displayed differing levels of migration; with LN229 and U138 showing significantly higher migration levels than T98G (Figure 2.6).

![Graph showing migration levels of cell lines](image)

**Figure 2.6: High grade glioma cell lines show variable levels of migration.**

Wound healing assay was carried out on LN229, LN18, T98G, U118 and U138 cell lines.

Distance migrated by cell lines during 8 hours was quantified. Images of the same field in the wound were taken at t = 0hr and t = 8hrs. Distance migrated is calculated by subtracting the
width (w) of the wound at t = 8hrs from the width at t = 0hrs. The experiment was repeated 3 times. One way ANOVA (Sigma plot) followed by Tukey’s test was performed to do pairwise multiple comparisons to calculate significance. *p<0.05 was considered significant.

Our results highlight variability in Cx43 protein expression, localization and GJIC communication in these high grade human glioma cell lines. We also demonstrate that the high grade glioma cell lines vary in how fast they migrate, suggesting all GBM cell lines may not be equally motile. We would need to use other migration assays such as the spheroid migration assay or the transwell assay to examine the full migratory potential of these cells.

2.3.2 Characterization of Cx43 mutants in LN229 human glioma cell line

Cx43 has been implicated in affecting tumor growth and migration [113, 115, 121, 134, 135, 185], however the mechanism by which it does so is still unclear. To better understand the mechanism responsible for Cx43’s role in these processes we generated Cx43 mutants that obstruct different functions of the protein. From our characterization of 10 different human glioma cell lines we observed that LN229 has the lowest Cx43 expression (Figure 2.3A and B); in addition, it has mutations that commonly occur in GBM (Table 2.1), which made it a good cell line for characterizing the function of various Cx43 mutants. We chose this cell line to over-express wildtype Cx43, the channel dead mutant T154A, the C-terminal truncation mutant TrCx43, and the extracellular loop mutant C61S. Site-directed mutagenesis was used to make T154A and C61S mutants. Western blotting confirmed over-expression of all constructs in LN229 cells (Figure 2.7A and B). Immunofluorescence showed proper localization of mutant
proteins except for C61S mutant, which predominantly localized to the perinuclear region (Figure 2.7 C). This was reflected in the GJIC results which showed increased coupling in wildtype Cx43 and TrCx43 mutant but very low coupling in C61S mutant (Figure 2.8 A and B). This suggests that the cysteine residues on the extracellular loops are critical for proper trafficking through the secretory pathway and localization of the protein to the cell membrane. The T154A channel-dead mutant produced the lowest levels of GJIC indicating that the mutation does indeed obstruct the channel function.
Figure 2.7: Expression and subcellular localization of Cx43 wildtype and mutant protein in LN229 cells.

A. Western blotting using anti-Cx43 antibody (Sigma) showed successful expression of all constructs except for TrCx43. TrCx43 was not detected because the C-terminal tail is the epitope for the Sigma antibody. The putative phospho-isoforms of Cx43 are denoted P0, P1, and P2. P0 is the non-phosphorylated form of Cx43, P1 and P2 are the predicted phosphorylated forms of Cx43 that migrate slower than P0. B. Anti-Cx43 antibody (Upstate) was used to detect TrCx43 expression since the epitope for this antibody is amino acid 131-152 in the cytoplasmic loop of
Cx43. C. Wildtype Cx43, TrCx43, and T154A proteins were observed at cell-cell contacts forming punctate plaques (green) (see arrows). The C61S mutant protein was predominantly localized to the perinuclear region in intracellular vesicles (see arrow). The scale bar is 10 µm.

Figure 2.8: GJIC is reduced in LN229 cells expressing Cx43 mutant constructs.
A. Donor cells (yellow, see arrows) were labeled with DiI (red) and Calcein AM (green). Passage of Calcein to recipient cells through gap junctions labelled them green. B. Wildtype Cx43 had significantly higher number of coupled cells than control cells expressing the empty vector. The T154A and C61S mutants had a significantly lower number of coupled cells than the wildtype Cx43. The experiment was repeated 3 times. One way ANOVA was used to calculate the significance; *p<0.05, **p<0.001. The scale bar is 50 µm.

We quantified proliferation using a growth assay in which we seeded 30,000 cells for each condition and counted cells at days 1, 3, 5-10 to determine the growth curves. We found no difference between the growth curves of LN229 cells transfected with the different constructs (Figure 2.9 A). A wound healing assay was used to quantify migration. Equal number of cells was seeded for each condition on uncoated plates with DMEM high glucose containing 5% serum. Images of the wound were captured at t = 0hr and t = 8hrs. The distance migrated was calculated by subtracting the width at t = 0 hr from the width at t = 8hrs. We observed no difference between control and mutants (Figure 2.9 B).
Figure 2.9: Proliferation and migration are not mediated by Cx43 expression in LN229 cells.

A. A growth assay was performed to examine if the different mutations of Cx43 resulted in changes in proliferation. No differences were observed between the various cell lines. B. A
wound healing assay was performed with each of the cell lines to examine migration over 8 hours. No significant difference was observed between LN229 control cells (Empty) and LN229 cells expressing wildtype and the various Cx43 mutant s. The experiments were repeated 3 times.

Our migration data shows LN229 has the highest levels of migration (Figure 2.5), however it has the lowest Cx43 expression (Figure 2.2 A and B), thus it is possible that the LN229 cell line carries mutation in key genes that drive migration regardless of Cx43 expression.

2.4 Discussion

As early as the 1960s Loewenstein observed a decrease in GJIC in cancer, and hypothesized a role for gap junctions in regulating cell growth [174, 186]. Subsequently, Budunova et al. demonstrated that non-mutagenic tumor promoting agents were able to decrease GJIC [86]. In addition, molecules such as retinoids and carotenoids supressed carcinogen induced tumorigenesis while increasing Cx43 and thus GJIC [187-189]. The observation of reduced GJIC was also observed in cancers of different organs such as skin, liver, bladder and brain suggesting that this may be a common phenomenon of carcinogenesis [78]. Although these studies verified the hypothesis formed 40 years ago that a reduction in GJIC in cancer is correlated with increased cell proliferation, the mechanism responsible was still unclear.

In the 1990s Naus and colleagues published several studies that implicated Cx43 expression in regulating glioma cell growth. Using the rat glioma C6 cells as their model system they demonstrated a negative correlation between Cx43 expression and cell proliferation, both in
vivo and in vitro [112, 113]. Interestingly, treatment of C6 cells with C6-Cx43 cell conditioned medium also reduced cell proliferation suggesting that Cx43 could be modulating cell growth by two mechanisms: (1) by passage of molecules through gap junctions and (2) by indirectly increasing secretion of a growth inhibitory factor(s) [115]. Additional studies showed that decrease in GJIC in glioma was confirmed in primary human glioma samples and over-expressing Cx43 reduced cell growth in T98G and U251 human glioma cell line [63, 114]. Interestingly, cell growth was not the only process affected by Cx43 expression, since over-expression of Cx43 in C6 cells increased migration while reducing expression via anti-Cx43 shRNA decreased migration; the C-terminal tail of Cx43 was implicated in mediating migration [134]. In addition, C6 cells over-expressing Cx43 invaded a larger area of the brain parenchyma than control cells [67, 133]. Over-expression of Cx43 in the human glioma cell line LN18 increased migration [135], however a reduction of Cx43 in the human glioma cell line U251 increased migration [143].

The aforementioned studies implicated Cx43 expression in two processes of gliomagenesis, cell growth and migration. Majority of these studies used the rat C6 glioma cell line to investigate Cx43’s role in gliomagenesis. The C6 glioma cell line was generated artificially by injecting adult Wistar-Furth rats with the mutagen N-nitrosomethylurea [109]. The C6 cells share some histopathological features with human GBM in that they display focal invasion into brain tissue when implanted in Wistar rats [190]. Examination at the cellular level showed signs of necrosis, nuclear polymorphism, and high mitotic rates similar to human GBM [191]. The genetics of the C6 cell line are similar to humans in that mutations are observed in the p16/CDKN2A/NK4A locus however it expresses a wild type p53 along with PTEN expression; p53 is a tumor suppressor gene and is one of the most frequently mutated genes in human GBM
Mutation or deletion of the tumor suppressor gene p53 is a key signalling event in the genesis of GBMs [100]; therefore the presence of wildtype p53 in C6 glioma cells highlights a caveat of using this cell line as a model system. This suggests that the evolution of C6 glioma may be quite different from that of human GBMs; to circumvent this problem we made use of human GBM cell lines that were cultured from patients that suffered from GBM.

The Sanger Cancer Institute recently sequenced the exome of several well-established cancer cell lines; these included the human GBM cell lines in our panel. Through this work they were able to identify several mutations in the cell lines, hence permitting us to make an informed selection for an in vitro GBM model system. We have compiled the mutations present in the different cell lines in Table 2.1.

Although previous studies have used human glioma cell lines to examine Cx43 and gliomagenesis, they did not perform a thorough characterization of Cx43 protein and function in these cell lines. Instead these studies used low endogenous Cx43 expression determined by Western blots as the main criteria for selecting an in vitro human GBM model system [114]. We expanded the previous findings by characterizing Cx43 expression and function in a panel of human glioma cell lines. Through our characterization of a panel of high grade human glioma cell lines we discovered that Cx43 expression levels vary between the cell lines. This was surprising since the prevailing dogma was that as tumorigenicity increases the levels of Cx43 protein decrease. This suggests that although there may be an overall decrease in Cx43 expression in high grade glioma, a population of tumor cells can still express Cx43 at relatively high levels.

We found that in addition to localizing at cell-cell contacts a large population of Cx43 was localized in intracellular vesicles near the perinuclear region. In fact, in cell lines LN18 and
T98G the majority of the Cx43 protein was found in intracellular vesicles. Consequently, GJIC varies between these human glioma cell lines, with LN18 and T98G having the lowest GJIC. The remainder of the cell lines had GJIC proportional to Cx43 protein expression. Thus, our results suggest that the decrease in expression is not the only factor to consider when examining Cx43’s role in gliomagenesis. This aberrance in localization could also be a contributing factor in gliomagenesis. Aberrant accumulation of Cx43 in the cytoplasm has been observed in other cancers both in vitro and in vivo [78]. Intracellular localization of Cx43 has been reported in prostate, skin, and liver cancer tissues [87, 193, 194]. In addition, intracellular localization of Cx43 has been observed in prostate cancer cell lines suggesting that this mislocalization could also be contributing to the decreased GJIC observed in human prostate cancer [195]. It should be noted that the authors did not co-stain with specific markers of the endomembrane system to determine where exactly Cx43 was accumulating in the cell. Mutant Cx43 proteins associated with genetic diseases such as oculo-dento-digital dysplasia display intracellular accumulation [78, 196]. However, the Cancer Cell Line sequencing database did not report mutations in the Cx43 gene for any of the cell lines we screened (Table 2.1). We need to do co-staining with various makers of the endomembrane system to determine exactly which compartment of the cell Cx43 is accumulating in. From this we could deduce if Cx43 is being accumulated due to a problem in trafficking or increased degradation. For example, if Cx43 is predominantly localizing to lysosomes of glioma cells, organelles that are part of the degradation pathway, then we could deduce that perhaps Cx43 is being degraded faster in glioma cells than in wildtype astrocytes.

As mentioned earlier several studies using both the C6 glioma cell line and various human glioma cell lines implicated Cx43 expression in both cell growth and migration, however
the exact mechanism remained unclear [113-115, 134, 176, 185]. We were interested in studying the role of Cx43 in glioma migration and its contribution to gliomagenesis. We specifically wanted to know what function of Cx43 was responsible for glioma migration therefore we generated mutants that were specific to the different domains of Cx43. The dominant negative channel-dead mutant T154A, the extracellular loop mutant C61S and the C-terminal truncation mutant TrCx43 were generated to examine the different functions of Cx43. We chose the human glioma cell line LN229 for characterizing Cx43 mutants by over-expression as it had the lowest levels of Cx43 that sparsely localized at cell-cell junctions. In addition to the low endogenous Cx43 levels LN229 also has mutations in genes that are components of core signalling pathways in GBM such as p53 gene (Table 2.1).

We noticed that with the exception of the C61S mutant, expression of wildtype Cx43, T154A and TrCx43 mutants localized at areas of cell-cell contacts. A prominent population of C61S mutant protein was observed at the perinuclear region of the cell. This was further corroborated by the reduced GJIC observed in cells expressing C61S. Our data suggest that the extracellular loops are not only important for docking gap junctions between cells but also play a role in proper trafficking and localization of the protein. The extracellular loops have been implicated in proper localization of Cx43 protein to the plasma membrane. Mutations in the second extracellular loop of Cx43 have been shown to disrupt proper localization of the protein and the mutant proteins accumulate in intracellular vesicles [197]. Given that the C61S mutant protein was not able to localize properly at cell-cell junctions we were unable to use this mutant to examine the role of extracellular loops in glioma migration. Unlike the C6 cell line, over-expressing wildtype Cx43, T154A, and TrCx43 in LN229 did not affect proliferation or cell migration.
As mentioned before the C6 rat glioma cell line was generated by treatment with a mutagen, whereas LN229 human glioma cell line was cultured from GBM obtained from a patient; the difference in species and how the cells were derived could account for why we did not observe a phenotype in LN229 cells. Interestingly, of the cell lines examined, LN229 cells have the lowest Cx43 protein expression and relatively high migration levels, whereas cell lines with higher Cx43 levels had comparable migration levels to LN229. This further suggests that Cx43 expression level is not the only factor that modulates glioma migration.

