# The Role of Pannexins, a Novel Gap Junction Family, in Glioma Oncogenicity and Neuronal Differentiation

by

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B.Sc., The University of British Columbia, 2003

# A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

## DOCTOR OF PHILOSOPHY

in

# THE FACULTY OF GRADUATE STUDIES

(Cell and Developmental Biology)

### THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

April 2010

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### Abstract

Gap junctions are unique intercellular channels assembled from the canonical gap junction family, connexins (Cxs). These channels connect the cytosols of adjacent cells, allowing direct passages of small ions and molecules for intercellular communication and homeostasis within tissues. A novel family of gap junction proteins, pannexins (Panxs), with low sequence similarity to the invertebrate gap junctions, innexins, was recently discovered in chordates. Similar to Cxs, Panxs are also capable of forming functional hemichannels as well as intercellular channels. Aberrations in gap junctions have been associated with abnormal CNS development and diseases including gliomas. The main purpose of this thesis was to determine if Panxs play a functional role under pathological and normal CNS conditions, each of which is represented by gliomas and neuronal differentiation, respectively. A loss of Panx expression was found in the C6 glioma cell line when compared to its normal counterparts, primary astrocytes. Restoring Panx1 and Panx2 expression in C6 glioma cells by stable transfection induced a dramatically flattened morphology, which is similar to the flat and polygonal shape of cultured astrocytes. Both Panx1 and Panx2 also significantly suppressed the neoplastic phenotype of C6 glioma cells, including in vitro growth, anchorage-independent growth, and in vivo tumorigenesis monolaver in immunodeficient mice. Interestingly, while Panx1 reduced cell motility in C6 glioma cells, Panx2 did not elicit a similar effect. Panx1 and Panx2 exhibited a distinct subcellular localization. Panx1 was detected at the plasma membrane and perinuclear regions, whereas Panx2 was only found in membrane-bound compartments within the cytosol, hence suggesting mechanistically different tumor-suppressive pathways employed by the two Panxs. Furthermore, it was determined that Panx1 and Panx3, but not Panx2, increased neurite numbers and further enhanced neurite outgrowth in PC12 cells during nerve growth factor-induced neuronal differentiation. In conclusion, findings from this thesis suggest a functional role of Panxs in normal and pathological conditions of the CNS, and merit critical future investigations to explore their underlying mechanisms and therapeutic implication in diseases.

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# List of Abbreviations

А	Adenine
aCSF	Artificial cerebrospinal fluid
AKT	v-akt murine thymoma viral oncogene homolog 1
AMP	Adenosine monophosphate
APC	Adenomatous polyposis coli
ATP	Adenosine 5'-triphosphate
BCA	Bicinchoninic acid
BCAN	Brevican
BiP	Binding immunoglobulin protein
С	Cytosine
cAMP	Cyclic adenosine monophosphate
CBX	Carbenoxolone
CCN	CTGF, CYR61, and NOV
CDK	Cyclin-dependent kinase
CDKN	Cyclin-dependent kinase inhibitor
CHI3L1/YKL40	Chitinase 3-like 1
CL	Cytoplasmic loop
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CTGF	Connective tissue growth factor
Cx	Connexin
Cyr	Cysteine rich
DAPI	4',6-diamidino-2-phenylindole
DBcAMP	Dibutyrl-cAMP
DCC	Deleted in colorectal cancer
DIC	Differential interference contrast
DLL	Delta-like
DMEM	Dulbecco's modified minimal essential medium
DNA	Deoxyribonucleic acid
EEA1	Early endosome antigen 1

EGFP	Enhanced green fluorescence protein
EGFR	Epidermal growth factor receptor
EL	Extracellular loop
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FFA	Flufenamic acid
G	Guanine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBM	Glioblastoma multiforme
GDNF	Glial cell line-derived neurotrophic factor
GEO	Gene Expression Omnibus
GFAP	Glial fibrillary acidic protein
GЛС	Gap junctional intercellular communication
GM130	Golgi matrix protein of 130 kDa
Grp78	Glucose regulated protein 78
HA	Hemagglutinin
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IGFBP	Insulin-like Growth Factor Binding Protein
IL	Interleukin
Inx	Innexin
IP <sub>3</sub>	Inosital triphosphate
JAK	Janus tyrosine kinase
LOH	Loss of heterozygosity
MAGUK	Membrane-associated guanylate kinase
MAP2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase
MCP-1	Monocytic chemotactic protein 1
MDCK	Madin-Darby canine kidney
MDP	Muramyl dipeptide
MDM	Murine double minute

MEK	Mitogen-activated protein kinase kinase
MFG-E8	Milk fat globule-EGF factor 8 protein
NeuN	Neuronal nuclei
NCBI	National Center for Biotechnology Information
NF-ĸB	Nuclear factor kappa-light-chain-enhancer
NGF	Nerve growth factor
NMDA	N-methyl D-aspartate
NMDAR	N-methyl D-aspartate receptor
NO	Nitric Oxide
Nod2	Nucleotide-binding oligomerization domain 2
NOV	Nephroblastoma overexpressed
OGD	Oxygen/glucose deprivation
Olig2	Oligodendrocyte transcription factor 2
Р	Postnatal
Panx	Pannexin
PBS	Phosphate-buffered saline
PCAN	Proliferating cell nuclear antigen
PDGF-A	Platelet-derived growth factor A
PDZ	PSD95/disc large/ZO-1 homology domain
PET	Polyethylene terephthalate
PI3K	Phosphoinositide 3-kinase
PTEN	Phosphatase and tensin homologues
RAP	Ras-related protein
Ras	Rat sarcoma
RB	Retinoblastoma
REMBRANDT	Repository of Molecular Brain Neoplasia Data
RIPA	Radioimmunoprecipitation
RNA	Ribonucleic acid
RPL19	Ribosomal protein L19
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
SH2	Src-homology 2
SILAC	Stable isotope labeling with amino acids in cell culture

STAT	Signaling transducer and activator of transcription
TOP2A	Topoisomerase II alpha
TP53	Tumor protein 53
Т	Thymine
TNF	Tumor necrosis factor
U	Uracil
VEGF	Vascular endothelial growth factor
WGA	Wheat germ agglutinin
ZO	Zonula occludens

# Acknowledgements

I offer my enduring gratitude to my supervisor, Dr. Christian Naus, for his continuous guidance, support and friendship. I would also like to extend my appreciation to my advisors Drs. Joy M. Richman, Calvin D. Roskelley, and Michael T. Underhill for their positive support, suggestions and advice throughout my Ph.D. study.

My sincerest gratitude also goes to my friends and mentors who have kept me sound and made me scientifically cognizant throughout this time in my life, including John Bechberger, Lynne Bechberger, Dr. Linda Matsuuchi, Dr. Wun Chey Sin, Dr. Vincent Chen, Dr. Marc Mesnil, Dr. Jiahn-Chun Wu, Cima Cina, Michael Kozoriz, Steve Bond, Dr. Sophie Crespin, Annie Aftab, Dave Bates, and Max Le Vasseur. I would also like to acknowledge the friendship and assistance from the faculty members, staffs, students in the department, as well as other members in the Naus Lab.

Special thanks are owed to my parents and brother, who have supported me in every possible way throughout my years of education.

# Dedication

This thesis is dedicated to:

my parents, David and Helen, who have encouraged and supported me in every possible way throughout my life;

my brother, William, and Rachel, who have always infused me with confidence and trust;

my supervisor, Chris, who has provided me fatherly guidance, support, and encouragement in life and career;

my very close friends, John and Lynne Bechberger (aka Lab Dad & Lab Mom), who have always been so caring, forbearing, and supportive to their "Asian son" (me) both inside and outside the lab setting;

last but not least, my "big sister", Sin. I appreciate very much for her constructive criticisms, which literally whipped me into shape, wishing "iron could turn into steel at once". And for that I am very grateful.

### **Co-Authorship Statement**

The following thesis contains material from previously published articles co-authored by Charles P. Lai, John F. Bechberger, Dr. Roger J. Thompson, Dr. Brian A. MacVicar, Dr. Roberto Bruzzone, and Dr. Christian C. Naus.

All experimental work presented in this thesis was performed by Charles P. Lai with the assistance of Dr. R. J. Thompson in the whole-cell patch-clamp dye loading assays (Chapter 2) and Dr. R. Bruzzone in the pRK 5 expression vectors of Panxs (Chapter 2). John F. Bechberger, Dr. B. A. MacVicar, and Dr. Christian C. Naus provided consultation.

**Chapter 1** 

Historical Review,

**Objectives, Rationale and Hypotheses** 

#### **1.1 HISTORICAL REVIEW**

#### **1.1.1 Gap Junctions and Hemichannels**

Gap junctions are intercellular membrane channels that directly connect the cytosols of two adjacent cells. These channels allow the direct passage of small ions and molecules with a molecular weight less than 1 and 1.2 kDa in vertebrates and invertebrates, respectively, and function in maintaining intercellular communication and homeostasis within and between tissues (Simpson et al., 1977, reviewed in Simon and Goodenough, 1998). The intercellular channels are found in almost every cell in vertebrates except in fully differentiated skeletal muscle cells (Balogh et al., 1993; Schmalbruch, 1982) and highly mobile cells, including circulating erythrocytes (Locovei et al., 2006a) and mature sperm (Batias et al., 1999; Decrouy et al., 2004). Gap junctional intercellular communication (GJIC) arises during embryogenesis, continues throughout adulthood, and plays an important role in development, cellular differentiation, and cell growth (reviewed in Naus et al., 2005; Willecke et al., 2002). Conversely, disturbance of GJIC has been implicated in developmental diseases and tumorigenesis (see *Gap Junctions and Cancer*) (reviewed in Naus et al., 2005; Sohl and Willecke, 2004; Wei et al., 2004).

A gap junction is assembled by the docking of two hemichannels, or connexons; one connexon is contributed by each of the coupling cells at the plasma membranes. This forms an intercellular channel with the membranes separated by a 2 and 3 nm intercellular gap in vertebrates and invertebrates, respectively, and the channels was therefore termed *gap junction* to describe the unique junctional appearance (Revel and Karnovsky, 1967; Flower, 1972). A later study demonstrated that a gap junction further traverses through the plasma membrane, extending less than 2 nm into the cytoplasmic sides (Unwin and Zampighi, 1980). A connexon is made of a hexamer of connexins (Cxs) (see *Connexins*), the basic subunits of gap junctions, forming a closed cylindrical structure (Figure 1.1) (Unwin, 1986). The gap junction type often



**Figure 1.1. Schematic diagram of hemichannels and gap junctions.** Different types of gap junction channels can be formed by a change in the composition of connexin subunits and connexons.

varies between cell types and depends on its Cx constituents. The variation in gap junction composition can occur at two structural levels: connexins and connexons (Figure 1.1). A connexon can be made of the same (*homomeric*) or mixed (*heteromeric*) types of Cxs. Subsequently, a gap junction can be composed of the same (*homotypic*) or mixed (*heterotypic*) types of connexons. Therefore, a gap junction composed solely with Cx43 is a homomeric, homotypic gap junction.