Through our characterization of human glioma cell lines we discovered that not all high grade glioma cells lines have low levels of Cx43 expression. In addition, most of the human glioma cell lines have a certain amount of Cx43 protein in the perinuclear region which may also be contributing to the low GJIC observed in GBM. We used LN229 as a model for characterizing Cx43 mutants by over-expression and found that C61S does not localize properly to cell-cell junctions. The dominant negative channel dead mutant T154A and C-terminal truncation mutants localized in an immunofluorescence pattern that was similar to that of wildtype Cx43. We learned from our findings that a suitable in vitro cell line to study the function of Cx43 in glioma migration should express Cx43 at cell-cell junctions and establish functional gap junctions (Chapter 3). In addition it should be noted that the panel of cell lines have multiple mutations in key regulatory genes (Table 2.3) hence it is possible that manipulating Cx43 expression can have variable effects depending on the genetic mutations already present in the cell line.
<table>
<thead>
<tr>
<th>Human Glioma Cell Line</th>
<th>WHO Grade Designation</th>
<th>Tumorigenic</th>
<th>Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>A172</td>
<td>IV</td>
<td>No</td>
<td>CDKN2A, PTEN, ARHGAP26, ARID2, CNOT3, EGFR, FANCA, NUMA1, RB1, WRN</td>
</tr>
<tr>
<td>LN18</td>
<td>IV</td>
<td>yes</td>
<td>TP53, p16, p14ARF, APC, BCR, BRCA2, CAMTA1, CD274, EP300, FOXP1, JAK1, JAK3, MED12, MLL3, MLLT4, MYST3, NOTCH1, PTPRC, ROS1, TCF12, TCF7L2, TIF1, TRAF7</td>
</tr>
<tr>
<td>T98G</td>
<td>IV</td>
<td>No</td>
<td>PTEN, APC, BLM, BRCA2, CTNNB1, ERC1, FBXO11, IL7R, MLL3, PCM1, PDE4DIP, PER1, PTPRC, SETD2, SRSF2, SRSF3, TCF12, TP53</td>
</tr>
<tr>
<td>U118</td>
<td>IV</td>
<td>yes</td>
<td>CDKN2A, PTEN, TP53, AKAP9, BCL11A, CARS, CCND2, DNMT3A, ECT2L, ERCC3, IL7R, JAK2, MECOM, PDGFRA, TRRAP</td>
</tr>
<tr>
<td>U138</td>
<td>IV</td>
<td>No</td>
<td>CDKN2A, PTEN, CDKN2C, AFF3, ATRX, CLTCL1, NF1, NOTCH2, NSD1, PCM1, TRAF7, XPC</td>
</tr>
<tr>
<td>U87</td>
<td>IV</td>
<td>yes</td>
<td>CDKN2A, PTEN, CDKN2C, AFF3, ATRX, CLTCL1, NF1, NOTCH2, NSD1, PCM1, TRAF7, XPC</td>
</tr>
<tr>
<td>Human Glioma Cell Line</td>
<td>WHO Grade Designation</td>
<td>Tumorigenic</td>
<td>Mutations</td>
</tr>
<tr>
<td>-----------------------</td>
<td>-----------------------</td>
<td>------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Hs683</td>
<td>III</td>
<td>No</td>
<td>SMARCA4, EBFR, ERBB2, ETV5, MEN1, NPM1, PDE4DIP, SF3B1, TP53</td>
</tr>
<tr>
<td>LN229</td>
<td>IV</td>
<td>yes</td>
<td>TP53, p16, p14ARF, ALDH2, CACNAID, COL1A1, ERBB1, FBXO11, H3F3A, JAK3, KDR, LIFR, MAF, NCOA1, PDE4DIP, PHF6, PTPN11, RAD21, SOCS1, TPR, TRRAP</td>
</tr>
<tr>
<td>Sw1088</td>
<td>III</td>
<td>yes</td>
<td>CDKN2A, PTEN, TP53, AFF4, ARHGAP26, ARID1A, BIRC3, CREBBP, FANCA, FANCF, H3F3B, MLL3, MYD88, NRAS, PER1, PTCH1, PTPRC, RANBP17, SETBP1, SUFU, TRIM33</td>
</tr>
<tr>
<td>U251</td>
<td>IV</td>
<td>Data not found</td>
<td>FANCD2, GAS7, IL7R, MLL3, NF1, NUP214, PAX7, PDE4DIP, PTEN, TP53</td>
</tr>
</tbody>
</table>

Table 2.1: Grade and mutation of human glioma cell lines that were screened.

These cell lines were obtained from ATCC, and the mutation information was obtained from [http://cancer.sanger.ac.uk/cancergenome/projects/cell_lines/](http://cancer.sanger.ac.uk/cancergenome/projects/cell_lines/).
<table>
<thead>
<tr>
<th>Mutants</th>
<th>Affected region</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular loop</td>
<td>Mutate conserved cysteine on the extracellular loop</td>
<td>Blocked gap junction due to lack of adhesion; trafficking defects</td>
<td>[180]</td>
</tr>
<tr>
<td>C61S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Third TM domain</td>
<td>Mutate conserved threonine on the third transmembrane domain.</td>
<td>Disruption of pore → No hemichannel function</td>
<td>[177]</td>
</tr>
<tr>
<td>T154A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-terminal</td>
<td>Truncation of C-terminal tail at positions 242-382</td>
<td>Loss of important phosphorylation sites, and protein interaction sites.</td>
<td>[128, 135, 181]</td>
</tr>
<tr>
<td>TrCx43</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.2: Summary of Cx43 mutants used in this study.**
<table>
<thead>
<tr>
<th>Human glioma cell lines</th>
<th>Cx43 protein expression (Western blot)</th>
<th>Cx43 subcellular localization (Immunocytochemistry)</th>
<th>GJ coupling (Preloading assay)</th>
<th>Migration in wound healing assay – 8 hours’ time point</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN229</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Highest migration distance</td>
</tr>
<tr>
<td>LN18</td>
<td>Low</td>
<td>Low – localization in cytoplasmic vesicles</td>
<td>Low</td>
<td>Moderate</td>
</tr>
<tr>
<td>U87</td>
<td>High</td>
<td>High- localization to both cytoplasmic vesicles and the cell-cell junctions</td>
<td>high</td>
<td>Cells don’t form confluent monolayer therefore were not amenable to wound healing.</td>
</tr>
<tr>
<td>U118</td>
<td>High</td>
<td>High- localization to both cytoplasmic vesicles and the cell-cell junctions</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>U138</td>
<td>High</td>
<td>High- localization to both cytoplasmic vesicles and the cell-cell junctions</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>U251</td>
<td>moderate</td>
<td>moderate- localization to both cytoplasmic vesicles and the cell-cell junctions</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td>A172</td>
<td>Moderate</td>
<td>moderate- localization to both cytoplasmic vesicles and the cell-cell junctions</td>
<td>Moderate</td>
<td>Experiment not done</td>
</tr>
<tr>
<td>Hs683</td>
<td>Expression is not consistent between passages</td>
<td>localization to both cytoplasmic vesicles and the cell-cell junctions</td>
<td>moderate</td>
<td>low</td>
</tr>
<tr>
<td>Sw1088</td>
<td>Expression is not consistent between passages</td>
<td>localization to both cytoplasmic vesicles and the cell-cell junctions</td>
<td>Experiment not done</td>
<td>Experiment not done</td>
</tr>
<tr>
<td>T98G</td>
<td>Low</td>
<td>Low – localization in cytoplasmic vesicles</td>
<td>Low</td>
<td>Lowest migration</td>
</tr>
</tbody>
</table>

Table 2.3: Summary of the panel of human glioma cell lines that were screened.
Chapter 3: Reduction in gap junction intercellular communication promotes glioma migration

3.1 Introduction

Glioblastoma multiforme (GBM) is a fatal glioma thought to arise from glial precursor cells or dedifferentiated astrocytes [198]. The World Health Organization (WHO) categorizes gliomas into four grades based on histological differences: grade 1: pilocytic astrocytoma; grade 2: diffuse astrocytoma; grade 3: anaplastic astrocytoma; grade 4: glioblastoma multiforme [99, 199]. The current treatment for glioma is resection of the tumor, followed by chemotherapy and radiation therapy [200, 201]. Even with such radical treatment, patients with GBM suffer from recurring tumors which arise due to the invasive nature of glioma cells. In addition to the histological changes, several molecular changes take place in the process of gliomagenesis [103, 202, 203].

Previous studies have shown a decrease in the expression of gap junction protein Cx43 in high grade gliomas [64, 78, 107, 114]. Our lab has also shown a decrease in Cx43 expression in high grade human gliomas in tumor microarrays (see section 4.3). Cx43 is the major gap junction protein in astrocytes; gap junctions directly link the cytoplasm of adjacent cells thus establishing a glial syncytium. Gap junctions between cells allow for passage of ions and small molecules such as Ca\(^{2+}\), cAMP, ATP, glucose and glutamate [204, 205]. The channel function of Cx43 has been shown to be regulated by phosphorylation of the C-terminal tail [206], which has several
phosphorylation sites that serve as substrate to a number of kinases including Src, MAPK and PKC kinases [76].

Deciphering the role of Cx43 in glioma migration is complicated by the fact that two types of gap junctions exist *in vivo*: homocellular gap junctions formed between glioma cells, and heterocellular gap junctions formed between glioma and host cells. *In vivo* studies with rats have shown that glioma cells can establish gap junctional intercellular communication (GJIC) with astrocytes in the brain, which aids in their invasion [67, 207]. *In vitro* studies have shown that blocking the channel activity by carbenoxolone in GL15 human glioma cell line increased migration on ECM proteins but decreased migration on astrocytes and brain slice cultures [133]. In addition, U251 human glioma cells showed an increase in migration when Cx43 expression was decreased, but showed an increase in migration when brain slices were used as a substrate [121, 143]. These findings show paradoxical roles for homocellular and heterocellular gap junctions.

In addition to the channel function of Cx43, the C-terminal tail has also been implicated in modulating migration. The C-terminal tail of Cx43 has several phosphorylation sites that are involved in regulating the protein’s life cycle, channel function and an association with the actin cytoskeleton [181, 208, 209]. We have previously shown that in rat C6 glioma cells the C-terminal tail was responsible for modulating migration [134]. In addition, the C-terminal tail has also been shown to cause changes in the actin cytoskeleton [135]. We have also shown that the C-terminal tail is needed for neuronal migration [182]. In addition, Cx43 and its C-terminal has been shown to influence B-cell spreading and adhesion [210], a process which consists of dynamic changes in the actin cytoskeleton.
To understand the role of homocellular gap junctions in glioma migration we used short hairpin RNA to reduce endogenous Cx43 in the human glioma cell line U118. We chose U118 glioma cell line for two reasons: (1) the cells express Cx43 that make gap junctions at cell-cell contacts (Figure 2.3 and 2.4), (2) U118 mutation profile consisted of mutation in p53 and loss of PTEN, both important drivers of gliomagenesis (Table 1.3). We show that reducing Cx43 increases migration, and also changes the migration pattern from collective to single cells. We used specific mutants to determine the domain of Cx43 responsible for influencing migration. The T154A is a dominant negative channel mutant that significantly blocks gap junction communication [177]. The C-terminal mutant TrCx43 truncates the tail at amino acid 242, eliminating the key phosphorylation sites and protein-protein interaction sites [135]. We found that obstructing the channel function increased migration. Previous studies with mammary breast epithelial cells have shown that reducing Cx43 causes erratic migration with cells moving in a more detached manner than collectively [169]. We wanted to determine if the increase in relative migration observed in U118 glioma cells with reduced Cx43 was due to changes in migration patterns. Using real-time imaging and single cell tracking we found reducing Cx43 changed the migration pattern from collective to single cells. Our results suggest that Cx43 may play a role in collective migration of glioma cells.

3.2 Materials and methods

3.2.1 Cell lines and shRNA constructs

Human glioma U118 cell line was obtained from American Type Culture Collection
(ATCC). GIPZ lentiviral Control and shRNA constructs 1, 3, 5, 6, 7 were obtained from Open Biosystems. The mature anti-sense sequence for construct 6 was TGAGTACCACCTCCACCGG, and construct 7 was TAAATACCAACATGCACCT. The shRNA vectors also expressed GFP protein for the purpose of selection. Fugene transfection reagent (E2311, Promega, Madison, WI) was used to deliver the shRNA constructs to the cells. The transfected cells were selected by addition of puromycin (0.5 µg/ml) and the expression of GFP. Fluorescence activated cell sorting (FACs) (Life Sciences Institute Flow Cytometry, University of British Columbia) was used to obtain higher percentage of cells expressing GFP and thus the shRNA construct.

3.2.2 Site directed mutagenesis and expression of wildtype and mutant Cx43

Cx43, TrCx43 and T154A cDNAs were inserted into pMSCVpuro vectors (Clontech Laboratories). The point mutation for T154A cDNA was generated by using the Qiagen site directed mutagenesis kit. The primer sequence for T154A was GGCTTGCTGAGACCTACATCATCAGCATCC (mutation underlined). The mutant construct was validated by sequencing (NAPS Unit, Michael Smiths Laboratories, University of British Columbia). Plasmids pMSCVpuro-Cx43, pMSCVpuro-TrCx43, pMSCVpuro-T154A, and the empty vector pMSCVpuro were transfected in to HEK293 packaging cell line using Lipofectamine 2000 (Invitrogen), and the viral titres were collected as per Crespin [135]. The viral titres were placed on U118 cells expressing ShRNA6 and ShRNA7. Puromycin (1.5 µg/ml) (Sigma) was used to select the cells expressing the constructs.
3.2.3 **Western blot**

Cells were cultured to confluence in 100 mm dishes. At 100% confluence the cells were washed with cold PBS and lysed in 500 µL radioimmune precipitation lysis buffer (RIPA) containing phosphatase inhibitors (Sigma) and protease inhibitors (Roche) [134]. DNA was sheared by sonication. Protein was quantified by using the colorimetric BCA Protein Assay Kit (Pierce) and 30 µg of protein per cell line was loaded on to 10% acrylamide gels. Gel electrophoresis was carried out at 100 V. The proteins were transferred to Immuno-Blot PVDF (polyvinylidene fluoride) (Bio-Rad, Hercules, CA) at 15 V. The membranes were blocked in 5% non-fat milk in 0.1% Tween20. The antibodies used were anti-Cx43 (rabbit, Sigma, 1:4000; targets the C-terminal tail of Cx43; Cx43NT1, mouse P1E11 clone, Fred Hutchinson Cancer Research Center, Seattle, WA, 1:50, targets the N-terminal of Cx43 from aa 1-20), and anti-GAPDH (mouse, HyTest Ltd., 1:10,000). Enhanced chemiluminescence (Pierce) was used to visualize the protein bands. ImageJ software (National Institute of Health) was used to perform densitometry on the x-ray films to quantify intensity of protein bands.

3.2.4 **Immunofluorescence on monolayer**

Cells on coverslips were fixed in 4% paraformaldehyde in PBS buffer for 10 minutes at room temperature. The cells were rinsed with PBS twice and incubated in blocking solution (2% BSA + 0.3% Triton X-100) for 30 minutes. Samples were incubated with primary antibodies in working solution (1% BSA + 0.3% Triton X-100) for 1 hour at room temperature. The antibodies used were anti-Cx43 (rabbit, Sigma, 1:400; rabbit, Abgent, San Diego, CA, 1:50). Coverslips
were washed three times (10 minutes each) with PBS. Coverslips were then incubated with appropriate secondary antibodies for 1 hr, after which they were washed and mounted on microscope slides with Prolong Gold containing DAPI. Confocal microscopy using the Leica SP5 system was used to collect images of the cells.

3.2.5 Spheroid migration assay

Cells were cultured as spheroids in low attachment round bottom 96 well plates. Spheroids were grown in DMEM-F12 with B27 supplement (1X, Invitrogen) EGF (20 ng/ml, Sigma), FGF (10 ng/ml, Sigma), Glutamax (100X, Life technologies, Carlsbad, CA) for 2 days. The spheroids were then seeded on fibronectin (10 ug/ml) coated coverslips and allowed to migrate for 8 hours. After 8 hours the spheroids were imaged using the Zeiss Axiovision microscope and the migration was quantified by subtracting the area of spheroid from area of migrated cells and dividing by area of spheroid. The method was modified from [127, 211]. One way ANOVA (Sigma plot) followed by Dunn’s Method to do pairwise multiple comparisons was used to calculate significance. P-values <0.05 was considered significant.

3.2.6 Real-time imaging

Spheroids were cultured and placed on fibronectin coverslips as mentioned above. Real-time imaging of migration was performed from t = 0 hr to t = 8 hrs on the Leica SP5 microscope. ImageJ software plugin MtrackJ was used to track single cells as they migrated. The nucleus was used to track the cells. The x-y coordinates of the tracks were used to calculate distance between
migrating cells, speed, and directionality. One way ANOVA (Sigma plot) followed by Holm-Sidak method to do pairwise multiple comparisons was used to calculate significance for distance between cells as they migrate, and the directionality; P-values <0.05 was considered significant. The student t-test was used to calculate the significance for cell speed; P-values<0.05 was considered significant.

3.2.7 Wound healing migration assay

Cells (1x10^6) were seeded in 6 well plates; on day 2 the monolayer of cells was scratched. The same field in the wound was imaged at t = 0 hours and at t = 8 hours with the Zeiss Axiovision microscope. The distance migrated was calculated by subtracting the width at t = 0 hr from the width at t = 8hrs[134]. Images were analyzed by the Axiovision software. One way ANOVA (Sigma plot) followed by Student-Newman-Keuls Method to do pairwise multiple comparisons was used to calculate significance. P-values <0.05 was considered significant.

3.2.8 Adhesion assay

Cell-ECM adhesion was examined by performing an adhesion assay using methods modified from [212, 213]. Cells were serum starved overnight and seeded the next day on fibronectin coated coverslips (1D, 12 circle, Fisher Scientific, Waltham, MA). After an hour the coverslips were washed and agitated two times on a shaker at 300 rpm for 5 minutes and then fixed and stained with DAPI. Zeiss epifluorescence microscope was used to image the adhered cells. Image J was used to count the adhered cells. The experiment were repeated 4 times with
n=13 coverslips (30 field of views were captured per coverslip) for all conditions. One way ANOVA (Sigma plot) followed by Student-Newman-Keuls Method to do pairwise multiple comparisons was used to calculate significance. P-values <0.05 was considered significant.

3.2.9 **Aggregation assay**

Cells were serum starved overnight. Agar coated plates were prepared by adding 50 µl of hot agar (Sigma) to each well in a 96-well plate. The plates were allowed to set for 1 hr at 4°C. Cells at 20,000 cell/100 µl were seeded per well in the agar coated plates and incubated for 24 hours at 37°C. The agar coating would prevent the cells from adhering to the plastic surface of the plate; hence the cell aggregates formed due to cell-cell adhesion only. GFP fluorescence was used to image the aggregates since the cells expressing the shRNA constructs will also express GFP. Images of the aggregates were acquired through epifluorescence microscopy and the size of the aggregates was quantified by ImageJ software. Only aggregates >3000 um² were counted in the analysis. This method was taken from [214]. One way ANOVA (Sigma plot) followed by Dunn’s Method to do pairwise multiple comparisons was used to calculate significance. P-values <0.05 was considered significant.

3.2.10 **Preloading assay**

GJIC was assessed using the preloading assay [184]. Briefly, parental U118 cells were cultured to 100% confluence to serve as recipient cells. Control, ShRNA6 and ShRNA7 cells were loaded with preloading solution (1 µl of Calcein Red-Orange to 1 ml of 0.3 M glucose), and
incubated for 20 minutes at 37°C. The loading solution was removed and the cells were washed three times with 0.3 M glucose. Cells were trypsinized and titrated until they were completely detached. Donor cells were seeded on to the recipient cells and incubated for 2 hours at 37°C. Zeiss Axiovision epifluorescence microscope was used to image the coupled cells. The donor cells appeared yellow due to the expression of GFP and presence of Calcein Red-Orange. Passage of Calcein Red-Orange to the recipient cells labelled them red. One way ANOVA (Sigma plot) followed by Dunn’s Method to do pairwise multiple comparisons was used to calculate significance. P-value<0.05 was considered significant.

3.3 Results

3.3.1 Reducing Cx43 changes the migration pattern from collective to single cell

We screened a panel of human glioma cell lines with several of the key mutations found in GBM for Cx43 expression, subcellular distribution and GJIC (Figure 2.2-2.4). In most human glioma cell lines we examined, Cx43 expression was mainly intracellular, however for the U118 cell line Cx43 also localized to the plasma membrane at cell-cell contact sites (Figure 2.3). In addition, U118 cell line displayed the highest GJIC indicating that Cx43 at cell-cell junctions made functional gap junctions (Figure 2.4 A and B). The aforementioned characteristics plus the mutations in the p53 and PTEN gene made U118 a suitable cell line to study Cx43 in glioma migration. We used wound healing and spheroid migration assays to investigate changes in migration due to Cx43 expression. The spheroid migration assay was carried out on fibronectin, an ECM protein that is suggested to be secreted by host cells around blood vessels in GBM.
facilitating invasion [215-219]. We used this assay for the majority of this study since the 3D structure of a spheroid is more akin to the structure of tumors in vivo. As well it was a useful assay for real-time imaging since it allowed us to examine how tumor cells migrate away from a 3D tumor core. A panel of five ShRNA constructs that targeted different sites of the Cx43 gene was used to knockdown Cx43 expression in U118 human glioma cells (Figure 3.1 A). Two different ShRNA constructs, ShRNA6 and ShRNA7, produced the highest degree of Cx43 protein expression knockdown in U118 cells as demonstrated by Western blot and immunocytochemistry (Figure 3.1 B and C). Furthermore GJIC was significantly reduced in U118 cells expressing ShRNA6 and ShRNA7 constructs, and therefore were chosen for the migration studies (Figure 3.2 A and B).
Figure 3.1: Expression of a panel of anti-Cx43 ShRNA constructs reduces Cx43 expression.
A. Five different anti-Cx43 shRNA constructs (obtained from Open Biosystems) were transfected in U118 cell line using Fugene reagent (Promega). Varying levels of protein knockdown were produced. ShRNA6 and ShRNA7 had the highest level of Cx43 protein reduction. B. U118 cells expressing ShRNA6 and ShRNA7 constructs were FACs sorted (carried out by Justin Leung in Life Sciences Institute Flow Cytometry, University of British Columbia) by GFP expression to obtain a higher population of cells expressing the ShRNA constructs; reduced Cx43 is shown by Western blot. ImageJ was used to quantify the intensity of the bands. The numbers represent the average of 3 blots. C. Reduction in Cx43 (red) was also observed by immunofluorescence. Cells expressing the ShRNA constructs were GFP positive (green), the nuclei were stained with DAPI (blue); scale bar = 50μm.
Figure 3.2: Reducing Cx43 expression decreases GJIC.

A. Control, ShRNA6 and ShRNA7 cell lines (expressing GFP (⁎) were loaded with Calcein red-orange and appear yellow (arrow). Passage of Calcein red-orange to recipient parental U118 cells
was an indication of GJIC; scale bar = 50µm. B. A significant reduction in coupled cells was quantified for both ShRNA6 and ShRNA7 cells indicating that GJIC had been reduced. This experiment was repeated 3 times, 200 cells were counted per condition. One way ANOVA (Sigma plot) followed by Dunn’s Method to do pairwise multiple comparisons was used to calculate significance, **p < 0.001.

In the wound healing assay, U118 cells expressing ShRNA6 and ShRNA7 migrated further than the control cells after 8 hours (Figure 3.3 A and B). This result was confirmed with a spheroid migration assay that had the advantage of providing a 3D architecture similar to in vivo tumors with a core and a defined border. In this assay, cells from ShRNA6 and ShRNA7 glioma spheroids migrated further than control cells on fibronectin (Figure 3.4 B and C). To confirm the increase in migration is due to the decrease in Cx43 in the knockdown cells, we expressed full-length Cx43 in ShRNA6 and ShRNA7 cells (Figure 3.4 A) and observed a reduction in the relative migration comparable to control cells (Figure 3.4 B and C). The ShRNA constructs were designed to bind to the 3’ UTR region of the endogenous Cx43 mRNA so it only targets the endogenous mRNA but has no effect on exogenous wild-type Cx43 translated from cDNA plasmid.
Figure 3.3: Reducing Cx43 expression increases migration in a wound healing assay.

A. Control and ShRNA 6 and 7 cells were grown to confluence and then scratched. The cells were imaged at $t = 0$ (when the cells were scratched) and at $t = 8$ hours. After 8 hours ShRNA6 and ShRNA7 cells migrated farther in the wound (wound area highlighted in red). Scale bar = 100 µm. B. Compared to control cells ShRNA6 and ShRNA7 increased migration distance by
50% and 45%, respectively. This experiment was repeated three times with n=12 per condition. One way ANOVA (Sigma plot) followed by Student-Newman-Keuls Method to do pairwise multiple comparisons was used to calculate significance, * p<0.05.
Figure 3.4: Reducing Cx43 expression increases migration in a spheroid migration assay.

A. Western blot analysis shows successful expression of exogenous full-length Cx43 cDNA lacking the 3’UTR in U118-ShRNA6 and U118-ShRNA7 cells. Using densitometry we found
the Cx43 expression in control and rescue cells to be 4 times higher than ShRNA6 and ShRNA7 cells. B. Expression of Cx43 in the knockdown cells produced relative migration levels similar to control cells. Scale bar = 100 µm. C. Compared to control cells ShRNA6 and ShRNA7 cells increased migration 46% and 30%, respectively. The experiments were repeated three times with control (n=81 spheroids), ShRNA 6 (n=81 spheroids), ShRNA 7 (n=88 spheroids), ShRNA 6-Cx43 (n= 54 spheroids) and ShRNA 7-Cx43 (n=54 spheroids). One way ANOVA (Sigma plot) followed by Dunn’s Method to do pairwise multiple comparisons was used to calculate significance, * p<0.05.