Gap junction channels can be quickly formed within 3-30 minutes after cells established physical contacts (Valiunas et al., 1997). Following its formation, gap junction channels coalesce to form high-density punctate structures, which are often referred to as gap junctional *plaques* (Bukauskas et al., 2000). The clustering of gap junctions is a dynamic process with rapid turnover, which involves new channels aggregating from the periphery of the plaques as aged channels move to the centre for subsequent internalization and/or degradation (Gaietta et al., 2002; Laing and Bever, 1995; Laing et al., 1997). This phenomenon coincides with the short half-life of Cxs, which lasts for only 1-5 hours (Beardslee et al., 1998; Fallon and Goodenough, 1981; Laird et al., 1991; Laird et al., 1995). Through these channels, small ions (*i.e.* Ca<sup>2+</sup> and K<sup>+</sup>) (Beblo and Veenstra, 1997; Saez et al., 1989), nucleotides (Pitts and Simms, 1977), and secondary messengers (*i.e.* IP<sub>3</sub> and cAMP) (Murray and Fletcher, 1984; Saez et al., 1989) can readily diffuse between coupled cells, hence facilitating direct intercellular communication. Furthermore, based on its Cx subunit composition, gap junction permeability is selective to molecules by size and charge; Cx40 and Cx43 have a 8-fold preference for cations whereas Cx32 has a slight (~6%) anionic preference (Beblo and Veenstra, 1997; Suchyna et al., 1999; Wang and Veenstra, 1997, reviewed in Nicholson et al., 2000).

Hemichannels, by contrast, were once envisioned as solely an intermediate structure during gap junction formation. However, emerging findings have suggested Cx hemichannels as plasma membrane channels that serve as a direct conduit between the cytosol and the

extracellular space for diffusion of metabolites such as adenosine 5'-triphosphate (ATP) (Belliveau et al., 2006; Stout et al., 2002). Notably, the release of ATP via hemichannels was shown partly responsible for  $Ca^{2+}$  wave propagation in primary astrocytes, where an increase in intracellular  $Ca^{2+}$  concentration ([ $Ca^{2+}$ ]<sub>i</sub>) under stimuli propagates as a "wave" into surrounding cells (see Astrocytes) (Stout et al., 2002). However, the existence of in vivo Cx hemichannels has been a matter of debate due to three primary concerns (reviewed in Spray et al., 2006): 1) Functional studies of Cx hemichannels were often performed in  $Ca^{2+}$ -free solution, which has been suggested to be non-physiologically relevant in vivo (Hofer and Dermietzel, 1998; Li et al., 1996; Stout et al., 2002; Ye et al., 2003); 2) Gap junction blockers such as carbenoxolone and flufenamic acid used to inhibit Cx hemichannels in functional studies were also shown to affect other transmembrane proteins including ionotropic (ligand-gated) P2X<sub>7</sub> receptors (P2X<sub>7</sub>R), which mediate ATP release independent from Cx43 hemichannels (Suadicani et al., 2006); 3) In addition to Cxs, other hemichannel "candidate" proteins may also facilitate hemichannel-like activities. In fact, recent studies have suggested a novel family of gap junction proteins, pannexins (Panxs), as the constituents of hemichannels (see *Pannexins*).

### 1.1.1.1 Connexins

Connexins (Cxs), the canonical family of gap junction proteins, are suggested as polytypic integral membrane proteins with four transmembrane domains, two extracellular loops (EL), a cytoplasmic loop (CL), and cytoplasmic N- and C-termini based on hydropathy plots and proteolysis experiments (Zimmer et al., 1987; Yeager and Gilula, 1992; Zhang and Nicholson, 1994; Hertzberg et al., 1988; Yancey et al., 1989) (Figure 1.2). Each EL domain contains three conserved cysteine residues which stabilize the EL for the docking of connexons during gap



**Figure 1.2.** Schematic diagram representing predicted topology of Cx and Panx proteins. Both Cx and Panx are tetraspan transmembrane proteins with two extracellular loops (EL), one cytoplasmic loop (CL), and N- (NT) and C-termini (CT). Cxs contain three conserved cysteine residues (*yellow regions*) in each extracellular loop, whereas Panxs contain only two such residues. While an interloop disulfide bond has been demonstrated in Cxs, intraloop disulfide bonds remain to be shown (Foote et al., 1998; John and Revel, 1991). It is not yet known whether Panx forms intraloop disulfide bonds. Cx topology was determined from hydropathy plots and proteolysis experiments (Hertzberg et al., 1988; Yancey et al., 1989; Yeager and Gilula, 1992; Zhang and Nicholson, 1994; Zimmer et al., 1987). Panx topology is currently based on *in silico* prediction from hydropathy plots (Panchin, 2005; Yen and Saier, Jr., 2007).

junction channel formation (Foote et al., 1998; Perkins et al., 1998). Amongst the Cxs, the highest degree of diversity occurs in the length and sequence of the CL, as well as the size and post-translational modification of the C-terminus (reviewed in Beyer et al., 1990; Lampe and Lau, 2004; Willecke et al., 1991). Conversely, the most well conserved regions are found within the four transmembrane domains, the two EL domains, and the N-terminus (reviewed in Beyer et al., 1990; Willecke et al., 2002).

Two nomenclatures are currently used for Cx proteins and genes (Sohl and Willecke, 2003). The first nomenclature abbreviates connexins with "Cx" and terms each Cx gene based on their origin of species and the predicted molecular weight in kDa. For example, mCx43 represents mouse connexin43 with 43 kDa protein size. Alternatively, in the second "Gja/Gjb" nomenclature, connexins are shortened with "Gj" and are divided into subgroups ( $\alpha$ ,  $\beta$ , or  $\gamma$ ) based on sequence similarities and length of the cytoplasmic domains. This later system was also adopted by the NCBI (National Center for Biotechnology Information) database. Although both nomenclatures are adequate, each has its own caveats. For instance, a Cx protein named after its predicted size can vary from its actual size in later studies. Similarly, the predicted sequence length used in the "Gj" nomenclature can be erred by different 5'-untranslated regions after alternative and/or consecutive splicing events (Sohl and Willecke, 2003). For the purpose of this thesis, the more commonly used "Cx" nomenclature is applied.

To date, Cxs consists of 20 and 21 members in human and mouse, respectively (reviewed in Sohl and Willecke, 2004). Cx expression is tissue-specific, and a cell may express more than one type of Cx, hence conferring the flexibility to theoretically form different types of gap junctions to carry out its functions. In the CNS, astrocytes and neurons express many types of Cxs, and mutations in these genes have been associated with different neuropathological conditions, including cancer, neurodegenerative disease, brain ischemia, and epilepsy (reviewed in Naus et al., 2005; Niemann et al., 2006; Vinken et al., 2006).

#### 1.1.1.2 Pannexins

In recent years, a novel family of gap junction proteins, Panxs (pan – all, throughout; nexus – connection, bond), with low sequence similarity to the invertebrate gap junctions, innexins (Inxs; <u>invertebrate connexin</u> analogs), has been discovered in chordates (Baranova et al., 2004; Bruzzone et al., 2003; Dykes et al., 2004; Panchin et al., 2000; Potenza et al., 2003). The identification of Inxs arose from the initial effort to clone Cxs from invertebrates, which failed after several attempts and subsequently led to the discovery of this family of invertebrate gap junction proteins unrelated to Cxs (Barnes, 1994; Krishnan et al., 1993; Phelan et al., 1998; Phelan and Starich, 2001; Starich et al., 1993; Watanabe and Kankel, 1992).

Correspondingly, Panxs share no similarity with Cxs in their primary amino acid sequences (Yen and Saier, Jr., 2007). However, Panx and Cx families possess resemblances in their predicted topologies based on hydropathy plots (Panchin, 2005; Yen and Saier, Jr., 2007). Similar to Cxs, Panxs have a predicted topology of four membrane-spanning domains, two ELs, a CL, and cytoplasmic N- and C-termini (Figure 1.2) (Yen and Saier, Jr., 2007). Intriguingly, whereas Cxs contain three regularly spaced cysteines in each of the two ELs, Panxs, like Inxs, only have two such residues and thus resemble Inxs in this aspect (Hua et al., 2003). Several studies have suggested the importance of the cysteine residues in the formation of functional Cx-based gap junctions and hemichannels (Bao et al., 2004b; John and Revel, 1991; Dahl et al., 1992; Foote et al., 1998; Perkins et al., 1998), and therefore the variation in the number of cysteine residues alludes to mechanistic differences in Panx and Cx functions (See *Pannexin Hemichannels*).

Currently, three Panx members (Panx1, Panx2 and Panx3) have been identified in vertebrates (Baranova et al., 2004). Previous studies on Inx mutants in *Drosophila* have demonstrated Inx-specific functions, including synaptogenesis in the giant-fiber system, epithelial organization and morphogenesis, and germ cell differentiation processes (Bauer et al.,

2005). Although it remains to be seen whether Panxs can be regarded as vestigial Inxs that have survived in higher animals, this implies that, other than Cxs, Panxs may also play a functional role in chordates (Bauer et al., 2005; Yen and Saier, Jr., 2007; Barbe et al., 2006). In fact, emerging studies since then have uncovered numerous novel functions of Panxs (See *Pannexin Hemichannels*).

Panxs expression is tissue-specific based mostly on transcriptional analyses of Panx mRNA expression in rodents. Panx1 was found to be most ubiquitously expressed and detected in various tissues, including the brain, spinal cord, retina, thyroid, skeletal muscles, blood endothelium, erythrocytes, kidney, and prostate (Bruzzone et al., 2003; Panchin et al., 2000; Penuela et al., 2007; Ray et al., 2005; Ray et al., 2006; Vogt et al., 2005; Weickert et al., 2005). Panx2, on the other hand, was predominately detected in the brain and retina (Baranova et al., 2004; Bruzzone et al., 2003; Dvoriantchikova et al., 2006; Litvin et al., 2006; Vogt et al., 2005). Panx3 is present in the cartilage, skin, and brain (in a lesser amount) (Bruzzone et al., 2003; Penuela et al., 2007; Ray et al., 2005; Vogt et al., 2005; Weickert et al., 2005). Interestingly, Panx1 and Panx2 transcripts detected in the brain were found to be co-localized with neuronal nuclei (NeuN), a marker for most neuronal cell types (Ray et al., 2005; Mullen et al., 1992; Vogt et al., 2005). Zappala et al. also showed Panx1 expression in Bergmann glia of the cerebellum as revealed by its co-localization with glial fibrillary acidic protein (GFAP), an astrocyte-specific intermediate filament (Zappala et al., 2006; Eng et al., 2000). Overall, these findings suggest a role of Panxs in the brain, and further detailed characterizations with the recent availability of Panx-specific antibodies will provide novel insight into identifying their respective functions.

#### **1.1.1.2.1** Pannexin Gap Junctions

Despite the presence of Cxs, Panxs remained highly conserved in vertebrates, hence suggesting that Panxs carry specialized, functional roles that are distinct from the Cxs. Analogous to Cxs, Panx hemichannel and intercellular channel formation are also Panx-specific (Bruzzone et al., 2003). Using the *Xenopus* oocyte expression system, rat Panx1 and Panx1/Panx2 were found to form functionally different hemichannels and intercellular channels in oocytes via electrophysiological experiments (Bruzzone et al., 2003; Boassa et al., 2008). By contrast, Panx2 and Panx3 alone did not exhibit any channel activity (Bruzzone et al., 2003).