Next, we examined whether there were changes in migration patterns between ShRNA6, ShRNA7 and control cells. Time lapse imaging of the spheroids at a higher magnification revealed that the control cells differ in speed, directionality and cell-cell association when compared to ShRNA6 and ShRNA7 cells (Figure 3.5). The distance between adjacent nuclei is significantly shorter in the control cells compared to ShRNA6 and ShRNA7 cells, indicating a change in migration pattern from collective to single cell (Figure 3.5 A). In control spheroids the cells seem to adhere to each other longer than in the knockdown cells. As expected, control cells migrated at a slower speed than ShRNA6 and ShRNA7 (Figure 3.5 B). Interestingly, we observed higher percentage of migrating control cells returning to the spheroid when compared to ShRNA6 and ShRNA7 cells (Figure 3.5 C).
Figure 3.5: Reducing Cx43 expression changes cell migration pattern in U118 cells.
A. Individual cells migrating away from the spheroid were tracked. Single cell tracking was done using ImageJ MtrackJ plugin; the nuclei of the cells were tracked. The distance between the nuclei of migrating cells was measured over an 8 hour time period. On average control cells migrated with shorter distance between them than ShRNA6 and ShRNA7 cells. B. The speed of cells was calculated by measuring the total distance travelled by cells divided by the total time. ShRNA6 and ShRNA7 cells migrated faster and farther than the control cells. C. Reducing Cx43 influenced the direction of migration with a higher percentage of control cells returning to the spheroid (control = 49%, ShRNA6 = 14%, and ShRNA7 = 13%). D. Cell tracks for control, and ShRNA6 cells at t = 0, 4, and 8 hours show difference in migration pattern. Control cells are migrating in a collective manner whereas the ShRNA6 cells are migrating in a more detached manner. At t = 8 hrs control cells are still in the field of view whereas ShRNA6 cells have left the field of view. The data shown here is from 3 experiments with Control n=153 cells, ShRNA6 n= 174 cells, and ShRNA7 n= 195 cells. One way ANOVA (Sigma plot) followed by Holm-Sidak method to do pairwise multiple comparisons was used to calculate significance for distance between cells as they migrate, and the directionality; *p<0.05, **p<0.005 was considered significant. The student t-test was used to calculate the significance for cell speed; *p<0.05 was considered significant. Scale bar = 50 μm.

3.3.2 Reduction in gap junctional intercellular communication increases migration

To determine the function of Cx43 responsible for mediating migration, we expressed the dominant negative channel dead mutant T154A in ShRNA6 and ShRNA7 cells (Appendix 1 A). We have previously demonstrated that expressing T154A mutant significantly reduces GJIC
Unlike the expression of full length wild type Cx43 (Figure 3.4), expression of T154A mutant in ShRNA6 and ShRNA7 cells did not reduce migration levels to that of control cells. Rather we observed migration levels comparable to ShRNA6 and ShRNA7 cells (Figure 3.6 A and B). This implies that for a cell to increase its migration rate it must either decrease Cx43 channel activity or reduce Cx43 protein expression; this coincides with the reduction in Cx43 protein expression observed in high grade glioma.

The C-terminal domain of Cx43 has been implicated in modulating cell migration and is known to interact with cytoskeletal proteins. To determine if the C-terminal tail played a role in glioma migration we used a mutant in which the C-terminal tail has been truncated after amino acid position 242 (TrCx43) (Appendix 1 B). This mutant lacks the phosphorylation and protein-protein interaction sites but still retains a significant level of channel activity [135, 220]. The expression of the TrCx43 mutant in ShRNA6 and ShRNA7 cells produced migration levels comparable to control and ShRNA6-Cx43 and ShRNA7-Cx43 (Figure 3.6 A and B). Our findings suggest that the association of Cx43 C-terminal tail with the cytoskeleton is probably not critical in controlling migration in U118 glioma cells. It is interesting to note that the lowered channel activity of the C-terminal tail mutant TrCx43 [220] rescues the knockdown phenotype as well as the wild type, indicating that a significant reduction in channel activity is needed to increase cell migration.
Figure 3.6: A reduction in Cx43 channel activity increases migration.

The expression of T154A mutant in the ShRNA6 and shRNA7 cells did not reduce migration levels to that of control cells. An increase in migration comparable to the knockdown cells was observed (A. shRNA6 27%, shRNA6-T154A 20%, B. shRNA7 23% and ShRNA7-T154A 21%). The TrCx43 mutant produced migration levels similar to control cells. The experiment was repeated three times with Control (n=61 spheroids), ShRNA 6 (n=55 spheroids), ShRNA6-T154A (n=58 spheroids), ShRNA6-TrCx43 (n=61 spheroids); Control (n=62 spheroids),
ShRNA7 (n=61 spheroids), ShRNA7-T154A (n=60 spheroids), and ShRNA7-TrCx43 (n=58 spheroids). One way ANOVA (Sigma plot) followed by Dunn’s Method to do pairwise multiple comparisons was used to calculate significance. *p<0.05 was considered significant. Scale bar = 100 μm.

To further confirm that the channel function is involved in migration, we used a complementary approach by carrying out the spheroid migration assay on parental cells in the presence of a gap junction blocker carbenoxolone (CBX) (150 μM) and its inactive analog glycyrrhizinic acid (GZA) (150 μM) (Figure 3.7 A). The presence of CBX significantly increased migration by 35% when compared to untreated control cells (Figure 3.7 B). The inactive analog was unable to produce a significant increase (14.5%) (Figure 3.7 B). Taken together, our results highlight that it is the gap junction channel function of Cx43 that is playing a role in cell migration.
Figure 3.7: Blocking gap junction communication with a pharmacological inhibitor increases migration.

A. Spheroid migration assay was carried out on control cells and cells that were treated with 150 μM CBX, after 8 hours the cells were imaged and migration quantified. B. Inhibiting gap junction communication with CBX (150 μM) increases migration levels by 31%. The inactive analog GZA was unable to produce the same degree of increase in migration (14.5 %). This experiment has been repeated 3 times with n= 44 spheroids for each condition. Student t-test was performed to calculate significance, *p<0.05 was considered significant. Scale bar = 100 μm.

3.3.3  **A reduction in Cx43 decreases cell-ECM adhesion**

We observed a switch from collective to single cell motility when Cx43 is reduced and
hence it is possible that cell-cell adhesion is affected. Since the docking of Cx43 hemichannel is known to increase intercellular adhesion [67], we examined whether the increase in migration is due to a weakening of intercellular adhesion. To examine changes in intercellular adhesion we carried out a slow aggregation assay in which there is no substrate to which cells can adhere, thus only testing cell-cell adhesion. We found no significant change in the average size of the aggregates between control and ShRNA6 and ShRNA7 cells (Figure 3.8 A and B). A reduction in Cx43 has been shown to change the subcellular localization and expression of the cell adhesion molecule N-cadherin [221], however we did not observe a change in the expression nor the subcellular localization of N-cadherin (Appendix 2 A). Our results suggest that reducing Cx43 expression in glioma cells does not affect their ability to form intercellular adhesions; however it is still unclear whether the stability of the adhesions is affected.
Figure 3.8: Intercellular adhesion is not affected after knocking down Cx43.

A. Control, ShRNA6, and ShRNA7 cells were seeded in agar coated 96 well plates for 24 hrs. GFP positive aggregates were imaged since that is a marker for ShRNA construct expression. Control cells produced aggregates of comparable size to ShRNA6 and ShRNA7 cells. B. The average size of aggregates showed no significant difference. The size of the aggregates was quantified by ImageJ software. Only aggregates >3000 um² were counted in the analysis. The experiment was repeated three times with n = 72, and 500 aggregates per condition. Scale bar = 100 μm.
In addition to intercellular adhesions, the strength of cell-ECM adhesion also affects how fast a cell will migrate [148, 150]. Therefore we investigated whether decreasing Cx43 expression leads to changes in cell-ECM adhesion by carrying out an adhesion assay with fibronectin as the ECM component. We noticed a significant decrease in adhesion for ShRNA6 and ShRNA7 cells compared to control cells after 1 hour (Figure 3.9 A and B). Western blot analysis showed no change in the expression of total β1-integrin, FAK and phospho-FAK, key proteins in cell-ECM adhesion (Appendix 3). Immunocytochemistry analysis of active β1-integrin, FAK and phospho-FAK on spheroids showed no change in the subcellular localization (Appendix 2 B and C). Our findings suggest that decreasing Cx43 decreases cell adhesion to fibronectin which may be involved in the increased migration observed in the spheroid migration assay.
Figure 3.9: A reduction in Cx43 decreases cell adhesion to fibronectin.

A. A large number of control cells adhered to fibronectin coated coverslips versus ShRNA6 and ShRNA7 cells (DAPI in blue). B. Adhesion to fibronectin was reduced in ShRNA6 and ShRNA7 cells by 21% and 34%, respectively. The experiment were repeated 4 times with n=13 for all conditions. *p<0.05 was determined by One way ANOVA (Student-Newman-Keuls Method).

Scale bar = 100 μm.
3.4 Discussion

Aggressive high grade gliomas have been reported to exhibit low expression of Cx43 [63]. A feature of aggressive glioma is their enhanced ability to migrate to healthy parts of the brain. The role of Cx43 in glioma migration is not clearly defined. There is a general agreement that heterocellular Cx43 gap junctions formed between glioma-astrocytes and glioma-endothelial cells facilitate invasion [67, 133, 207, 222]. However, the role of homocellular Cx43 gap junctions between glioma cells is debatable and dependent on the system used [133-135, 143]. Overall the role of homocellular gap junctions in glioma migration has been examined by using 2D monolayer migration assays. Therefore, we decided to use the stringent approach of loss-of-function and rescue experiments in a 3D spheroid model that better mimics invading glioma cells in vivo. To further create an assay more akin to in vivo we used the ECM protein fibronectin which is up-regulated in GBM [215]. We reduced endogenous Cx43 expression by two independent ShRNAs and then rescued it by expressing wild type or mutated/truncated Cx43; using two different migration assays we show that decreasing Cx43 expression increases glioma migration.

Our study is the first to show that reducing Cx43 in glioma cells changes the mode of migration from collective to single cell motility. In fact, the same phenotype has been observed in a study in breast epithelial cells, where reducing Cx43 expression increased cell migration by facilitating single cell motility rather than collective [169]. An increase in cell speed correlates with a switch from collective to single cell motility [223], and indeed reducing Cx43 expression increased the speed of glioma cells thus explaining the increase in relative migration. We also observed a higher percentage of control glioma cells returning to the spheroids which suggests
that reducing Cx43 allows the cells to migrate in a persistent directional manner and cover a larger area.

Cx43 is a multi-modular protein that couples cells chemically through its channel and physically through its extracellular loops. The protein’s C-terminal tail has phosphorylation sites that regulate the life cycle of the protein and also affect the channel function [133, 134, 209]. As well, the C-terminal tail is known to interact with cytoskeletal proteins [69, 181] and influence neuronal and glioma migration [134, 182]. Given that previous studies have implicated Cx43 as an adhesion molecule it is possible that the expression of Cx43 at the cell-cell junctions could be aiding cell adhesion in collective migration [67, 224]. Using specific mutants that obstructed the C-terminal tail and the channel function we demonstrated that blocking the channel function of Cx43 increased glioma migration.

To further confirm that blocking the channel function increased migration we treated control cells with CBX to pharmacologically block the channel function and indeed we observed an increase in migration compared to untreated cells. This indicates that for cells to increase migration a significant reduction of gap junctional intercellular communication must occur. Gap junctions can pass small molecules that are important in many signaling pathways, such as glucose, IP$_3$ and ATP, as well as ions such as Ca$^{2+}$ and H$^+$ [21, 225]. The passage of such important metabolites and second messengers through gap junctions assist cells in tissues to maintain homeostasis and to function in a coordinated manner. Interestingly, adrenal cells transfected with Cx43-GFP migrate in a collective sheet pattern and retain gap junctions as they migrate [159, 168]. Thus it stands to reason that the presence of gap junctions between glioma cells could be aiding to coordinate collective cell migration and when gap junctions are absent the cells transition to single cell motility (Figure 3.11).
A positive correlation between Cx43 expression and cell-cell adhesion in the rat glioma C6 cell line has been reported previously [67], however when we examined intercellular adhesion in the various U118 cells using an aggregation assay we observed no difference. Our method measured the ability of cells to form adhesions but did not measure the strength of the intercellular adhesions. Hence it is possible that reducing Cx43 weakens intercellular adhesions, permitting the cells to detach and move as single cells. Although we did not observe changes in the subcellular localization or expression of N-cadherin, the main protein that facilitates intercellular adhesion, it remains a possibility that Cx43 affects collective migration by influencing the dynamics of intercellular adhesion molecules.

Cell-ECM adhesions are formed when integrins on the cell surface bind ECM proteins such as fibronectin; they facilitate migration by providing traction and organizing signaling pathways that are involved in migration. A decrease in cell-ECM has been shown when Cx43 is decreased in the U251 human glioma cell line [143]. Similarly, we found that glioma cells with reduced Cx43 were less adhesive to fibronectin than control cells. Expression and turnover of integrins influence the strength of cell-ECM adhesions; however we found no change in the expression of total β-1 integrins. We also did not observe changes in the localization of active β-1 integrin. Since there were no changes observed in protein expression and localization it is possible that there are changes in β-1 integrin turnover that could be promoting increased migration. Cell-ECM adhesion provides traction for the cell and affects the speed of migration [148, 156]; our results suggest that reducing Cx43 lessens the traction on fibronectin allowing cells to migrate faster.

Our study highlights a new role for Cx43 as a determinant of migration patterns in gliomas. In addition we show that this is mediated by the gap junction channel function of Cx43.
Collective migration requires cells to remain physically connected and chemically synchronized, given that gap junctions meet both of these criteria, Cx43 may be an important protein in this mode of migration.