Previous morphological and ultrastructural studies in crayfish axons and rodent spinal cords have identified fine, rosette-like gap junctional puncta that are noticeably different from the classic Cx gap junction plaques, which are comparably larger and with brighter punctate staining (Peracchia, 1973; Peracchia and Dulhunty, 1976; Rash et al., 1998). Panx1 and Panx3 signals are observed in finer puncta at cell-cell junctional regions (Lai et al., 2007; Locovei et al., 2006a; Penuela et al., 2007; Penuela et al., 2009), inferring that the rosette-like structure are gap junctions formed by Panxs instead of Cxs (Shestopalov and Panchin, 2008). Further ultrastructural analysis using Panx-specific antibodies and electron microscopy will be required to test this hypothesis.

In fact, utilizing electron microscopy and Panx1 with tetracysteine tag, Boassa *et al.* recently showed that Panx1 is dispersed across the plasma membrane at cell-to-cell contacts and has an intercellular space of 20-50 nm in Madin-Darby canine kidney (MDCK) cells (Boassa et al., 2007). Cx43-based gap junctions, on the other hand, appeared as concentrated punctate plaques and have a smaller intercellular gap (*i.e.* 2-5 nm) (Boassa et al., 2007). Furthermore, it was found that exogenous Panx1 formed limited intercellular channels when compared to Cxs (Boassa et al., 2007; Lai et al., 2007). Therefore, whether Panx forms gap junctions and carries a

functional role via intercellular communication remains a matter of debate and awaits future investigations.

#### 1.1.1.2.2 Pannexin Hemichannels

Increasing studies have reported functional Panx hemichannels via patch-clamp and dye uptake experiments, and therefore hemichannels have been advocated as the prevailing functional structure of Panxs (Bao et al., 2004a; Silverman et al., 2009; Huang et al., 2007b; Thompson et al., 2008; Reigada et al., 2008; Iglesias et al., 2009; Bruzzone et al., 2003; Locovei et al., 2006a; Penuela et al., 2007; Pelegrin and Surprenant, 2006). By contrast, limited evidence of Panx gap junctions has been found, particularly in cultured neurons and glia (Huang et al., 2007a). The notion of Panxs predominantly as hemichannels is further supported by 1) their predicted structure and 2) glycosylation status of Panxs. Structurally, EL domains of Panxs are longer in length ( $\sim 50 - 68$  a.a.) and have only 2, instead of 3, cysteine residues when compared to the Cxs (~30 a.a.) (Baranova et al., 2004; Panchin et al., 2000). In Cxs, the cysteine residues form interloop disulfide bonds between the two ELs of each Cx subunit, creating one β-hairpin per each EL (Figure 1.2). Thus, each connexon possesses a total of 12  $\beta$ -hairpins with 2  $\beta$ hairpins from each Cx subunit, which allows their interdigitation (docking) with the other 12 βhairpins from the opposing connexon, forming a tight seal between the consequent two  $\beta$ -barrel structure of connexons (gap junction) at the extracellular "gap" of a gap junction (Foote et al., 1998; John and Revel, 1991; Perkins et al., 1998). Conversely, Panxs' reduced number of cysteine residues (i.e. reduced intramolecular disulfide bonds) along with the greater length in ELs (*i.e.* structural hindrance to the docking of pannexons) implies its preferred formation in hemichannels (Boassa et al., 2007; Boassa et al., 2008). Furthermore, Panxs were demonstrated as glycoproteins where glycosylation occurs at the first (Panx2 and Panx3) or second (Panx1)

extracellular loops (Penuela et al., 2007; Penuela et al., 2009). It was suggested that the resultant carbohydrate moieties from glycosylation interfere with the docking of two pannexons, thus preventing the formation of intercellular channels under specific physiological conditions (Boassa et al., 2007; Penuela et al., 2007; Penuela et al., 2009).

Additionally, Peneula *et al.* demonstrated that all three Panxs alone were able to form functional hemichannels (Penuela et al., 2009), which is in contrast to an earlier report that showed only Panx1 and Panx1/2 were able to form hemichannels (Bruzzone et al., 2003). The discrepancy in findings may be attributed to the difference in species of Panx constructs (rat Panx cDNAs in Bruzzone *et al.*; mouse Panx cDNAs in Peneula *et al.*), as well as cell models used (*Xenopus* oocytes in Bruzzone *et al.*; human embryonic kidney (293T) cells in Peneula *et al.*) (Bruzzone et al., 2003; Penuela et al., 2009). Specifically, it would be of interest to examine if a variation in Panx glycosylation status was present in the different experimental conditions and consequently contributed to the difference in findings. Peneula *et al.* also showed that co-expressing Panx1 and Panx2, but not Panx1 and Panx3, resulted in co-localization and co-immunoprecipitation of both proteins, and further reduced channel activity when compared to homomeric Panx1 hemichannels using dye uptake experiments (Penuela et al., 2009). This suggests that Panx2 may function by modulating Panx1 channel activity (Penuela et al., 2009).

To date, one of the most observed functions of Panx1 lies in its hemichannel activity which allows transmembrane passage of ATP in response to physiological and pathological stimuli, thereby facilitating paracrine and/or autocrine signaling (Locovei et al., 2006a; Locovei et al., 2006b; Pelegrin and Surprenant, 2006). Although many studies have reported that Cx hemichannels facilitate ATP release (reviewed in Saez et al., 2005), it has now been considered that Panx hemichannels are also involved in this process (Bargiotas et al., 2009; D'hondt et al., 2009; Dahl and Locovei, 2006; Scemes et al., 2007). This supposition is further supported when Panx and Cx channels showed sensitivity, albeit at varying degrees, to the same pharmacological

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blockers; whereas Cx hemichannels were inhibited by both carbenoxolone (CBX) and flufenamic acid (FFA). Panx hemichannels were more sensitive to CBX and only modestly inhibited by FFA when compared to Cxs at the same concentration (Bruzzone et al., 2005; Srinivas and Spray, 2003). CBX is a synthetic derivative of 18-glycyrrhetinic acid, which is a saponin derived from liquorice root (Davidson and Baumgarten, 1988). The mode of inhibitory action of CBX remains to be elucidated but it has been suggested that CBX intercalates into the plasma membrane, binding to Cxs to induce a conformational change which results in channel closure (Davidson and Baumgarten, 1988; Goldberg et al., 1996). FFA is a nonsteroidal antiinflammatory drug proposed to induce hemichannel closure of Cxs, and perhaps Panx, by binding to a modulator site presumably within the plasma membrane, but not directly to the channel's pore (Srinivas and Spray, 2003). Some Cx mimetic peptides previously used in Cx hemichannel inhibition studies also attenuated Panx channel currents (Wang et al., 2007). These findings suggest that previous reports on Cx hemichannel activities demonstrated by using "Cxspecific" inhibitors may be confounded by the presence of Panx hemichannels. Overall, findings to date advocate that Panxs preferentially form hemichannels to elicit their functions.

#### **1.1.1.2.3 Regulations and Functions**

In very recent years, many novel functions of Panxs, namely Panx1, have been uncovered. This section will cover some of the major regulatory mechanisms of Panx hemichannels, as well as their functional implications under both normal and pathological conditions (Figure 1.3).

#### 1.1.1.2.3.1 Calcium Concentration, pH, and Membrane Potential

Most Cx hemichannels were shown to be gated by external divalent cations, such as  $Ca^{2+}$  and  $Mg^{2+}$ , with a low and high frequency of channel opening under physiological  $[Ca^{2+}]_0$  (1.3-



**Figure 1.3. Regulatory mechanisms of Panx1 hemichannel.** Activation of P2X and P2Y purinergic receptors by extracellular ATP can directly and indirectly trigger Panx1 hemichannel opening by P2X<sub>7</sub>R-Panx1 interaction and P2YR-elicited increase in  $[Ca^{2+}]_i$ , respectively. These pathways result in extracellular ATP-stimulated ATP release from cells. Panx1 can also be activated by mechanical stress, membrane depolarization, and oxygen/glucose deprivation (OGD) during an ischemic event. In addition to ATP, Panx1 hemichannels are also permeable to  $Ca^{2+}$ , dyes, and other small molecules, including bacterial toxins and antigens. Specifically, the internalization of bacterial endotoxins and antigens via opened Panx1 hemichannels was found important in initiating acute inflammatory response. B, binding; C, cleavage; CM, covalent modification; IE, influence on expression; T, transition into open channel configuration; Tn, transport; ?, unspecified mechanism. (Used with permission from Springer Science + Business Media: Cellular and Molecular Life Sciences, Shestopalov and Panchin. 2008. Pannexins and gap junction protein diversity. 65(3): 376-94.).

1.8 mM, *in vitro*; 1-1.3 mM, *in vivo*) and low  $[Ca^{2+}]_o$  (~0 mM), respectively, using dye uptake and electrophysiological measurements (Ebihara and Steiner, 1993; Li et al., 1996; Pfahnl and Dahl, 1999; Quist et al., 2000; Valiunas, 2002). The inhibitory effect of extracellular Ca<sup>2+</sup> was demonstrated by atomic force microscopy as a result of induced connexon conformation change (Muller et al., 2002), likely via a direct binding of Ca<sup>2+</sup> to a Ca<sup>2+</sup> -binding site on the external vestibule of connexon pores (Gomez-Hernandez et al., 2003).

Unlike Cx hemichannels which open under low  $[Ca^{2+}]_o$ , Panx1 hemichannels are insensitive to  $[Ca^{2+}]_o$  as shown by patch-clamp experiments in Panx-expressing *Xenopus* oocytes (Bruzzone et al., 2005). By contrast, Panx1 hemichannels were opened by  $[Ca^{2+}]_i$  in a linear fashion where higher  $[Ca^{2+}]_i$  resulted in a greater inward membrane current and  $Ca^{2+}$  washout ceased the channel activity (Locovei et al., 2006b). This suggests that Panx1 hemichannels open to mediate ATP release during agonist-induced  $Ca^{2+}$  signaling (see *Mechanosensitivity and Vasodilation*) (Figure 1.3). However, it has not yet been identified as to how the increased  $[Ca^{2+}]_i$ triggers the opening of Panx1 hemichannels.

Low intracellular pH, on the other hand, has been found to induce both Cx and Panx1 channel closure (Duffy et al., 2004; Gonzalez-Nieto et al., 2008; Locovei et al., 2006b; Morley et al., 1997; Yu et al., 2007). A cytoplasmic pH sensor of connexons has been shown responsible for the inhibitory effect of intracellular acidification by using excised inside-out patch from *Xenopus* oocytes and recording hemichannel currents at different pH (Trexler et al., 1999). A mode of low pH<sub>i</sub>-induced inhibition of Panx hemichannels remains to be elucidated.