**Figure 3.10: Cx43 expression facilitates collective migration.**

A. Endogenous expression of Cx43 protein forms gap junctions between glioma cells (Cx43 in red). Gap junctions physically connect the cells and allow exchange of molecules which facilitates collective migration. B. Decreasing Cx43 expression in glioma cells weakens the connection between them and reduces the passage of molecules thus promoting single cell motility.
Chapter 4: Characterizing the role of Cx43 channel function in invasion *in vivo*

4.1 Introduction

Gliomas are the most lethal intracranial tumors due to infiltration of small tumor clusters in healthy brain tissue that preclude complete surgical resection [98]. In the brain, chronic inflammation due to the presence of glioma cells results in the recruitment of cell types such as astrocytes and microglia, and a glial scar consisting of reactive astrocytes usually encases gliomas and brain metastases [226-228]. Although the physiological role of reactive astrocytes is to protect healthy neurons from cell death [229], they also appear to increase the resistance of tumor cells against chemotherapeutic drugs [228]. Reactive astrocytes express numerous proteins that confer a survival advantage to tumor cells in a paracrine fashion [230-232]; the secretion of chemokines by hypertrophic astrocytes [233, 234] provides a permissive microenvironment for tumor survival and expansion [235, 236]. Specifically, reactive astrocytes have been shown to secrete Interleukin 6 that helps tumor cells to resist apoptosis [237], connective tissue growth factor and metalloproteases which facilitate glioma invasion [238-240].

The gap junction protein connexin43 (Cx43) is widely expressed in adult astrocytes [241], and exhibits increased expression in reactive astrocytes induced by various brain pathologies [242, 243]. Reduced Cx43 expression in tumor cells is generally linked to accelerated growth *in vitro*, but its role in cell migration is not well defined [121]. Over-expression of Cx43 has been reported to promote migration in channel-dependent and independent manners [67, 134, 135]. On the other hand, others have shown that inhibition of
GJIC enhances cell motility but the opposite is true when glioma cell motility is examined in the presence of stromal cells such as astrocytes [133, 244]. Therefore, optimal migration appears to occur with low homocellular GJIC and high heterocellular GJIC. Cx43-mediated GJIC also appears to alter astrocyte morphology in an in vitro co-culture of glioma cells with astrocytes [145, 245], suggesting that bi-directional signaling exists between glioma cells and astrocytes; however it remains unclear how these interactions modify the invasive properties of glioma cells in vivo. In addition to GJIC, Cx43 strengthens adhesive connections via its extracellular loops [67, 68, 224] and also regulates cytoskeletal dynamics via the interaction of its C-terminal tail with various intracellular signaling molecules [147]. The C-terminal of Cx43 also contains protein-protein interaction sites [124] that may serve a role in hemichannel gating [246-248].

Upregulation of Cx43 has been detected in astrocytomas and peri-tumor parenchyma [249-251]. This increase in Cx43, which could be due to an upregulation of Cx43 mRNA by the astrocytes [252] or changes to its antigenicity and subcellular distribution [243, 253], raises the question of whether Cx43 contributes to the microenvironment at the tumor-host interface and participates in the formation of an invasive niche [254, 255]. Specifically, we wanted to determine how heterocellular gap junctions between glioma and host cells may be influencing glioma invasion.

While the U118 human glioma cell line was a suitable model for discerning the role of homocellular gap junctions it was not ideal for in vivo experiments to study the contributions of heterocellular gap junctions in glioma invasion as it requires using nude mice with compromised immune system. The immune system has been shown to play a role in gliomagenesis, specifically immune cells such as lymphocytes, neutrophils, and macrophages have been observed in GBM tissues [102]. In addition within the tumor microenvironment immune cells
called microglia can penetrate the tumor and cause oxidative damage that would subsequently cause genetic and epigenetic changes that contribute to the process of gliomagenesis [256]. Specifically, microglia cells have been shown to influence the invasion of glioma cells a key feature of high grade malignancy. Gliomas show reduced invasion on microglia depleted brain slices when compared to control brain slices [257, 258]. Thus given that immune cells play an important role in glioma malignancy we wanted to use an in vivo glioma model with an intact immune system. Therefore we employed a syngeneic mouse intracranial model using the mouse GL261 glioma cells and C57BL/6 mice. In addition to an intact immune system we also had the advantage of discerning the role of astrocytic gap junctions as we had access to Cx43 conditional knockout mice made in the C57BL/6 background. Using a syngeneic intracranial mouse model, we demonstrate that Cx43-mediated intercellular communication between astrocytes has a considerable role in the dissemination of glioma cells into the brain parenchyma. We further show that Cx43-mediated intercellular communication between glioma cells and astrocytes is not essential for astrocytic Cx43 to mediate its pro-invasive effect in vivo.

4.2 Materials and methods

4.2.1 Cell lines

I maintained mouse GL261 glioma cells (NCI-Frederick DCTD) in DMEM supplemented with 10% FBS and transfected with pcDNA-mCherry plasmid with lipofectamine 2000 (Invitrogen). I generated the T154A mutant as described previously [220] and stably transfected into mCherry-expressing GL261 cells. Dr. Wun Chey Sin performed the Cx43
knockdown with ShRNA in pGIPZ vector carrying a turboGFP tag (Open Biosystem). turboGFP is cloned from \textit{Pontella} plumata. Its excitation/emission max is 482/502 nm therefore it is visible sooner than the other GFP protein; it is also brighter \cite{259}. V3LmM\_526066 was the most effective ShRNA construct while ShRNA construct V3LmM\_526068 had no effect and was used as a control.

4.2.2 \textbf{Immunofluorescence}

The following protocol was carried out by me and Dr. Wun Chey Sin. Cryosections of brain tissue 10 \(\mu\)m thick were briefly washed with PBS and blocked for 1 hr. at room temperature in 2\% BSA and 0.3\% Triton X-100 in PBS. Sections were then incubated overnight at 4 \(^\circ\)C in 1\% BSA and 0.1\% Triton X-100 in PBS with either anti-Cx43 (rabbit, Sigma, 1:800), anti-GFAP (G9269, rabbit, Sigma, 1:200,) or anti-GFAP (G3893, mouse, Sigma, 1:800). Anti-GFAP was used at a concentration to detect only reactive astrocytes with upregulated GFAP expression \cite{242}. Sections were then probed with Alexa Fluor-conjugated secondary antibodies (1:500, Invitrogen), mounted in Prolong antifade mounting media (Invitrogen) with 4’ 6-diamidino-2-phenylindole (DAPI) or TO-PRO-3 (Invitrogen) and visualized by confocal microscopy with either an Olympus Fluoview1000 or a Leica TCS SP5 II Basic VIS system. The extent of astrogliosis (denoted by the distance from the tumor border to the outermost edge of glial scar with detectable GFAP staining) was determined by tracing the area of the glial scar using Image J software, and divide this area with the length of the tumor border in at least 3 separate sections.
4.2.3 Western blot

I carried out the Western blot protocol from section 2.2.2 with the following modifications. GL261 cell pellets or glioma tissues were lysed in a RIPA buffer containing 0.1% SDS, 1% IGEPAL, 0.5% Sarkosyl, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl supplemented with protease inhibitors (Roche Applied Science) and phosphatase inhibitors (Sigma). Rabbit Anti-Cx43 (1:4000, Sigma) and anti-GAPDH (1:10,000, Hytest) were used as primary antibodies, followed by either anti-rabbit-HRP (1:4000) or anti-mouse-HRP (1,10000) (Sigma) as secondary antibodies. Protein bands were detected with Amersham ECL western detection reagents (GE Healthcare).

4.2.4 Quantification of invasiveness of intracranial GL261 cells

The following protocol was carried out by me and Dr. Wun Chey Sin (for data in Appendix 5). The invasiveness of GL261 cells was determined using 3 different measurements with Image J available at http://rsbweb.nih.gov/ij/. Firstly, an infiltrating tumor edge was identified by the presence of multiple high cellular density layers, visualized by increased staining of either cresyl violet or TO-PRO-1 (Invitrogen), indicative of the presence of invading cells in a 200 μm width band of brain parenchyma circumscribing the tumor mass [260]. The invasive index was presented as a percentage of the length of infiltrative edge over the circumference of the tumor border. Secondly, invasiveness was measured as the distance from the edge of the tumor to the 5 most distantly invaded cells around the circumference of the tumor [261]. Each individual measurement from each cell is shown as a separate point. Finally, the
density of invading glioma cells within a 200 μm width band of brain parenchyma was also manually counted and expressed as number of cells per mm$^2$ of tumor border. Images were analyzed blind (without knowing the sample being analyzed). At least 3 distinct tumor sections located in different regions of the tumor were analyzed for each sample.

4.2.5 Preloading assay

I evaluated GJIC between glioma cells and astrocytes as per the method described previously [134]. Donor GL261 cells, pre-loaded with 10 μM of lipophilic dye Dil (Sigma) and 5 μM Calcein-AM (Molecular Probes) that is permeable to gap junctions, were allowed to adhere onto a monolayer of recipient cells of either GL261 cells for 8 hr., or wild type mouse astrocytes overnight at 37 °C in 150 μM of carbenoxolone (CBX) to inhibit premature transfer of Calcein dye. Unattached donor cells and CBX were removed by rinsing with the culture media and the monolayer was incubated for a further 2 hr at 37 °C to allow dye transfer (i.e. coupling) to occur. Epifluorescence microscopy was used to visualize the total number of cells directly or indirectly coupled to a single donor cell.

4.3 Results

4.3.1 Astrocytic Cx43 facilitates detachment of glioma cells from the tumor core

Reactive astrocytes are one of the major cell types found in gliomas [226, 227, 262], and they are often indistinguishable from glioma cells in pathological samples [262]. Cx43 is
upregulated in reactive astrocytes in response to various cellular insults [242, 263] and in tumor-associated astrocytes [264]. These host-derived cells were identified by their lack of red fluorescence when we implanted mcherry-labeled mouse GL261 glioma cells into the striatum of syngeneic C57B/6 mice (Appendix 6 A).

Dr. Wun Chey Sin carried out experiments to determine whether upregulation of Cx43 in reactive astrocytes affects the behavior of glioma cells, she used a Nestin-Cre mouse line crossed with Cx43 floxed mice to selectively eliminate expression of Cx43 in host astrocytes. The knockout was confirmed by the lack of Cx43 immunoreactivity at the glioma periphery in Cx43-null brain (Appendix 6 B). Analysis of multiple cresyl violet–stained coronal sections revealed that tumors in the absence of astrocytic Cx43 were more likely to be circumscribed with well-defined boundaries, lacking the jagged periphery of an invasive edge (Appendix 6 C). Indeed, we observed a significant reduction in the percentage of infiltrative tumor edge in Cx43-null (39.6 ± 3.5; n=5) compared to control (61.2 ± 5.3; n=6) brains. In addition, the density of mCherry-positive GL261 cells in brain parenchyma adjacent to the tumor core was significantly lower in Cx43-null (22.5 ± 6.8 per mm²; n=5) than in the wild-type (72.4 ± 15.7 per mm²; n=5) mice (Appendix 6 D). Therefore, astrocytic Cx43 appears to mediate signaling pathways that facilitate glioma invasion.

4.3.2 **Reduction of Cx43 in tumor cells attenuate glioma invasion *in vivo***

Cx43-deficient brains have attenuated overall astrocytic intercellular communication [265]. Therefore, the reduced ability of glioma cells to infiltrate brain parenchyma in the absence of astrocytic Cx43 may be due to elimination of heterocellular GJIC between glioma cells and
astrocytes previously shown to be critical in enhancing glioma invasion [67, 133, 143, 145, 222, 244]. Although GL261 glioma cells expressed low levels of Cx43 compared to mouse astrocytes (Figure 4.1 A), they retained the ability to communicate with each other and with astrocytes in a Cx43-dependent manner; I assessed this by a dye-coupling assay that measured the transfer of Cx43-permeable Calcein dye from donor cells to recipient monolayer (Figure 4.1 B). Inhibition of coupling by carbenoxolone (CBX), a gap junction channel blocker, confirmed the transfer of dye between cells was due to the presence of functional gap junctions (Figure 4.1 C). To investigate whether elimination of heterocellular GJIC would decrease glioma invasion, Dr. Sin further reduced Cx43 level in glioma cells by shRNA. Successful knockdown was confirmed by the decrease of Cx43 protein (Figure 4.1 D) and gap junction plaques (Figure 4.1 E) in Cx43shRNA-transfected cells. Implantation of GL261 cells with reduced Cx43 into wild type mice produced tumors with a significant reduction in the percentage of infiltrative edge (38.4 ± 6.05; n=8) compared to parental GL261 tumors (59.7 ± 6.05; n=8) (Figure 4.1 F). There was also a corresponding decrease in the number of mCherry-positive GL261 cells in brain parenchyma adjacent to the tumor formed by Cx43shRNA-transfected GL261 cells (19.7 ± 4.7 per mm²; n=6) when compared to parental GL261 tumors (38.4 ± 8.1 per mm²; n=5) (Figure 4.1 G).
Figure 4.1: Reduction of Cx43 in glioma cells attenuates glioma invasion.
A. Reduced expression of Cx43 in GL261 cells compared to mouse wild-type astrocytes. GAPDH was used as a loading control. B. Transfer of gap junction-permeable Calcein dye (green) from donor GL261 cells labeled with lipophilic membrane dye DiI (red) to recipient monolayer that were either GL261 cells or mouse astrocytes. The confluence of the monolayer is confirmed with a DIC image as shown in the merged picture. CBX is an inhibitor of GJIC. C. Percentage of adhered donor GL261 cells that are coupled to the confluent monolayer of either GL261 cells or mouse astrocytes. Data were pooled from at least 3 independent experiments. CBX is an inhibitor of GJIC. D. Reduction of Cx43 protein (25% of Control as determined by Image J) in Cx43shRNA transfected GL261 cells. GAPDH was used as a loading control. E. Reduction of Cx43-positive plaques (red) in Cx43shRNA cells. F. Knockdown of Cx43 in glioma cells attenuates glioma invasion. Quantification of glioma invasion by the percentage of tumor border with infiltrative cells. *p < 0.05. G. Decreased number of mcherry-positive GL261 glioma cells in the brain parenchyma in Cx43shRNA (n=6) compared to wild-type brains (n=5). *p < 0.05. Data are shown as (mean ± SEM). Experimental data in panels B-C were obtained by me. Panels A, D-G were obtained by Dr. Wun Chey Sin.

4.3.3 Cx43 mediates glioma invasion in a channel-independent manner

Cx43 has been demonstrated to affect cell migration via channel-dependent and independent mechanisms [67, 68, 147, 224]. To disrupt GJIC I expressed a dominant-negative channel-defective T154A mutant containing intact extracellular loops and C-terminal tail [177, 220] in GL261 cells (Figure 4.2 A). The T154A mutant localized at cell-cell contacts in GL261 cells (Figure 4.2 B) and reduced the coupling ability of GL261 cells to a level that is comparable
with the use of CBX inhibitor (Figure 4.2 C), agreeing with our previous observation with T154A expressing human glioma cells [220].

Similar to our previous results with the Nestin-Cre line (Appendix 5 C), implantation of either Mock or T154A-transfected cells into Cx43-null brains generated with a GFAP-Cre mouse line (Figure 4.2 D) exhibited a tumor mass with smooth border (Figure 4.2 E), indicating the lack of infiltrating glioma cells. The percentage of infiltrative edge in mock-transfected tumors decreased from (53.0 ± 7.0; n=9) in wild type to (33.4 ± 16.0; n=3) in Cx43-null brains (Figure 4.2 F). Similarly, the percentage of invasive edge in T154-transfected GL261 gliomas was reduced in Cx43-null brains (37.7 ± 8.1; n=3) (Figure 4.2 F). In contrast, I did not observe a reduction of glioma invasiveness (59.9 ± 7.64; n=8) when T154A expressing GL261 cells were implanted in wild-type brains (Figure 4.2 F).

I next examined the number of GL261 cells dispersed in the brain parenchyma adjacent to the tumor core, and the distance they migrated from the tumor periphery. The majority of the dispersed cells were located within a 200 µm infiltrative zone that shows enhanced Cx43 and GFAP expression (Figure 4.2 G). The number of fluorescent parental GL261 cells in brain parenchyma within the region of astrogliosis (Figure 4.2 G) was reduced in Cx43-null mice (11.4 ± 1.5 per mm²; n=3) when compared to wild-type mice (23.6 per mm² ± 3.6; n=6) (Figure 4.2 H). The reduction in the number of invading tumor cells in the absence of astrocytic Cx43 was also observed in mouse brains implanted with T154A-transfected GL261 cells (18.5 ± 3.0 per mm²; n=6) (Fig 4.4H), mirroring our previous results (Figure 4.2 F). Interestingly, T154A-expressing cells migrated significantly farther (122.65 µm ± 19.75) than parental GL261 cells (66.39 µm ± 10.46) in both wild-type and Cx43-null mice (Figure 4.2 I). However, we did not observe significant difference in the distance migrated by parental GL261 cells in wild-type and
Cx43-null brain parenchyma (Figure 4.2 I), indicating that astrocytic Cx43 facilitates the dissemination of tumor cells from the glioma core but does not enhance the migration of detached tumor cells.
Figure 4.2: Cx43 promotes glioma invasion in a channel-independent manner.

A. Expression of channel-deficient T154A mutant in GL261 cells. GAPDH was used as a loading control. B. Localization of wild-type Cx43 and Cx43-T154A (white arrow) protein as gap junction plaques at cell-cell contacts. C. Reduced coupling of T154A-expressing GL261 cells compared to Mock-transfected cells. Donor cells are yellow (arrow) due to overlap of Calcein AM (green) and DiI (red) merged with a DIC image highlighting the recipient
monolayer. CBX is an inhibitor of GJIC. D. Expression of Cx43 (green) adjacent to GL261 glioma cells (red) in Cx43fl/fl (WT) mice but not in GFAP-Cre: Cx43fl/fl (Cx43-null) mice. E. Representative cresyl violet-stained sections of Mock transfected cells in Cx43fl/fl (Mock-WT) and GFAP-Cre: Cx43fl/fl (Mock-Null) brains, and T154A-transfected cells in wild-type (T154A-WT) and Cx43-null (T154A-Null) brains. Scale bar, 500 μm. F. Quantification of glioma invasion by the percentage of tumor border with infiltrative cells. (Mock-WT: n=9; Mock-Null: n=3; T154A-WT: n=8; and T154A-Null: n=3) *p < 0.05 (Student’s t-test). Data are shown as (mean ± SEM). G. Representative images showing the presence of mcherry-positive GL261 glioma cells (magenta) in the brain parenchyma with increased GFAP immunoreactivity (green) expressed by the reactive astrocytes. H. Fewer mcherry-positive glioma cells leave the core in Cx43-null brains but there is no significant difference between Mock and T154A expressing cells in wild type mice. *p < 0.05 (One-way ANOVA followed by pairwise comparison with Student-Newman-Keuls method). I. Glioma cells expressing Cx43-T154A mutant migrate significantly farther away from the tumor edge compared to control GL261 cells. *p < 0.05 (Student’s t-test). (Mock-WT: n=6; Mock-Null: n=3; T154A-WT: n=6; and T154A-Null: n=3). Data are shown as (mean ± SEM). The data represented in panels A-D was obtained by me and F-I were obtained by both me and Dr. Wun Chey Sin. Data in panel E was obtained by Dr. Wun Chey Sin.

4.4 Discussion

Reactive astrocytes constitute a major component of the host environment encountered by invading glioma cells in the brain parenchyma [227, 230, 258, 266] and are well positioned to
regulate tumor cell invasion. Indeed, astrocytes are believed to enhance the invasiveness of glioma cells by paracrine action [267], such as by secreting matrix metalloproteases [238, 240] and extracellular matrix associated proteins such as connective tissue growth factor [239] and chondroitin sulfate proteoglycans [268]. Here we demonstrate for the first time that the absence of a gap junction channel protein Cx43, normally upregulated in activated astrocytes, reduces the ability of glioma cells to invade into the brain parenchyma (Appendix 6). This is supported by our findings from two independent immune-competent conditional knockout mouse lines in which Cx43 has been eliminated in the reactive astrocytes (Appendix 6 and Figure 4.2).

A number of mechanisms have been proposed for Cx43-mediated cell migration [121, 142], but the role of astrocytic Cx43 in glioma invasion has never been extensively explored. Here, we investigate the role of reactive astrocytes in glioma invasion in a syngeneic mouse with an intact immune system [175, 269] by intracranial implantation of GL261 mouse cells which exhibit histological characteristics of human gliomas [270, 271]. We demonstrate for the first time that astrocytic Cx43 is a major player in the regulation of glioma invasion by stromal cells in the invasive niche of the microenvironment. This is especially relevant clinically since 90% of glioma recurrence occurs within 2 cm from the primary gliomas in a human brain [272], which translates into a very short distance well within the 200 μm gliosis zone exhibiting enhanced astrocytic Cx43 in a mouse brain based on the difference in their brain mass [273].

It is not clear how the absence of Cx43 alters the signaling pathways in reactive astrocytes; our results with Cx43-null mice suggest Cx43 may mediate its effects via either the channel activity or the C-tail. With regard to channel activity, we suspect Cx43-mediated intercellular GJIC is critical for its role in promoting glioma invasion. A recent study has highlighted the deregulation of CXCL12 (SDF-1), a chemokine with a prominent role in glioma
progression [274], in a Cx43/Ca\(^{2+}\)-dependent manner [275]. Therefore, we speculate attenuated calcium signaling observed in Cx43-null astrocytes in brains [246, 276, 277] may account for the reduction in glioma invasion.

Investigations on the specific role of Cx43 in glioma invasion has been hindered by the fact that alteration in tumoral Cx43 level not only disrupts homocellular communication, but also perturbs the formation of heterocellular channels between glioma cells and astrocytes. In addition, the opposing effects of homocellular and heterocellular GJIC on glioma migration [133], further complicate the role of tumoral Cx43 in glioma progression. Our findings suggest that heterocellular GJIC does not play a major part in the presence of astrogliosis within an intact brain because glioma cells expressing a channel-defective T154A mutant did not exhibit reduced invasiveness (Figure 4.2 E-G). Interestingly, an attenuated response in invasion was observed in Cx43ShRNA tumors; the discrepancy can be attributed to the presence of intact C-terminal tail in the T154A mutant which has been shown to be important in cell motility [134, 135, 278]. In addition, the extracellular loops present in the T154A mutant may facilitate its increased motility in the brain parenchyma by providing additional intercellular adhesion [67, 68, 224, 279].

The juxtaposition of astrocytes with cancer cells in low grade gliomas raised the interesting question whether Cx43 expressed by the tumor-associated astrocytes contributes to glioma invasion since increasing Cx43 levels in these gliomas appears to be associated with poor prognosis. Our results demonstrate a role of Cx43 at two distinct sites in the invasive niche: astrocytic Cx43 facilitates the ability of glioma cells to make the initial dissemination into the brain parenchyma but does not appear to have a role in regulating the migration of glioma cells in the brain parenchyma, since there is no difference in the distance travelled by invading cells in wild-type and Cx43 null brains (Figure 4.2 I). Instead the ability of glioma cells to migrate
depends on the level of tumoral Cx43 and appears to be enhanced in the absence of GJIC, as demonstrated for channel-deficient T154A expressing glioma cells (Figure 4.2 I).

Our findings reveal the unexpected role of astrocytic Cx43 at the glioma-brain parenchyma interface. It is intriguing to hypothesize that astrocytic Cx43 facilitates glioma recurrence after resection by promoting dissemination of glioma cells, since Cx43 expression is expected to be significantly upregulated in reactive astrocytes due to tissue injury during surgery [242]. It has long been known that the interaction between tumor cells and their microenvironment contribute to the resistance of tumor cells to therapeutic treatment [280-282]. Our results highlight the potential to manipulate astrocyte signaling by targeting Cx43 expression and function to limit local infiltration of glioma cells into the brain parenchyma.
Chapter 5: Conclusion

5.1 Overview of research undertaken

The overall objective of this thesis was to identify the mechanism responsible for Cx43 mediated glioma migration and invasion. The genomic and molecular heterogeneity of GBM in conjunction with the diversity of gap junctions that can form (heterocellular vs. homocellular), as well as the various connexins which could be expressed, complicate deciphering the role of Cx43 in glioma migration. I undertook two different approaches to discern the role of homocellular and heterocellular gap junctions in glioma migration. Firstly, I screened a panel of human GBM cell lines for Cx43 protein expression, subcellular localization, GJIC and migration to establish an in vitro model to study the effects of homocellular gap junctions in glioma. We selected the U118 human glioma cell line to examine the effects of homocellular gap junctions on glioma migration as this line had the classic mutations of GBM (i.e., p53 and PTEN) and Cx43 protein properly localizing to the plasma membrane. Subsequently, using rigorous loss of function and rescue experiments we showed that the channel function of Cx43 is important in glioma migration. Secondly, we set up an in vivo model by intracranial implantation of mouse glioma cells in syngeneic mice to examine both homocellular and heterocellular gap junctions in glioma invasion. Our work demonstrates that obstructing the channel function does not reduce the number of glioma cells that invade. However it does increase the distance migrated by glioma cells, corroborating our in vitro data and suggesting that reducing GJIC may facilitate recurring tumors at multiple foci which is currently an impediment in treating GBMs.
5.2 Integrating the findings of this thesis work and its significance to the field

5.2.1 Discerning the importance of homocellular gap junctions in glioma migration

Previous *in vitro* studies using rat C6 glioma and human glioma cell lines have implicated Cx43 in glioma migration, however there is inconsistency between these reports in terms of how Cx43 mediates migration [134, 135, 244]. For example, Bates et al. compared migration rates between two C6 clones, C6-high (C6-H) and C6-low (C6-L), and found C6-H cells to have a higher migration rate than C6-L [134]. In addition, reduction of Cx43 by ShRNA resulted in decreased migration of the C6-H cells and implicated the C-terminal tail of Cx43 in modulating migration [134]. It should be noted that the authors relied on the over-expression approach to study the C-terminal truncation mutant [134]. The approach of over-expression produces proteins at levels substantially higher than found under physiological conditions and could affect signalling pathways that would otherwise be unaffected. This is likely for Cx43 since it can influence signalling pathways by both the channel function and its C-terminal tail. In addition, since Cx43 oligomerizes to form hexamers, over expression of mutant protein in the presence of endogenous wildtype Cx43 can influence the stoichiometry of the hexamers which can produce misleading results. As well only one ShRNA construct was used to test the effects of Cx43 reduction in C6 glioma cells. To ensure this was not due to off-target effects a better approach would have been to use two shRNA constructs that target Cx43 mRNA.