Panx hemichannels are sensitive to membrane potential as demonstrated by voltageactivated membrane current and ATP release at 20 mV depolarizing-steps in *Xenopus* oocytes expressing Panxs using patch-clamp and luciferin/luciferase experiments, respectively (Figure 1.3) (Bao et al., 2004a; Bruzzone et al., 2003). Panx1 hemichannels exhibited larger membrane currents with increasing depolarization similar to Cx hemichannels. However, Panx1 reached peak current with 10- to 50-folds faster kinetics and showed larger unitary conductance, weak voltage-gating, and multiple subconductance states when compared to Cxs (Bao et al., 2004a; Bruzzone et al., 2003; Paul et al., 1991; White et al., 1999). Interestingly, Panx1/Panx2 heteromeric hemichannels presented reduced current amplitude and slower kinetics of pore opening when compared to Panx1 hemichannels, suggesting that Panx2 acts as a modulator of Panx1 channels since Panx2 alone did not exhibit channel activity when expressed (Bruzzone et al., 2003). Panx3 did not present channel activity when expressed alone or in combination with Panx1 in the *Xenopus* oocyte system (Bruzzone et al., 2003). By contrast, later studies showed low and high amount of dye uptake in 293T cells following exogenous expression of Panx2 and Panx3, respectively, when compared to GFP-transfected cells (Penuela et al., 2007; Penuela et al., 2009). Since glycosylation status of Panxs have been shown responsible for their proper trafficking to the plasma membrane (Penuela et al., 2007; Penuela et al., 2009), the difference in findings may be a result of varying degree of Panx glycosylation between the cell lines used.

### 1.1.1.2.3.2 Initiation of Inflammatory Response

Activation of Panx1 hemichannel has been closely associated with acute inflammatory response to bacterial surface antigens, endotoxins, and other pro-inflammatory stimuli (*i.e.* TNF- $\alpha$ , IFN- $\gamma$ ). IL-1 $\beta$  is transcriptionally activated by pro-inflammatory stimuli, but its release as the mature 18-kDa form by macrophages requires proteolytic processing by activated caspase-1 (Thornberry et al., 1992), which is cryopyrin inflammasome-dependent (Mariathasan et al., 2006). Activating the inflammasome complex first involves the entry of pro-inflammatory molecules into the cytosol, and this step is facilitated by a secondary stimulus such as exogenous ATP (Surprenant et al., 1996; Sluyter et al., 2004; Di Virgilio, 1995). ATP treatment on macrophages activates ionotropic P2X<sub>7</sub>R, a cation-selective ion channel, and this activation was demonstrated by an increase in ionic current passage across the plasma membrane (Surprenant et al.)

al., 1996; Sluyter et al., 2004; Di Virgilio, 1995). Interestingly, the activation of P2X<sub>7</sub>R was also accompanied by the formation of a non-selective, large pore shown to be permeable to molecules up to 900 Da, including ethidium bromide and YoPro-1 (Surprenant et al., 1996; Di Virgilio, 1995). The large pore was suggested to facilitate the entry of pro-inflammatory molecules into macrophages, but its identity was unidentified. Given that expression of P2X<sub>7</sub>R alone in *Xenopus* oocytes showed only cation channel activity whereas co-injection of P2X<sub>7</sub>R and murine macrophage mRNA exhibited further presence of a large pore, it was speculated that additional cellular components were required for the pore formation (Nuttle et al., 1993). Following the discovery of Panxs, Panx1 hemichannel was found to present similar channel properties to the P2X<sub>7</sub>R pore, including passage of the same dyes, as well as sensitivity to cytoplasmic acidification and gap junction channel blockers (Locovei et al., 2007). In fact, Panx1 was later demonstrated as the constituting component of the pore using Panx1-specific siRNAs and co-expression of P2X<sub>7</sub>R and Panx1 in *Xenopus* oocytes (Locovei et al., 2007).

Ensuing studies using Panx1-specific siRNA and mimetic peptides to inhibit Panx1 channel activity showed that Panx1 hemichannel opening followed by ATP-stimulated P2X<sub>7</sub>R is required to facilitate the entry of pro-inflammatory molecules, including marine toxin (maitotoxin),  $K^+/H^+$  antiport ionophore nigercin, and muramyl dipeptide (MDP). The entry of pro-inflammatory molecules then leads to caspase-1 activation and the subsequent maturation (cleavage) and release of IL-1 $\beta$  (Figure 1.3) (Kanneganti et al., 2007; Marina-Garcia et al., 2008; Pelegrin and Surprenant, 2006; Pelegrin et al., 2008). Specifically, a recent study by Silverman *et al.* demonstrated that activation of the inflammasome by high [K<sup>+</sup>]<sub>0</sub> treatment in neurons and astrocytes, but not low [K<sup>+</sup>]<sub>0</sub> as suggested in macrophages and monocytes, resulted in Panx1's association with P2X<sub>7</sub>R-containing inflammasome complex and the subsequent activation of caspase-1 for an inflammatory response (Silverman et al., 2009). Furthermore, an *in silico* study by Shestopalov and Panchin using independent microarray data sets from Gene Expression

Omnibus Profiles (<u>http://www.ncbi.nlm.nih.gov/projects/geo</u>) found that *PANX1* transcript is upregulated by 3-7 fold with pro-inflammatory stimuli, including TNF- $\alpha$ , INF- $\alpha$ , IFN- $\gamma$ , lipopolysaccharide, cold, and systematic inflammation, when compared to controls (Shestopalov and Panchin, 2008). Importantly, this correlation was detected in multiple mammalian cell lines and tissues, such as microglia and monocyte-derived dendritic cells, suggesting that Panx1 is an integral component of the inflammatory response in different systems (Shestopalov and Panchin, 2008).

### 1.1.1.2.3.3 Mechanosensitivity, Vasodilation, and Astrocytic Intercellular Ca<sup>2+</sup> Wave

In the blood circulatory system, Panx1 hemichannels of erythrocytes can be triggered by mechanical stress and/or low glucose to release ATP as shown by using an [ATP]-sensitive luciferin/luciferase assay (Locovei et al., 2006b). This may subsequently activate metabotropic P2Y receptors on both erythrocytes and endothelial cells in close proximity (Figure 1.4) (Locovei et al., 2006a). The activation of P2Y receptors may result in an autocrine, positive feedback mechanism to enhance Panx1 hemichannel opening since erythrocytes responds to P2Y receptor-induced elevation in  $[Ca^{2+}]_i$  (Locovei et al., 2006a; Locovei et al., 2006b). Panx1 hemichannels may also function in a paracrine fashion by activating P2Y receptor of endothelial cells via released ATP to initiate an intercellular  $Ca^{2+}$  wave, resulting in the activation of nitric oxide (NO) synthase and the subsequent release of NO to the smooth muscles at the precapillary sphincter region to induce vasodilation (Figure 1.4) (Locovei et al., 2006a). Although ATP was demonstrated to induce Panx1 hemichannel opening, it was also shown to inhibit Panx1 channel activity in erythrocytes at a slightly higher  $[ATP]_0$  than that is required for P2X<sub>7</sub>R and P2Y<sub>2</sub>R activation, suggesting that ATP also functions in a negative feedback manner to regulate Panx1 channel activity (Oiu and Dahl, 2009). Given that positively charged amino acids are involved in

Figure 1.4. Schematic representation of Panx-mediated long-range Ca<sup>2+</sup> wave propagation in blood endothelium to induce NO-mediated vasodilation. Panx1 hemichannels open under stimuli such as mechanical stress and/or glucose deprivation during an ischemic event, resulting in the release of ATP. The released ATP activates P2Y receptors of endothelial cells (blue boxes), which induce the release of intracellular  $Ca^{2+}$  by activating IP<sub>3</sub> receptors at the endoplasmic reticulum (ER). The elevation in  $[Ca^{2+}]_i$  triggers the opening of Panx1 hemichannels in endothelial cells to release ATP, resulting in paracrine activation of P2Y receptors of neighboring cells and the consequent  $Ca^{2+}$  wave propagation. The opened hemichannels may also serve as a direct conduit for  $Ca^{2+}$  release, facilitating  $Ca^{2+}$  gradient regulation given Panx1's sensitivity to  $[Ca^{2+}]_i$ . Alternatively, direct mechanical stimuli to endothelial cells have also been demonstrated to open Panx1 hemichannels, resulting in an increase of endothelial  $[Ca^{2+}]_i$  via  $Ca^{2+}$  influx. This hypothesized  $Ca^{2+}$  wave propagation was suggested responsible for the activation of endothelial nitric oxide (NO) synthase and the subsequent release of NO to the smooth muscles at the precapillary sphincter region to mediate vasodilation. A direct passage of  $Ca^{2+}$  and IP<sub>3</sub> via Panx and/or Cx gap junctions may also facilitate  $Ca^{2+}$  wave propagation within a tissue. B, binding; T, transition into open configuration; Tn, transport; Z, catalysis; ?, unspecified mechanism of activation; white star, activated IP<sub>3</sub> receptor at the ER. (Used with permission from Springer Science + Business Media: Cellular and Molecular Life Sciences, Shestopalov and Panchin. 2008. Pannexins and gap junction protein diversity. 65(3): 376-94.).


ATP recognition by P2X receptors (Ennion et al., 2000; Jiang et al., 2000), Qiu and Dahl hypothesized and further demonstrated that arginine, a positively charged amino acid, in the putative extracellular loops at amino acid position 75 of mouse Panx1 was required for ATP-mediated Panx1 channel inhibition using mutational analysis (Qiu and Dahl, 2009).

Intercellular Ca<sup>2+</sup> waves are also found in astrocytes of the CNS, and it was shown to play an important role in neuron-astrocyte bidirectional communication. Similar to the proposed model of Panx-mediated Ca<sup>2+</sup> waves in endothelial cells, previous reports suggested that Cx hemichannels facilitate this phenomenon in astrocytes by serving as a direct conduit at the plasma membrane for the release of ATP into the extracellular space following ATP-induced purinergic receptor activation (Cotrina et al., 1998; Guthrie et al., 1999; Stout and Charles, 2003; Stout et al., 2002). It was also proposed that gap junction directly facilitates the Ca<sup>2+</sup> waves via intercellular communication (Charles et al., 1992). However, these earlier studies tested Cx's involvement in  $Ca^{2+}$  wave propagation mainly by using pharmacological gap junction blockers. which have now been shown to also inhibit Panx1 channels, as well as  $P2X_7R$  (Bruzzone et al., 2005; Suadicani et al., 2006). Through the use of spinal cord astrocytes derived from neonatal P2X<sub>7</sub>R-null mice, Suadicani et al. showed that activation of P2X<sub>7</sub>R pore complex is required to mediate the ATP release and resultant amplification of  $Ca^{2+}$  wave in astrocytes (Suadicani et al., 2006), which is similar to the observations made during inflammatory responses (see Initiation of Inflammatory Response) (Pelegrin and Surprenant, 2006; Pelegrin et al., 2008). Moreover, it was shown that knockdown of Panx1, but not of Cx43, inhibited amplification of  $Ca^{2+}$  wave in astrocytes (Scemes et al., 2007; Suadicani et al., 2006). These findings led to the hypothesis and later identification of Panx1 as part of the  $P2X_7R$  pore complex by abolishing ATP release of Cx43-null astrocytes with Panx1-specific siRNAs treatment (Iglesias et al., 2009). Although previous studies reported a direct correlation between Cx43 expression level and the amount of ATP release (Cotrina et al., 1998; Naus et al., 1997; Stout and Charles, 2003; Stout et al., 2002),

Iglesia *et al.* suggested that the correlation was a result of reduced cytoplasmic  $[ATP]_i$  in Cx43null astrocytes instead of the absence of Cx43 hemichannels (Iglesias et al., 2009). Overall, the studies support that Panx1, but not Cx, predominantly facilities Ca<sup>2+</sup> wave propagation via ATPinduced ATP release in astrocytes.

#### 1.1.1.2.3.4 Oxidative Mechanism, Ischemic Death of Neurons and Glia, and Epilepsy

Under oxygen/glucose deprivation (OGD) conditions during a stroke, neurons exhibit large conductance channels that result in neuronal swelling and  $Ca^{2+}$  dysregulation, leading to neuronal cell death. Using electrophysiological measurements, the large conductance channel was suggested to be a Panx1 hemichannel, which could be activated during OGD to induce neurolethal anoxic depolarization in pyramidal neurons (Figure 1.3) (Thompson et al., 2006). In accordance to this supposition, Domercq *et al.* demonstrated that Panx1 hemichannel opens to mediate ATP release under OGD in both cultured oligodendrocytes and isolated optic nerves, leading to P2X<sub>7</sub>R activation and the subsequent death of oligodendrocytes, myelin damage, and axonal dysfunction (Domercq *et al.*, 2009). Furthermore, Panx1 was suggested to mediate  $Ca^{2+}$ leakage from the ER (Vanden Abeele et al., 2006), which may also promote neuronal necrosis during an ischemic event (Vannucci et al., 2001).

Additionally, Panx1 expression was shown at postsynaptic sites in pyramidal neurons by its co-localization with postsynaptic density protein 95 (PSD95) (Zoidl et al., 2007). This led to the hypothesis and identification of Panx1's participation in the postsynaptic responses of hippocampal neurons during epileptiform seizure activity by opening its hemichannels under *N*methyl *D*-aspartate receptor (NMDAR) activation (Thompson et al., 2008). Notably, the opening of Panx1 in this scenario was suggested to be a consequence of  $[ATP]_i$  depletion during NMDAR activation, but not of an increase in  $[Ca^{2+}]_i$  as was the case in the blood circulatory system (Thompson et al., 2008). This raises the possibility that Panx1 hemichannel opening may be negatively regulated by [ATP]<sub>i</sub> (Thompson et al., 2008).

#### 1.1.2 Gap Junctions and Cancer

Gap junctions have been found to be down-regulated in many types of cancer including glioma, breast carcinoma, and prostate cancer. Reciprocally, restoring GJIC in cancer cell models reverts some of the transformed phenotype and reduces proliferation of the neoplastic cells (reviewed in Leithe et al., 2006; Mesnil, 2002; Mesnil et al., 2005). This suggests that Cxs function as tumor suppressors, and numerous studies have explored restoration of GJIC as a potential therapeutic route against cancer (reviewed in Trosko and Ruch, 2002). The exact functional pathway by which Cxs and gap junctions mediate tumor-suppressive effects remains to be elucidated. However, five main mechanisms have been proposed (Figure 1.5): 1) dispersal of pro-mitotic factors; 2) accumulation of anti-mitotic factors; 3) Cx-interacting proteins; 4) Cxinduced transcriptional change; 5) Cx hemichannels (reviewed in Naus et al., 2005). The first two mechanisms were suggested on the basis of canonical gap junction functions where they facilitate either 1) the dispersal of pro-mitotic factors to promote tumor phenotypes or 2) the spread of anti-mitotic signals to suppress neoplasm (Krysko et al., 2005). Methods employed in these studies often involve a combination of intercellular dve passage assay, pharmacological and peptide inhibition of gap junctions, and/or mutated Cx constructs to observe a change in oncogenicity. Although a number of transjunctional metabolites, including glutamate, ATP, and ADP were identified (Goldberg et al., 1998; Goldberg et al., 1999), the identity of transjunctional mitotic molecules remains largely unknown. 3) Gap junctions have also been suggested as a "nexus" at the plasma membrane to allow the docking of scaffolding and signaling molecules, some of which are involved in tumor-suppression, including catenins and CCN3

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- 1. Disperse pro-mitotic factors
- 2. Accumulate anti-mitotic factors
- 3. Cx interacting proteins
- 4. Transcription
- 5. Hemi-channel



**Figure 1.5. Proposed mechanisms of gap junction protein-mediated effects on cell proliferation and tumorigenesis.** (Used with permission from Springer Science + Business Media: Astrocytes in (patho)physiology of the nervous system, Lai and Naus. 2009. Connexins and pannexins: Two gap junction families mediating glioma growth control. 547-68.).

(Duffy et al., 2002; Fu et al., 2004). 4) In recent years, Cx expression was shown to induce a large-scale alteration of transcriptomes, suggesting Cxs as "hubs" in gene expression networks (Iacobas et al., 2007). Remarkably, growth genes were found significantly more likely to be altered by changes in Cx expression when compared to other gene categories (Kardami et al., 2007), hence further suggesting a role of Cxs in the cell growth regulation. Specifically, Cxs have been associated with transcription of several genes involved in proliferation and tumorigenesis, such as monocyte chemotactic protein 1 (MCP-1), cyclin A, D1, D2, cyclin-dependent kinase (CDK) 5 and 6 (Chen et al., 1995; Huang et al., 2002). 5) Although a functional role of hemichannel in neoplasia has not been shown, it is hypothesized they may also facilitate tumor-suppression as observed in Cx-expressing cancer cells by allowing a direct passage of apoptotic signals and/or chemotherapeutic agents from the extracellular space into the cytosol (Decrock et al., 2009; Goodenough and Paul, 2003). Altogether, gap junctions may mediate their tumor-suppressive effects via one or a combination of the proposed mechanisms, while other vet identified pathways remain to be discovered.

#### 1.1.2.1 Astrocytes

Glial cells are commonly perceived as mere supporting cells that provide structural support and neurotrophic substances to the seemingly more important character of the brain, the neuron. Glia, derived from the Greek word for glue, was initially used by Virchow in the 1840s as "neuroglia" to describe a cell type which forms a layer of connective tissue to bond nerve cells in the brain. Glial cells are categorized into two main groups: macroglia and microglia. Macroglia represents majority of the glial population whereas microglia, the immune effector cells of the brain derived from myeloid progenitor cells, are a smaller group within the population (reviewed in Ransohoff and Perry, 2009). In the central nervous system (CNS),

macroglia are further classified into oligodendrocytes, ependymal cells, radial glia, and the most abundant type of cells within the group, astrocytes.

Astrocytes are star-shaped cells with a dense array of processes that interpose between glial and neuronal cells. Mature astrocytes are commonly identified by their classic expression of GFAP, an intermediate filament in mature astrocytes (Eng et al., 2000). S100 $\beta$  has also been reported as an astrocytic marker, but it is less often used since it is not only expressed in a subset of mature astrocytes, but also in other neural-cell types (Steiner et al., 2007).

Traditionally, astrocytes are known for their housekeeping roles as sensors and regulators of the local microenvironment in the CNS, including extracellular ion concentration and glutamate homeostasis (reviewed in Gee and Keller, 2005; Nedergaard et al., 2003). However, emerging evidence from the past decade on the crosstalk between neurons and glia have discovered novel functions of astrocytes under both normal and pathological CNS conditions, including synaptic transmission, neuronal differentiation, axonal guidance, and synaptogenesis (reviewed in Araque, 2008; Seth and Koul, 2008). Astrocytes express a wide variety of ion channels, ionotropic nucleotide receptors, and neurotransmitter receptors (reviewed in Verkhratsky and Steinhauser, 2000). With these channels, astrocytes propagate gliotransmission through the astrocytic network in response to chemical stimuli such as glutamate and ATP from the neurons, thereby making neuron-astrocyte bidirectional communication possible (reviewed in Zhang and Haydon, 2005). A classic neuroglia crosstalk involves astrocytes responding to the release of neurotransmitters such as glutamate from the synapse with an increase in  $[Ca^{2+}]_i$ which subsequently results in the release of neuroactive substances, termed gliotransmitters, onto the neurons to modulate neuronal excitability and synaptic transmission (Dani et al., 1992; Pasti et al., 1997). Moreover, it was found that the elevation in  $[Ca^{2+}]_i$  was not limited to astrocytes at the synaptic sites after repeated neurotransmitter stimulation, but was propagated to neighboring astrocytes (intercellular  $Ca^{2+}$  wave). This resulted in glutamate-mediated  $[Ca^{2+}]_i$  elevations and

subsequent neuronal activity in neurons of surrounding regions, likely via a Ca<sup>2+</sup>-dependent release of glutamate from astrocytes following the astrocytic [Ca<sup>2+</sup>]<sub>i</sub> waves (Araque et al., 1998; Dani et al., 1992; Parpura et al., 1994; Pasti et al., 1997; Stout et al., 2002). Some of the major gliotransmitters include glutamate, ATP, and D-serine (Koizumi et al., 2005; Martineau et al., 2006; Zhang and Haydon, 2005). In addition to chemical signaling, electrical signaling is evident between neurons and astrocytes, as well as between astrocytes themselves. Importantly, gap junctions have been suggested to be closely involved in these processes by allowing a direct passage of ions between the cell types (Alvarez-Maubecin et al., 2000; Froes et al., 1999; Meme et al., 2009).

Other functional roles of astrocytes are mediated via their secretion of neurotrophins and uptake of neuronal waste products. Astrocytes secrete a number of neurotrophic factors and cytokines, including nerve growth factor (NGF) (Furukawa et al., 1986; Tarris et al., 1986), glial-cell-line-derived neurotrophic factor (GDNF) (Bohn, 2004), and ciliary neurotrophic factor (CNTF) (Sendtner et al., 1994), which serve as neuroprotective/regenerative agents to the neurons under CNS disturbances (Muller et al., 1995). To prevent neurotoxic levels of metabolic byproducts and neurotransmitters such as glutamate from accumulating at the synaptic cleft, astrocytes also act as caretaking cells by removing these agents via mechanisms such as glutamate and  $K^+$  uptake, and dissipating them through the astrocytic network (syncytium) formed by gap junctions between the cells (Swanson et al., 2004). Together, astrocytes serve an important role in maintaining and facilitating proper functioning of the CNS.

# 1.1.2.2 Gliomas

Astrocytes differ from neurons, in that they can be readily induced to divide, and this may contribute to the prevalence of gliomas, which account for more than 65% of all primary brain tumors (Muller et al., 1977). Gliomas are divided into three categories by the cell type from which

they originate: astrocytoma, oligodendroglioma and ependymoma. Amongst the three types, astrocytoma is the most predominant form, and as such it will be the main focus of this thesis on CNS neoplasm.