Crespin et al. over-expressed Cx43 and the C-terminal truncation mutant TrCx43 in the human glioma cell line LN18 and observed an increase in migration [135]. Our data shows that the LN18 cell line has the majority of the Cx43 protein localizing to the perinuclear region and a
small population at cell-cell contacts (Chapter 1); this worsens the problems with over-expression since Cx43 expression would increase both at the perinuclear region and at cell-cell contacts and may produce erroneous effects. Over expressing Cx43 would compromise the function of the endomembrane compartment Cx43 is localized to due to saturation. As well, if the Cx43 plays a functional role in the intracellular compartment than that function would be enhanced in addition to the gap junction function. For more specific conclusions we need to do co-staining with various markers of the endomembrane system. Oliveira et al. showed that blocking gap junctions using the pharmacological blocker CBX increased the migration of the human glioma cell line GL15 [133]. Strale et al. corroborated this finding by using two shRNA constructs to reduce Cx43 expression in the human glioma cell line U251 [244]. Nevertheless this study would have benefited from having a control condition in which exogenous Cx43 was expressed in the cells expressing shRNA to see if the phenotype could be rescued.

Given the deficiencies in the aforementioned studies we designed a more comprehensive approach in which we reduced endogenous Cx43 protein expression and subsequently rescued it by expressing exogenous wildtype Cx43, in comparison with the Cx43 mutants T154A (channel dead dominant negative) and TrCx43. We wanted to carry out our loss of function and rescue experiments in a glioma cell line that was more akin to human GBMs in that it was derived from patients and it should have mutations that are common in GBM such as PTEN and TP53 [103]. In addition we also wanted a cell line with Cx43 protein that localized properly to the plasma membrane at cell-cell junctions to form functional gap junctions. We screened a panel of human grade IV GBM cell lines that have been used extensively to study glioma characteristics and have also been sequenced by The Sanger Institute to identify mutations. We found variability in Cx43 protein expression, subcellular localization, GJIC, and migration between the glioma cell
lines of the same grade. Previous studies have shown a decrease in Cx43 expression and GJIC in high grade GBM tissues [63, 78, 121], however we show that glioma cell lines that have been classified as grade IV GBMs based on histopathology have varying levels of Cx43 expression. Our work highlights that Cx43 protein expression is heterogeneous in GBMs, that there might be niches in the tumor that have cells with abundant Cx43 expression. We also found two cell lines that expressed Cx43 predominantly at the perinuclear region and hence had very low levels of GJIC suggesting that the low GJIC observed in GBMs could also be due to changes in subcellular localization. The cysteine residues on the extracellular loops of Cx43 are important for proper trafficking since mutation in any or all of the cysteines displays increased accumulation in intracellular vesicles with a concomitant decrease in GJIC when compared to wildtype Cx43 [67, 180]. Thus it is possible that LN18 expresses Cx43 with a mutation in the extracellular loops, however exome sequencing of the cell line did not identify a mutation in Cx43 (Table 2.1). This characterization is the first to report this heterogeneity in Cx43 expression and subcellular localization in grade IV GBM cell lines (Figure 2.2 – 2.3).

From our screening we chose the U118 human glioma cell line as it met the aforementioned criteria. Using a specific approach of ShRNA knockdown to reduce endogenous Cx43 expression and rescue by expressing exogenous Cx43 (WT and mutants) we discovered that obstructing the channel function of Cx43 increased glioma migration. By examining migration in real-time we showed for the first time that glioma cells that express Cx43 compared with cells with reduced Cx43 migrate in a more attached manner akin to collective migration than cells with reduced Cx43. Consequently it is possible that Cx43 knockdown cells were able to cover a larger distance because they became detached from the cohort of cells and migrated faster and farther. This phenotype has also been observed using a wound healing assay, in breast
epithelial cells, where reduction of Cx43 promoted changes in cell adhesion, disruptions in cell polarity, enhanced single cell motility, and migration faster and farther than cells expressing normal levels of Cx43 [169]. Our findings suggest that gap junctions could play an important role in collective migration.

During collective migration the cohort of cells need to communicate with each other [159] and this can be facilitated by gap junction channels since they can chemically and electrically couple cells. Gap junctions are permeable to small ions such as Ca$^{2+}$ [283], second messenger molecules such as IP$_3$, and small metabolites such as glucose [21, 24], however it is unclear which one of these or other molecules could be important in migration. Nonetheless one can speculate that passage of Ca$^{2+}$ between cells could play a role in migration since Ca$^{2+}$ has been implicated in migration. In single cell motility Ca$^{2+}$ is found in microdomains with an increasing concentration at the leading edge of the cell [284]; alternatively cells that migrate collectively have leader cells that could have more Ca$^{2+}$ microdomains than the cells that are at the rear. Gap junctions between migratory cells may play a role in creating a Ca$^{2+}$ gradient in the ‘supra-cellular’ organization of cells formed during collective migration [Figure 5.1 A]. Given that collective migration is an important mode of migration during morphogenesis, wound healing and cancer invasion, it is possible that gap junctions are facilitating this mode of migration in the aforementioned processes.

5.2.2 **Role of Cx43 channel in the context of in vivo glioma invasion**

Investigating the role of Cx43 in glioma invasion *in vivo* is an entirely different scenario as heterocellular communication may also be playing an important role in glioma invasion.
Studies examining the interaction between glioma cells and host cells have demonstrated that glioma cells establish GJIC with host astrocytes and remodel the extracellular matrix as they invade the brain [67, 240, 245]. Specifically, C6 glioma cells over-expressing Cx43 communicated with astrocytes and invaded a larger area of the brain parenchyma than glioma cells with low Cx43 expression [67]; a better approach would have been to reduce endogenous Cx43 expression and rescue it with exogenous Cx43 cDNA. In addition, blocking gap junction communication with CBX in glioma cells co-cultured with astrocytes, or in organotypic brain slice cultures, showed a decrease in invasion suggesting that GJIC was important for this process [133]. While this showed the importance of the channel function in glioma invasion, using a Cx43 mutant that blocks the channel function would have been a more specific approach. These studies suggest a role for gap junction communication between glioma and astrocytes in facilitating invasion but the mechanism is unclear due to the deficiencies of the approaches taken.

We wanted to determine how homocellular (glioma-glioma) and heterocellular (glioma-astrocytes) gap junctions influence glioma invasion in vivo. Although the U118 human glioma cell line was an appropriate model for studying the role of homocellular gap junctions in glioma migration, it was not suitable for in vivo experiments as it required using nude mice with a compromised immune system and we wanted to use mice with intact immune systems. To circumvent this we employed a syngeneic intracranial mouse model using the GL261 glioma cells and C57BL/6 mice with an intact immune system. We found that reducing endogenous Cx43 protein expression in glioma cells showed a reduction in dissemination of these cells and thus reduced invasion. In addition, this was the first study that expressed the specific dominant negative mutant T154A in glioma cells to study the channel function in vivo. We found that
blocking the channel function in glioma cells increased the distance of glioma invasion with no
effect on the number of cells that disseminated. Our findings have two implications. Firstly, our
findings suggest that GJIC between glioma cells and astrocytes may determine the site of
secondary glioma occurrence. This is significant since recurrence of GBMs is the major hurdle in
treating the disease. Secondly, since we did not observe changes in the dissemination of glioma
cells when we reduced GJIC it is possible that gap junction independent functions such as the C-
terminal tail interactions with the cytoskeleton are playing a role in this process. In addition, this
is the first study to examine the role of heterocellular GJIC between glioma and astrocytes by
deleting endogenous Cx43 in astrocytes using conditional Nestin-Cre; Cx43 floxed knockout
mice. A reduction in glioma invasion was observed in Cx43 KO mice implicating astrocytic
Cx43 in facilitating glioma invasion.
A. Homocellular gap junctions between glioma cells promote collective migration \textit{in vitro} by passage of molecules, small metabolites and second messengers. B. \textit{In vivo} glioma cells communicate with each other through homocellular gap junctions and with astrocytes through passage of small metabolites and second messengers between glioma and astrocytes as the glioma cells invade the brain parenchyma.

\textbf{Figure 5.1: The proposed role of Cx43 gap junctions in glioma migration.}
heterocellular gap junctions. Heterocellular communication may play a more prominent role in facilitating invasion \textit{in vivo}.

\section*{5.3 Future directions of the research in this dissertation}

\subsection*{5.3.1 Selection of a suitable human glioma cell line to study Cx43’s role in glioma migration}

In chapters 1 and 2 we used grade IV immortalized human glioma cell lines to discern the role of Cx43 in glioma migration. The main advantage of using immortalized glioma cell lines is that their exomes have been sequenced by the Sanger Institute (http://cancer.sanger.ac.uk/cell_lines/cbrowse/all), and a complete list of all the mutated genes is publicly available. We made use of this information to exclude human glioma cell lines with EGFR mutations, since EGFR signaling is known to affect Cx43 dynamics. In addition, these cell lines have been extensively used to study glioma therefore there is a wealth of knowledge available on them in terms of culturing conditions, growth and migration assays. The sequencing of both primary GBM tissues and immortalized grade IV GBM cell lines highlights the genetic heterogeneity of this disease. The genetic heterogeneity between grade IV GBM cell lines may explain why manipulating Cx43 protein expression in different GBM cell lines yields contradicting migration phenotypes.

Thus far we have established the role of Cx43 in glioma migration using immortalized human glioma cell lines however for future experiments we would expand our work using primary GBM cell lines obtained from patients. Recent studies have shown that immortalized
glioma cells maintained in culture for long periods obtain additional mutations over time; this is true for human, rat and mouse glioma [285]. This presents a caveat since genomic alterations in immortalized glioma cell lines may not accurately represent the genomic landscape of GBMs in patients, in turn affecting the interpretation of data. Expanding our work by using primary human GBM cells would circumvent this caveat as primary cell lines have a short passage life and therefore their genetic makeup would be more similar to the GBM from which they were extracted.

Our experimental plan would entail screening a panel of primary GBM cell lines for Cx43 expression, subcellular localization, GJIC, and mutation of Cx43 gene GJα1. In addition, we would sequence the genome of the cell lines to identify the subtype of GBM they represent i.e. classical, proneural, mesenchymal, neural and proliferative. Upon completing this characterization, we would manipulate Cx43 expression by using loss of function and rescue approach in the selected cell lines and carry out tumorigenicity assays examining cell growth, migration and invasion. The results from these experiments would give a more comprehensive explanation of how Cx43 mediates tumorigenicity in different genetic subtypes of GBM.

5.3.2 3D spheroids in vitro models

In chapter 2 we relied on a 2D wound healing migration assay to screen the panel of human GBM cell lines for their inherent motility. In chapter 3 we expanded our work by utilizing the spheroid migration assay to examine changes in migration due to changes in Cx43 protein expression. The spheroid migration assay has the advantage of mimicking the 3D architecture of solid tumors with a necrotic center and hypoxic regions [211]. In addition, we
used a standardized method of culturing spheroids by using 96 well low attachment plates that allowed us to grow individual spheroids of the same size with high reproducibility. The spheroids were then placed on fibronectin coated coverslips to determine changes in migration. This method allowed us to place several spheroids in an array on a coverslip which facilitated easy imaging. For future experiments we can extend this method to automated imaging using the IncuCyte ZOOM system from Essen Bioscience (http://www.essenbioscience.com/essen-products/incucyte/) which facilitates high throughput drug screening and imaging of live cells. The IncuCyte ZOOM system contains an epifluorescence microscope in a cell incubator and has a hard drive that captures and processes image data. With the aid of this system we could conduct experiments that assess the efficacy of drugs on control (cells with endogenous Cx43 protein expression) glioma spheroids and Cx43 reduced glioma spheroids by assaying for oncogenic traits such as cell proliferation, migration and apoptosis. High throughput data could be obtained since the machine has compartments for 96 and 384 well plates.

The physiological environment of GBMs is quite complex, consisting of various ECM proteins, host cells such as astrocytes, neurons, oligodendrocytes and microglia, and endothelial cells that make up the blood vessels. Our spheroid assay can be expanded to incorporate the different constituents of the GBM microenvironment. The ECM components in the microenvironment consist of collagen I, collagen IV, fibronectin, and laminin [286]; we can extend our study to include the aforementioned ECM components to create a migration surface more akin to in vivo conditions. In addition, we can integrate the host cells such as astrocytes and endothelial cells in the spheroid assay to determine how GJIC between glioma and host cells influences migration.
5.3.3 Manipulating Cx43 expression

5.3.3.1 Over-expression experiments

In Chapter 2 of this thesis we examined Cx43 mediated migration by over-expressing exogenous Cx43 in human glioma cell line LN229 and although we did not see a change in migration phenotype, it is still important to state the caveats of this technique. Over-expressing exogenous proteins is generally carried out by using a very strong promoter such as the cytomegalovirus (CMV) promoter, this increases the amount of protein expressed beyond endogenous levels. To circumvent this we chose a cell line that had very low levels of endogenous Cx43, LN229, and although this lessened erroneous effects of over-expression it did not completely abolish them. In addition, when over-expression is being used to study mutant forms of the protein both the mutant and wildtype protein exist and the effect of the mutant can be masked unless it is a dominant negative mutant. This problem is more complicated with regard to Cx43 because connexin monomers oligomerize to form hexameric hemichannels; in an over-expression experiment this oligomerization would happen between wildtype and mutant Cx43, potentially masking the effect of the mutant. In addition the ratio of mutant to wildtype Cx43 protein in each hexamer would vary and also influence whether a phenotype is observed or not. If the mutant Cx43 has a dominant-negative phenotype such as T154A the effect of mixing with the endogenous Cx43 would be minimum, however if this not the case as with TrCx43 the true phenotype of the mutant can be masked due to oligomerization with endogenous Cx43. Thus the approach of over expression should be used with caution when studying connexins.
5.3.3.2 RNAi experiments

In chapter 3, we addressed the aforementioned issues by using ShRNAs to reduce endogenous Cx43 (in U118 cell line) and then rescuing the knockdown by expressing exogenous wildtype Cx43 and Cx43 mutants. The reduction of endogenous Cx43 by ShRNA minimizes the erroneous effects of over-expression and limits the diversity of the hexamers formed since the exogenous Cx43 (wildtype and mutant) is present at higher levels than endogenous Cx43. Using ShRNA to reduce endogenous proteins does however have the potential caveats of producing off-target effects. This can be controlled by using non-targeting ShRNA as a control, using multiple ShRNA constructs that target different parts of the gene, and rescuing by the wildtype protein to confirm the phenotype is not an artifact; we used all of these controls in the experiments conducted in Chapter 3.