Astrocytoma is classified by four malignancy grades which are recognized by the World Health Organization, with grade I being the least aggressive and grade IV being the most aggressive. These malignancy grades correspond directly to the prognosis of glioma patients (Doolittle, 2004), and tumor grades are assigned based on four main histological characteristics: degrees of hypercellularity and pleomorphism, number of mitotic figures, endothelial cell proliferation, and necrosis (Gonzales, 2001). Grade I-II tumors exhibit varying degrees of nuclear atypia; grade III tumors show both nuclear atypia and mitotic activity; and grade IV tumors demonstrate additional characteristics such as endothelial cell proliferation and/or necrosis (Gonzales, 2001). Using this system, astrocytomas are divided into four main categories, each reflecting its malignancy grade: pilocytic astrocytoma (grade I), diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III), and glioblastoma multiforme (GBM) (grade IV) (Table 1.1) (Dowling-Warriner and Trosko, 2000).

However, gliomas often have "grey areas" between the different grades, which were originally based only on morphological criteria. This may result in inaccurate grading and is detrimental to the prediction of gliomas' biological behavior and thus subsequent treatment strategies. To overcome this limitation, researchers have focused on increasing the accuracy of the classification system through cytogenetics by searching for distinct genomic alterations associated with each grade (Collins, 2007). To date, studies have identified numerous genetic abnormalities in gliomas, including the p53 tumor suppressor gene, epidermal growth factor receptor (EGFR), phosphatase and tensin homologues (PTEN), platelet-derived growth factor  $\alpha$  (PDGF- $\alpha$ ) and its receptor, PDGF receptor  $\alpha$  (PDGFR- $\alpha$ ) (Figure 1.6) (Hermanson et al., 1996; Ino et al., 1999a; Sansal and Sellers, 2004; Varley, 2003; Watanabe et al., 1997). Aberrations in

these genes often result in four main pathway dysfunctions: 1) alteration of p53-dependent functions (*i.e.* apoptosis, cell cycle progression, DNA repair); 2) constitutive activation of growth factor receptor signaling (*i.e.* PDGFR- $\alpha$ ; EGFR); 3) alteration of retinoblastoma 1 (RB1)-dependent cell-cycle regulation; 4) constitutive activation of PI3K/Akt signaling (reviewed in Reifenberger and Collins, 2004). Dysfunction of p53-dependent cell-cycle control and constitutive activation of growth factor receptor signaling are commonly observed amongst all glioma grades. Dysfunction of RB1-dependent cell-cycle regulation at G1/S phase transition occurs in grade III gliomas, whereas additional constitutive activation of PI3K/Akt signaling arises in grade IV gliomas (Figure 1.6).

WHO Grade	Tumor Type
Ι	Pilocytic astrocytoma
Ι	Subependymal giant cell astrocytoma
II	Diffuse astrocytoma
II	Pilomyxoid astrocytoma
II	Pleomorphic xanthoastrocytoma
III	Anaplastic astrocytoma
IV	Glioblastoma
IV	Giant cell glioblastoma
IV	Gliosarcoma

Table 1.1. Grading of astrocytic tumors according to World Health Organization.(Modified by permission from Springer Science + Business Media: Acta Neuropathologica,Louis et al. 2007. The 2007 WHO Classification of Tumours of the Central Nervous System.114(2): 97-109.).

Using gene expression profiling and immunohistochemistry on human tumor tissues, Phillips and colleagues further identified three molecular subclasses of high-grade astrocytomas (grade III and IV), each of which resembles different stages of neurogenesis (Phillips et al., 2006). In contrast to neurogenesis, high-grade astrocytomas appeared to gain malignancy by retrogressing from the proneural to the proliferative and mesenchymal (angiogenic) subtypes (Figure 1.7) (Phillips et al., 2006). The proneural subtype exhibited better prognosis with longer survival, whereas the proliferative and mesenchymal subclasses displayed poorer prognosis with equally shorter survival. A shift from proneural subtype towards the mesenchymal signature was also frequently observed in recurrent GBM (Phillips et al., 2006), corroborating with previous reports which suggested that neural stem and/or progenitor cells are potential origins of tumorigenic neural stem-like cells of GBM (Galli et al., 2004; Ignatova et al., 2002; Lenkiewicz et al., 2009). Elevated Akt pathway activation may result from PTEN deletion and EGFR amplification, and it has been reported to promote GBM formation by enhancing several cellular processes, including proliferation, angiogenesis, and migration (Knobbe et al., 2002; Pore et al., 2003; Su et al., 2003; Abe et al., 2003; Sonoda et al., 2001). Notably, a loss of PTEN locus as well as gains/amplification of EGFR locus was commonly detected in the proliferative and mesenchymal subtypes, hence suggesting that Akt signaling activation, at least in part, promotes aggressive phenotype in the two subtypes (Phillips et al., 2006). On the other hand, Delta-like 3 (DLL3), a ligand to the Notch receptors, was found to positively correlate with the longer survival of the proneural subtype in the presence of intact PTEN (Phillips et al., 2006). Since DLL3, in contrast to other Notch ligands, was shown to inhibit Notch signaling and consequently promote neurogenesis in Xenopus laevis (Ladi et al., 2005; D'Souza et al., 2008), Philips et al. hypothesized that DLL3 expression may repress the development of neoplasm in the proneural subtype by inducing a more differentiated phenotype (*i.e.* proneural subtype) (Phillips et al., 2006). Overall, it was suggested that DLL3 and PTEN expression can serve as markers to predict



Figure 1.6. Schematic representation of typical chromosomal and genetic aberrations, and resultant dysfunctional pathways associated with glioma grades. CCND, cyclin; CDK, Cyclin-dependent kinase; CDKN2A, cyclin-dependent kinase inhibitor 2A; DCC, deleted in colorectal cancer; EGFR, epidermal growth factor receptor; LOH, loss of heterozygosity; MDM, murine double minute; PDGF- $\alpha$ , platelet-derived growth factor  $\alpha$ ; PDGFR- $\alpha$ , platelet-derived growth factor receptor  $\alpha$ ; PTEN, phosphatase and tensin homologues; RB, retinoblastoma; TP53, tumor protein 53. (Modified by permission from Springer Science + Business Media: Journal of Molecular Medicine, Reifenberger. 2004. Pathology and molecular genetics of astrocytic gliomas. 82(10): 656-70.).

**Figure 1.7. Molecular subclasses of high-grade astrocytomas.** A) A comparison of major features between the high-grade astrocytoma subclasses. B) Hypothesized model of glioma progression in parallel to the stages during forebrain neurogenesis. During forebrain neurogenesis, undifferentiated neural stems cells progressively differentiate into neurons but also give rise to other cells types, including astrocytes and endothelial cells. In contrast to this process, high-grade glioma cells frequently exhibit a pattern where they gain malignancy by retrogressing from the proneural to the proliferative and mesenchymal (angiogenic) subtypes. BCAN, Brevican; DLL3, Delta-like 3; CHI3L1/YKL40, chitinase 3-like 1; Olig2, oligodendrocyte transcription factor; PCNA, proliferating cell nuclear antigen; TOP2A, topoisomerase II alpha; VEGF, vascular endothelial growth factor. (Used by permission from Elsevier: Phillips et al. Cancer Cell. 2006. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. 9(3): 157-73.).

Α	Proneural	Proliferative	Mesenchymal	
Histological grade	WHO grade III or WHO grade IV with or without necrosis	WHO grade IV with necrosis	WHO grade IV with necrosis	
Cellular morphology	Astrocytic or Astrocytic Oligodendroglial		Astrocytic	
Evolution of signature	Arises in 1° tumor, may persist or convert to Mes to Mes		Arises in 1° tumor or by conversion from other subtype	
Patient age	Younger (~40 yrs.)	Older (~50 yrs.)	Older (~50 yrs.)	
Prognosis	Longer survival	Short survival	Short survival	
Histological Markers	Olig2, DLL3, BCAN	PCNA, TOP2A CHI3L1/YKL40, CD4 VEGF		
Tissue similarities	Adult and Fetal Brain	HSC, lymphoblast	Bone, cartilage, smooth musc, endothelium, dendritic cells	
<b>Biological process</b>	al process Neurogenesis		Angiogenesis	
Analogous forebrain cell	Neuroblast	Neural Stem Cell and/or Transit Amplifying Cell	Neural Stem Cell	
Chromosome gain/loss	None	Gain of 7 & Loss of 10 or 10q	Gain of 7 & Loss of 10	
PTEN locus	PTEN intact	PTEN loss	PTEN loss	
EGFR locus	EGFR normal	EGFR amplified or normal	EGFR amplified or normal	
Signaling	Notch activation	Akt activation	Akt activation	



# **Glioma Progression**

survival of high-grade astrocytomas (Phillips et al., 2006).

Therapeutic strategies for treating gliomas include surgical resection, radiotherapy and chemotherapy. However, since gliomas are closely surrounded by normal brain tissues, specific targeting and complete eradication of the tumor cells is difficult and poses a high risk of side effects to patients (Butowski et al., 2006). In addition, invasive gliomas frequently recur after treatments due to extensive infiltration into surrounding tissues, resulting in death in nearly all cases (Reardon et al., 2006). Therefore, a further understanding of glioma biology is needed to yield accurate prognostic markers and novel therapeutic targets.

# 1.1.2.3 Connexins and Gliomas

In the past years, studies have focused on finding a correlation between Cx expression and glioma grade in tissues. A decrease in Cx43, the predominant Cx protein in astrocytes, was first observed in C6 glioma cells when compared to its normal counterpart, astrocytes (Naus et al., 1991). This observation was followed by studies showing a retardation of *in vitro* oncogenicity parameters and *in vivo* tumorigenesis in C6 and human glioblastoma cell lines following transfection of Cx43 and restored gap junctional coupling (Huang et al., 1998; Naus et al., 1992a; Zhu et al., 1991; Zhu et al., 1992). An inverse correlation between Cx43 expression and glioma tumorigenicity was hence suggested (Naus et al., 1999), and other studies since then have also made similar observations in human glioma cell lines (Table 1.2).

Gliomas were shown to exhibit increased glucose uptake to support their heightened metabolic activity (Di Chiro et al., 1982). This uptake in astrocytes was elevated when gap junctions were inhibited by blockers such as carbenoxolone, suggesting that gap junctions dissipate elevated [glucose]<sub>i</sub> across the astrocytic syncytium (Tabernero et al., 1996). Tolbutamide is a potassium channel blocker known to open gap junctions via induced plasma membrane depolarization (Granda et al., 1998), and dibutyrl-cAMP (DBcAMP) is a permeable cAMP analog also known to activate gap junctions by preventing Cx hypophosphorylation (Sato et al., 2003). Concurrently, treatment of C6 gliomas with tolbutamide or DBcAMP was shown to increase Cx43 expression and gap junctional coupling, respectively, thereby reducing the glucose uptake in C6 gliomas to suppress cell proliferation (Sanchez-Alvarez et al., 2005).