Even with attaining 84-89% ShRNA knockdown there is still residual endogenous Cx43 that can mix with exogenous TrCx43 mutants to generate mixed oligomers with wildtype Cx43 that have an intact C-terminal tail. Given that trafficking defects have not been reported with TrCx43, the mixed connexons would get trafficked to the plasma membrane and the intact C-terminal tails of the endogenous Cx43 could rescue the phenotype. The T154A mutant would form mixed connexons with the residual endogenous Cx43 as well but since it is a dominant negative mutant that renders the channel blocked there is less concern with the phenotype being undetected. In addition, we showed that the pharmacological blocker of gap junctions also increases migration thus further confirming our finding with the T154A mutant. Nevertheless to conclusively determine whether the TrCx43 mutant has an effect on migration we have to completely abolish the effects of mixing between exogenous and endogenous Cx43 we could
knock out the endogenous Cx43 gene in human glioma cell lines and then introduce exogenous Cx43 proteins. The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated system (Cas) can be used to target the Cx43 gene in glioma cells. There are two main components in this technique, guide RNA (gRNA) and an endonuclease called the CRISPR associated nuclease (Cas9); the gRNA forms a complex with Cas9 [287, 288]. Expression of gRNA and Cas9 in cells can cause targeted changes in the genomic sequence of the cell. The gRNA is designed to be complementary to the gene of interest thus allowing base pairing between the gRNA and genomic DNA [287, 288]. The base pairing facilitates Cas9 endonuclease to interact with the DNA to cause double stranded breaks. The repair of double stranded breaks often leads to insertion or deletions causing frameshifts and premature stop codons thus disrupting the gene [287, 288]. Using this system we could delete endogenous Cx43 and thus eliminate the caveat of mixed Cx43 oligomers and convincingly determine whether TrCx43 influences migration or not.

5.3.4 Use of pharmacological inhibitors

Pharmacological blockers have been used to study channel function of gap junctions. We used CBX, a well-known blocker of gap junctions to confirm the effects of the dominant-negative channel dead mutant T154A. Since two different approaches that block the channel function produced the same phenotype we are confident in implicating the channel function in influencing migration. Nevertheless, a caveat of using pharmacological blockers is that they can be non-specific and produce erroneous affects. As an additional control we could use the Gap26 mimetic peptide that specifically corresponds to the extracellular loops of Cx43 and blocks GJIC
[289]. If treatment with Gap26 increases migration just as the T154A mutant and CBX, then we can further conclude that decreasing GJIC increases glioma migration.

5.3.5 Other Cxs

With 21 Cx isoforms in the human genome, functional redundancy and compensation within this protein family needs to be taken into consideration. As mentioned in the introduction, Cxs can form heteromeric hemichannels and heterotypic gap junctions, therefore it is important to examine the expression of Cxs that can form the aforementioned channels with Cx43. GBMs are believed to arise from de-differentiated astrocyte or precursor glial cells. Since astrocytes express Cx43, Cx26 and Cx30, we would examine the expression of these Cxs in the human glioma cell lines. Cx26 has been implicated in neuronal migration along radial glia [68]; its role in glioma migration remains unexplored. If these Cxs are expressed in human glioma cell lines one could hypothesize that reducing their expression may also affect glioma migration.

5.3.6 Intracranial implantation

In chapter 4 we used intracranial implantation of GL261 glioma cells into C57BL/6 black mice to study the role of Cx43 in glioma invasion in vivo. The advantage of using this model is that it is syngeneic to the mouse thus we were able to examine gliomagenesis with an intact immune system, which is more akin to GBMs in patients. As mentioned in chapter 4 the immune system plays an important role in gliomagenesis in vivo, specifically microglial cells have been shown to promote invasion. Thus it was important for us to have a mouse model with an intact
immune system to recapitulate gliomagenesis as it occurs in vivo. Nonetheless a caveat with this technique is that injecting the tumor cells in the brain injures the brain and could elicit an immune response that is more pronounced than a glioma would elicit. In addition, this would affect the extent of the glial scar formed around the glioma which provides a permissive niche for glioma cells to invade. We have to take this caveat into consideration since we assessed glioma invasion which could be facilitated by a permissive niche. Another caveat of injecting unattached cells in the brain is accurately injecting equal number of cells for each injection thus creating variability in the volume of tumors formed. Tumor volume is an indicator of cell proliferation however with this aforementioned caveat we cannot use this method to determine if tumors formed by GL261 with reduced Cx43 influence cell growth. This can be circumvented by culturing tumor cells as spheroids and injecting the 3D spheroids in the mice. We can culture individual spheroids that are uniform in size and shape and minimize the variability between injections.

In conclusion, we showed that human glioma cell lines have variable expression of Cx43 and subsequently GJIC. We also observed an accumulation of Cx43 in intracellular vesicles in human glioma cell lines LN18 and T98G, since sequencing data did not detect mutations in Cx43 gene it is possible that there may be trafficking defects in these cells. We showed that reducing Cx43 in U118 increased relative migration levels, and real-time imaging of the spheroid migration assay showed that this was due to knockdown cells moving faster and in a detached manner. We also showed that cells with Cx43 turned around more than knockdown cells suggesting that reducing Cx43 promotes persistent migration. Expression of dominant negative T154A mutant and treatment of glioma cells with CBX yielded higher migration levels when compared to control cells suggesting that reducing GJIC causes an increase in migration of
glioma cells. In contrast, reducing Cx43 in GL261 mouse glioma cells reduced invasion in to the brain however expression of T154A did not reduce invasion. Interestingly, deleting Cx43 in host astrocytes also reduced invasion. Altogether our findings show opposing roles for homocellular and heterocellular communication in glioma invasion thus meriting further critical experimentation to determine the therapeutic repercussions.
References


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Appendix 1

Appendix 1: Expression of Cx43 mutants in U118 ShRNA6 and ShRNA7 cells.

Western blot analysis was done to examine the successful expression of mutants T154A and TrCx43 in ShRNA6 and ShRNA7 cells. A. We used anti-Cx43 (Sigma) that targets the C-
terminal domain of Cx43 to detect mutant protein T154A. Using densitometry (ImageJ) we found Cx43 expression in control cells to be 16 times higher than ShRNA6 and ShRNA7 cells. Cx43 expression in ShRNA6-T154A and ShRNA7-T154A cells was 9 and 16 times higher, respectively. B. Since the TrCx43 mutant lacks the C-terminal tail we could not use the Sigma anti-Cx43 antibody we used for detection of wildtype Cx43 or T154A. We used P1E11 anti-Cx43 antibody (Fred Hutchinson Cancer Research Center) that targets the N-terminal of Cx43 to detect TrCx43 expression in ShRNA6 and ShRNA7 cells. Since the TrCx43 is lacking the C-terminal tail it is of a smaller molecular weight, predicted to be 30 kDa. Cx43 expression in control cells was 6 times higher than ShRNA6 and ShRNA7 cells. Cx43 expression in ShRNA6-TrCx43 and ShRNA7-TrCx43 cells was 3 and 6 times higher, respectively. Please note that extraneous lanes were removed from the blot.
Appendix 2: Immunofluorescence analysis of N-cadherin, active β1-Integrin, and phospho-FAK.
Spheroid migration assay on fibronectin coated coverslips was carried out on Control, ShRNA6 and ShRNA7 cells. The spheroids were fixed with 4% paraformaldehyde and immunofluorescence was carried out. A. N-cadherin staining showed no gross changes in localization between control and knockdown cells. B. Active β1-integrin staining shows no gross changes in localization between control and knockdown cells. C. Phospho-FAK staining shows no gross changes in localization. * marks the position of the spheroid; scale bar = 20 µm.
Appendix 3: Western blot analysis shows no obvious change in the protein expression of total β1-integrin, total FAK and phospho-FAK in cells cultured as spheroids or monolayer. Western blot analysis was performed on control, ShRNA6 and ShRNA7 cells cultured as spheroids and monolayer. Samples were probed for Cx43, total β1-Integrin, total FAK, phospho-FAK. GAPDH was used as loading control. Ovcar3 cells were used as positive control for β1-
integrin, total FAK, and phospho-FAK (provided by Roskelley lab). Fibronectin stimulation was not used. The experiment was repeated twice.
Appendix 4

The supplemental methods for Chapter 4 are presented here. The following protocols were performed by John Bechberger and Dr. Wun Chey Sin.

Animal experiments

Cx43 conditional knockout mice were generated by crossing mice containing GFAP-Cre [290] or Nestin-Cre [291] with mice harboring floxed Cx43 alleles [292]. Mice of either sex were used in the experiment, maintained in an animal facility (Center for Disease Modelling, Life Sciences Institute) for 12 hr. light/dark cycle and were provided food and water ad libitum. These experiments were performed by John Bechberger.

Intracranial implantation of glioma cells

GL261 cells (25,000) were resuspended in 2 μl Hank’s balanced salt solution and injected intracerebrally into the striatum of adult C57BL/6 mice fit to a stereotaxic frame (David Kopf Instruments) at position 2.5 mm lateral to the midline, 1.0 mm anterior of the bregma, and 3.0 mm ventral from the dura. To minimize inflammatory response from damaged brain tissue due to needle injection [242], GL261 cells were injected into the brain at the rate of 0.5 μl/min. The needle was held in place for an additional 30 sec before removal. While every effort had been made to ensure equal number of cells was introduced into each mouse, there was variability in the resultant tumor sizes, which may be due to the presence of pencils of Wilson myelinated
fibers in the striatum affecting the initial “seeding” of the injected GL261 cells. These experiments were performed by John Bechberger.

**Histological preparation**

Tumors were either visualized by cresyl violet or TO-PRO-1 (Invitrogen) staining two weeks after intracranial implantation of glioma cells. Brains were removed after transcardial perfusion with 4% paraformaldehyde followed by immersion in the same fixative overnight at 4 °C. Harvested brains were dehydrated in 20% sucrose in phosphate-buffered saline (PBS) overnight and kept at 4 °C prior to sectioning through the entire tumor mass. Cryosections 20 µm thick were first rehydrated in graded alcohols, stained with cresyl violet (0.1% cresyl violet acetate and 0.25% glacial acetic acid in ddH₂O (double distilled H₂O), dehydrated in graded alcohols, mounted with xylene-based Cytoseal XYL mounting medium (Richard-Allan Scientific) and visualized with Leica FireCam microscope. TO-PRO1 staining was carried out at 1:1000 for 20 min at room temperature and the tumor was visualized with a Leica TCS SP5 II Basic VIS system. These experiments were carried out by Justin Leung, and Helen Chen.

**Immunohistochemistry**

Human brain tumor and normal tissue microarray slides (GL208 and GL2082 from US Biomax, Rockville, MD) were processed by Wax-it Histology Services Inc. (University of British Columbia, Canada) as described previously [220]. Rabbit Anti-Cx43 antibody (Sigma) was used at 1:4000. Slides were scanned with an Aperio microscope scanner, and the level of
Cx43 expression was analyzed with Image J software after color deconvolution and binary thresholding. Statistical comparisons were performed with one-way ANOVA. These experiments were carried out by John Bechberger and Dr. Wun Chey Sin.
Appendix 5

Supplemental figure for Chapter 4. The experimental data presented here was obtained by John Bechberger.

Appendix 5: Expression of Cx43 in primary human gliomas.

A. Cx43 immunoreactivity was quantified by Image J and expressed as arbitrary units. Number within column denotes the number of tissue core in each category. B. Representative pictures from normal, grade I, and grade IV gliomas showing enhanced Cx43 staining (brown) in grade I astrocytomas. The experimental data presented here was obtained by John Bechberger and Dr. Sin.
Appendix 6

Supplemental Figure for Chapter 4. The experimental data presented here was obtained by Dr. Wun Chey Sin.

Appendix 6: Astrocytic Cx43 promotes dissemination of glioma cells.

A. Increased Cx43 staining (white arrow) adjacent to GL261 cells (white asterisk) that were protruding into the brain parenchyma. Nuclei were stained blue with DAPI. B. Expression of Cx43 (green) adjacent to m-cherry expressing GL261 glioma cells (red) in control (Cx43fl/fl) but
not in Cx43-null (Nestin-Cre:Cx43fl/fl) brains. Nuclei were stained blue with DAPI. C. Cresyl violet-stained glioma sections showing circumscribed border in the Cx43-null brain compared to the control. Scale bar, 200 μm. Increased percentage of tumor border with infiltrative cells in control (n=6) compared to Cx43-null brains (n=5). *p < 0.05 (Student’s t-test). D. Increased number of mcherry-positive GL261 glioma cells in the brain parenchyma adjacent to the glioma border (white line) in Control wild-type (n=5) compared to Cx43-null brains (n=5). *p < 0.05. Data are shown as (mean ± SEM). The experimental data presented here was obtained by Dr. Wun Chey Sin.