In later studies, an emphasis was put on examining the correlation between Cx43 expression and glioma tumor grades. Using immunohistochemical analysis of paraffin-embedded tissue sections of grade I-IV human gliomas, Huang *et al.* first demonstrated an inverse correlation between Cx43 expression and human glioma grades (Huang et al., 1999). In accordance, Soroceanu *et al.* made similar observations using Western blot and immunohistochemical analyses on acutely isolated cells from biopsies of various glioma grades, and further demonstrated a decrease in gap junctional coupling via dye passage assay in primary cultures derived from GBM (Soroceanu *et al.*, 2001). Other studies also reported a similar correlation that Cx43 expression not only decreases with increasing glioma grade, but also with increasing proliferation activity of the tumors (Aronica et al., 2001; Pu et al., 2004). Overall, the findings support that a loss of Cx43 expression is involved in glioma oncogenicity.

Cell Line or Tissue	Connexin	Effect of Cx	Reference
	Expression	Transfection on	
		Proliferation	
Rat C6 glioma cells	Cx43 reduced	Reduced by Cx43 in	(Naus et al., 1992a;
		vitro and in vivo	Zhu et al., 1991)
Rat C6 glioma cells	Cx43 reduced	Reduced by Cx32 in	(Bond et al., 1994)
		vivo	
Rat C6 and 9L glioma	Cx43 reduced	Reduced by Cx30 in	(Princen et al., 1999)
cells	Cx30 absent	vitro	
Astrocytomas	Cx43 reduced with		(Huang et al., 1999)
	increasing grade		
Astrocytomas	Cx43 mRNA and		(Shinoura et al., 1996)
	protein variable		
	with grade,		
	especially in high		
	grade		
Astrocytomas	Cx43 reduced with		(Pu et al., 2004)
2	increasing grade		
Glioblastomas and	Cx43 reduced with		(Soroceanu et al.,
astrocytomas	increasing grade		2001)
Human glioma cell lines:	Cx43 variable		(Shinoura et al., 1996)
U87, U118, U137, U373,			
T98G, Hs68331, SNB-			
10			
Human glioma cell lines:	Cx43 variable		(Soroceanu et al.,
U138, CH235, D56,			2001)
U373, U251			,
Human glioma cell lines:	Cx43 reduced	Reduced by Cx43 in	(Huang et al., 1998)
U251, U87, U373, T98G		vitro and in vivo	
Human glioma cell lines:	Cx43 reduced		(Pu et al., 2004)
TJ899, TJ905, TJ8510			
Human glioma cell lines:	Cx43 increased in		(Oliveira et al., 2005)
GL15, 8MG	invasive gliomas		
Human glioma biopsy			
xenographs maintained			
in nude mice			
Primary brain tumors	Cx26 and Cx43		(Estin et al., 1999)
5	expressed		

**Table 1.2. Connexin expression in gliomas.** (Modified by permission from Springer Science +Business Media: Astrocytes in (patho)physiology of the nervous system, Lai and Naus. 2009.Connexins and pannexins: Two gap junction families mediating glioma growth control. 547-68.).

# **1.1.2.4 Connexins and Growth Factors**

With the evident inverse correlation between Cx43 expression and glioma grades, investigations have concentrated on the therapeutic implications of Cx43 in gliomas by altering its expression via transfection or chemical treatment (Bradshaw et al., 1993b; Bradshaw et al., 1993a; Goldberg et al., 2000; Huang et al., 1998; Naus et al., 2000). From these studies, it has been proposed that Cx43-elicited tumor suppression is mediated via a change in growth factor expression: increase growth-suppressing and decrease tumor-promoting growth factors (Table 1.3).

Cell Lines	Proliferation	Growth	Up/Down-	Reference
		Factor	regulated	
Rat C6 glioma	Reduced	IGFBP-3	Down	(Bradshaw et al.,
cells				1993b)
		IGFBB-4	Up	(Bradshaw et al.,
		IGF-I	Down	1993a)
		CCN1/CYR61	Up	(Naus et al., 2000)
		CCN3/NOV	Up	
		RPL19	Down	
		MFG-E8	Down	(Goldberg et al.,
				2000)
U251 human	Reduced	MCP-1	Down	(Huang et al., 2002)
glioblastoma				

**Table 1.3. Effects of Cx43 overexpression on growth factors, and glioma proliferation.** CCN stands for the assemblage of CTGF, CYR61, and NOV; CTGF, connective tissue growth factor; Cyr, cysteine rich; IGB, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; MCP-1, monocyte chemotactic protein 1; MGF-E8, milk fat globule-EGF factor 8 protein; NOV, nephroblastoma overexpressed; RPL19, ribosomal protein L19. (Modified by permission from Springer Science + Business Media: Astrocytes in (patho)physiology of the nervous system, Lai and Naus. 2009. Connexins and pannexins: Two gap junction families mediating glioma growth control. 547-68.).

Reciprocally, growth factors have been shown to modulate Cx43 expression, affecting cell growth (Aberg et al., 2003; Chatterjee et al.; Kennedy et al., 2003; Ozog et al., 2002; Tacheau et al., 2008; Ueki et al., 2001). One classic example was shown by a treatment of ciliary neurotrophic factor (CNTF) in combination with its receptor, CNTF receptor  $\alpha$ , which enhances Cx43 expression via the Janus tyrosine kinase/signaling transducer and activator of transcription (JAK/STAT) pathway, and was associated with reduced proliferation in C6 glioma cells (Ozog et al., 2002; Ozog et al., 2004).

# 1.1.2.5 Connexins, Migration and Invasion

Besides intercellular channel and hemichannel functions, gap junctions also elicit their functions via interactions with other molecules, including cell-cell junctional proteins. Indeed, the dynamic interaction between Cxs and zonula adherens junction proteins has been suggested to modulate glioma migration and proliferation (Lai and Naus, 2007; Olk et al., 2009).

Zona occludens-1 (ZO-1), a peripheral tight junction protein belonging to the MAGUK (membrane-associated guanylate kinase) family, was initially found to interact with Cx43's C-terminal tail via its second PSD95/disc large/ZO-1 (PDZ) homology domain, and later investigations also demonstrated a similar interaction at the C-terminus of other Cxs, including Cx36, Cx45, and Cx46 (Giepmans and Moolenaar, 1998; Kausalya et al., 2001; Laing et al., 2001; Li et al., 2004; Toyofuku et al., 1998). It appeared that ZO-1 facilitates gap junction turnover since Cx43 with a GFP-tagged C-terminus, which is incapable of interacting with ZO-1, formed larger gap junction plaques than endogenous Cx43 as a result of reduced turnover rate (Hunter et al., 2003; Hunter et al., 2005; Lauf et al., 2002). As the size of gap junctional plagues has been suggested as an important variable in regulating gap junction-mediated intercellular communication, ZO-1 may therefore modulate GJIC via its direct interaction with Cxs (Evans

and Martin, 2002). Furthermore, since ZO-1 links actin to tight and adherens junctions through its interaction with occludin and  $\alpha$ -catenin, respectively, its interaction with Cxs suggests ZO-1 may also link actin to gap junctions as a novel cytoskeletal anchorage site at the plasma membrane, hence affecting cell motility (Elias et al., 2007; Giepmans, 2004; Xu et al., 2006).

Instability and disorganization, but not reduced expression, of adherens junctional proteins were found associated with the invasive properties of T98G and U373 MG glioblastoma cell lines (Perego et al., 2002). Notably, Cxs were observed to co-localize with adherens junctional proteins, including E-, N-cadherins, and B-catenin (Jongen et al., 1991; Meyer et al., 1992; Fujimoto et al., 1997; Hertig et al., 1996; Ai et al., 2000). Abolishing adherens junctions with anti-cadherin antibody or  $[Ca^{2+}]_0$ -depletion prevented formation of gap junctions, showing that adherens junctions are a prerequisite of gap junction formation (Meyer et al., 1992; Hernandez-Blazquez et al., 2001; Matsuda et al., 2006; Fujimoto et al., 1997; Kostin et al., 1999). In accordance, Nambara et al. further demonstrated that the cytoplasmic loop of Cx43 is required for E-cadherin-regulated Cx43 trafficking using Cx43-CL deletion mutants (Nambara et al., 2007). On the other hand, pre-existing gap junctions may also recruit additional cadherins to the plasma membrane, regulating subsequent adherens junction formation, as well as cytoskeletal organization, since microtubules are anchored to the cadherins. While β-catenin is well known for its function in adherens junction by linking actin cytoskeleton to adherens junction via  $\alpha$ catenin, it also acts as a transcriptional co-factor downstream of the Wnt signaling pathway to regulate gene expression. As  $\beta$ -catenin binds to cadherins to form complexes, adherens junctions have been suggested to regulate Wnt-mediated gene transcription by sequestering cytoplasmic βcatenin and preventing it from translocating into the nucleus (reviewed in Barami et al., 2006). A similar interaction between Cxs and  $\beta$ -catenin has been shown by co-immunoprecipitation (Ai et al., 2000), and implies that Cxs, analogous to cadherins, may also regulate gene expression.

Altogether, the findings suggest that Cxs may influence cell motility by affecting the stabilization of adherens junctions at the plasma membrane.

Several studies have investigated the effect of Cx43 expression on cellular motility in gliomas. While the findings have been controversial in other systems (Olk et al., 2009), Cx43 appears to promote the migratory and invasive properties of gliomas. A study demonstrated that Cx43 overexpression induced migratory behavior of non-migratory rat C6 glioma cells following transplantation into the rat brain (Lin et al., 2002; Zhang and Haydon, 2005). In addition, another study using C6 subclones with low and high endogenous Cx43 expressions showed a direct correlation with migration where high Cx43 expression yielded increased motility (Bates et al., 2007). Bates et al. further demonstrated the enhanced motility requires the presence of the Cx43 C-terminus (Bates et al., 2007), suggesting that an interaction of the C-terminus with proteins such as ZO-1 is involved in regulating cell migratory properties. On the other hand, studies have also reported reduced Cx43 expression in high-grade, invasive human gliomas (Huang et al., 1999; Soroceanu et al., 2001). Interestingly, Oliveira et al. showed that homocellular interaction between gliomas supported cellular adhesion, whereas heterocellular interaction between glioblastoma and astrocytes enhanced glioma migration (Oliveira et al., 2005). Since normal astrocytes, but not glioma cells, in biopsied glioma tumors typically have high Cx43 expression which directly correlates with the invasiveness of gliomas, glioma cells were proposed to employ the Cx43-mediated heterocellular interaction between glioblastoma and astrocytes to enhance migration (Oliveira et al., 2005).

Furthermore, an inverse relationship between migration and proliferation was found in gliomas where reduced migration by a non-permissive substrate (vitronectin) increased proliferation, and vice-versa (Giese et al., 1996). Given that reduced Cx43 expression is generally associated with increased proliferation in high-grade gliomas (see *Connexins and Glioma*) and the fact that tumor cells are heterogeneous, it is possible that 1) majority of the

glioma population actively proliferates with reduced Cx expression, and 2) a subpopulation specializes in migration/invasion with higher Cx expression. These may be the alternating mechanisms by which Cxs influence tumor cell behavior, while multifold factors including genetic aberrations and growth factors stimuli, are certainly also involved. Overall, Cx43 may play an important role in the invasiveness of gliomas, possibly via its interaction with zonula adherens junctional proteins, as well as other Cx proteins in adjacent cells such as astrocytes.

# 1.1.2.6 Pannexins and Glioma

A role of Panxs in cancers has not been investigated prior to studies described in this thesis. Transcripts of Panx1, and especially Panx2, are abundantly expressed in the brain (see *Pannexins*). In my pilot study, endogenous transcriptional expression of Panx1, Panx2, and Panx3 was detected in rat primary astrocytes but not in the tumorigenic counterpart, C6 glioma cells, via reverse transcription (RT)-polymerase chain reaction (PCR) analysis. This finding indicates that a loss of Panx expression may participate in the development of C6 gliomas, and *Panxs* act as tumor-suppressor genes once expression is restored. Of particular interest is the presence of Panx1 transcripts in a panel of human glioma cell lines, suggesting that aberration in Panx1 transcription specifically applies to a subset of gliomas. However, a loss of Panx1 expression could also occur at the translational level, contributing to glioma transformation. Further study on Panx1 protein expression is required to test this supposition.

Panx2 transcript, unlike Panx1, are particularly abundant in the rodent brain and presumably brain-specific in humans (Baranova et al., 2004; Ray et al., 2005; Vogt et al., 2005). In parallel to the loss of Panx1 transcript in C6 cells, high-throughput cDNA microarray analysis of human brain tumor samples has shown an overall reduction of *Panx2* gene expression in gliomas (Litvin et al., 2006). Furthermore, a correlation between Panx2 up-regulation and post

diagnosis survival in patients with glial tumors was found using the brain cancer gene expression database REMBRANDT (Repository of Molecular Brain Neoplasia Data, <u>http://rembrandt.nci.nih.gov/rembrandt</u>) (Litvin et al., 2006). In addition, the *Panx2* gene is located within chromosomal region 22q13.3. where deletion was often found in human astrocytomas and ependymomas (Hu et al., 2004; Ino et al., 1999b; Oskam et al., 2000; Rey et al., 1993). Given that Panx2 alone was not able to form either hemichannel or intercellular channel in the *Xenopus* oocyte system (Bruzzone et al., 2003), Panx2 may elicit its function via interaction with Panx1 and/or other molecules.

Overall, recent findings advocate an inverse correlation between Panx expression and glioma tumorigenicity, and warrant upcoming investigation on the effect of Panxs in gliomas, as well as on identifying their underlying mechanism(s) in tumor suppression.

## **1.1.3 Gap Junctions and CNS Development**

Gap junctions are widely expressed in neocortex, and play an important role in brain development by providing a direct means of communication between cells via the passage of vital ions and signaling molecules. Indeed, Cx knockdown/knockout studies have demonstrated a role of Cxs in regulating proliferation and migration of neural progenitor cells (Cina et al., 2009; Elias et al., 2007; Fushiki et al., 2003). These functions of Cxs have been suggested to act via gap junctions, hemichannels, as well as serving as adhesive molecules (reviewed in Elias and Kriegstein, 2008). Importantly, Cxs have been implicated in neuronal differentiation via gap junctional and hemichannel activities (Bani-Yaghoub et al., 1997; Bani-Yaghoub et al., 1999a; Bani-Yaghoub et al., 1999b; Belliveau et al., 1997; Belliveau et al., 2006; Unsworth et al., 2007). Altogether, gap junctions are proposed as multifaceted regulators during embryonic cortical development.

### 1.1.3.1 Connexins and neuronal differentiation

Throughout neocortical development, Cx expression is temporally regulated and coincides with different stages during the neuronal differentiation period (Cina et al., 2007). In addition, Cx types are differentially expressed during this time. Cx43 was consistently observed throughout embryonic and postnatal development, with an increase in expression during early postnatal stages (Dermietzel et al., 1989; Nadarajah et al., 1997). By contrast, Cx26 and Cx32 showed an inverse expression pattern: Cx26 expression was prominent in the neuroepithelium of embryonic brain and later diminished between the third and sixth postnatal week, which was also when Cx32 expression appeared and increased (Dermietzel et al., 1989; Nadarajah et al., 1997). The types of cells expressing Cx also change during this period. During development, Cx43 and Cx26 are expressed in migrating neurons and radial glia, which have now been shown to be neuronal progenitors (Anthony et al., 2004; Dermietzel et al., 1989; Nadarajah et al., 1997). Yet, in mature brain, Cx43 is largely expressed in astrocytes, whereas Cx26 and Cx45 are abundantly expressed in neurons (Bittman and LoTurco, 1999; Maxeiner et al., 2003; Nadarajah et al., 1997; Nagy et al., 2001).

The pattern of diminishing Cx43 expression in neurons as development progresses was corroborated by *in vitro* studies. Using conditionally immortalized mouse hippocampal multipotent progenitor cells (MK31) that express abundant Cx43, Rozental *et al.* showed a significant decrease of Cx43 while Cx33 and Cx40 increased upon induced neuronal differentiation (Rozental et al., 1998). Another report ensued and further demonstrated that the decrease in Cx43 occurred during neuronal, but not glial, differentiation since a similar observation was only made when rat multipotent RT4 peripheral neurotumor was induced to differentiate into neurons and not glia (Donahue et al., 1998). In agreement with these findings, investigations with human pluoripotent teratocarcinoma NTera-2/clone D1 (NT2/D1) and mouse P19 embryonal carcinoma cells also showed reduced Cx43 expression and gap junctional

coupling following retinoic acid-induced neuronal differentiation (Bani-Yaghoub et al., 1997; Belliveau et al., 1997). Remarkably, pharmacological inhibition of gap junctional coupling using carbenoxolone on differentiating NT2/D1 and P19 cells successfully reduced the number of resultant mature neurons (MAP2 positive, microtubule-associated protein 2) and increased the proportion of immature neurons (vimentin-, nestin-, and cytokeratin-positive) (Bani-Yaghoub et al., 1999a; Bani-Yaghoub et al., 1999b).

In addition to gap junction intercellular channels, Cx hemichannels have recently been demonstrated to modulate neurite outgrowth (Belliveau et al., 2006). In the study, exogenous Cx43 and Cx32 expression in rat pheochromocytoma PC12 cells not only induced limited autodifferentiation but also significantly increased neurite outgrowth upon NGF-induced differentiation (Belliveau et al., 2006). It was then identified that Cx43 and Cx32 released ATP via hemichannels upon NGF stimulation, and subsequently activated purinergic receptors to induce the enhanced neurite outgrowth (Belliveau et al., 2006). Interestingly, a recent study found a similar neurite outgrowth enhancing effect for Cx31 in SH-SY5Y human epithelial-derived neuroblastoma cells (Unsworth et al., 2007). Yet, no apparent Cx31 signal at the plasma membrane was observed in this study, suggesting that the phenotype is mediated via intracellular signaling pathways (Unsworth et al., 2007). Altogether, these findings advocate a role of Cxs in neuronal differentiation processes via functional intercellular, hemichannels, and/or intracellular signaling pathways.

# 1.1.3.2 Pannexins and neuronal differentiation

Increasingly, results from functional studies have pointed to hemichannels as Panxs' preferred functional configuration (Bao et al., 2004a; Locovei et al., 2006a; Locovei et al., 2006b; Pelegrin and Surprenant, 2006; Penuela et al., 2007; Penuela et al., 2009; Thompson et al., 2008).

Specifically, Panx1 hemichannels were found to mediate ATP release and possess channel properties that are physiologically more suitable than Cx as a hemichannel, including activation by mechanical stress, elevation in  $[Ca^{2+}]_i$ , purinergic receptor stimulation, and insensitivity to physiological  $[Ca^{2+}]_o$  (reviewed in Scemes et al., 2007). Furthermore, pharmacological inhibitors such as carbenoxolone, as well as inhibiting mimetic peptides of Cx channels, which are commonly used in studies prior to the debut of the Panx family, have now been shown to also abolish Panx channel activities (Bruzzone et al., 2005; Wang et al., 2007). Therefore, previous functional studies on Cx hemichannels have now been considered fraught with possible misinterpretations with the use of non-specific channel blockers and the assumption of Cx hemichannels as the sole contributor of the observed activity (Bruzzone et al., 2005; D'hondt et al., 2009; Dahl and Locovei, 2006; Scemes et al., 2007; Shestopalov and Panchin, 2008). Nonetheless, findings from Cx hemichannels still provide valuable insight into the overall functional aspect of hemichannels, especially Panx hemichannels.

Involvement of Panxs during CNS development has remained largely unexplored to date. However, emerging evidence since Panxs' discovery has implied a potential role of Panxs in neuronal differentiation. Recent studies identified Panx1 mRNA to be distributed in different cell types of the brain, including neurons. Notably, its expression is significantly reduced from embryonic to adult stages (Ray et al., 2005; Vogt et al., 2005). Panx2 transcript, on the other hand, was present at a low level in prenatal brain but increased dramatically in postnatal (P) developmental stages starting from P7 (Vogt et al., 2005). Thus, Panx1 and Panx2 expression appear to be inversely regulated during rat brain development (Ray et al., 2005; Vogt et al., 2005). Additionally, Panx3 was also detected in the brain but to a lesser extent (Penuela et al., 2007). The inverse expression between Panx1 and Panx2 resembles that of Cx43 and Cx32 during neocortical development (Dermietzel et al., 1989; Nedergaard et al., 2003). A recent study by Belliveau *et al.* demonstrated that exogenous Cx43 and Cx32 induced limited autodifferentiation, and the Cx hemichannels allowed the release of ATP, which subsequently activated purinergic receptors and thereby enhanced neurite outgrowth in PC12 cells (Belliveau et al., 2006). While Panx1 has been reported to form ATP permeable hemichannels, less is known about Panx2 channel functions. Panx3 has been shown to form hemichannels to facilitate dye uptake into cells (Penuela et al., 2007; Penuela et al., 2009), yet a systemic function still remains to be identified. With the Panxs' unique expression pattern during brain development and their complementary channel properties, Panx presents itself as a strong candidate to play an important role during CNS development.

# **1.2 OBJECTIVES, RATIONALE AND HYPOTHESES**

Reduced gap junctional intercellular communication has been associated with oncogenicity in many types of cancers including gliomas, as well as in developmental diseases (Naus, 2002; Naus et al., 2005). The inverse correlation between functional gap junctions and disease is based on the physiological role of these channels to provide a direct means of intercellular communication, which facilitates tissue homeostasis by allowing exchange of metabolites and signaling molecules. Restoring gap junctional coupling allows diseased cells to re-establish cell-cell communication, thereby retarding pathological phenotypes. The general purpose of this thesis was to examine the role of a novel gap junction family, Panxs, in pathological and physiological conditions of the CNS, each of which was represented by gliomas and neuronal differentiation process, respectively. Based on previous findings by others (see *Historical Review*), it was generally hypothesized that *1*) functional gap junctions and/or hemichannels play a role in regulating normal cell growth and CNS development, and 2) restoring intercellular communication in gap junction-deficient cells regresses pathological phenotypes.

The purpose of the first and second studies in this thesis was to examine whether Panx expression is up- or down-regulated in gliomas, as well as the effect of Panx expression on glioma oncogenicity. Gliomas have generally been shown to exhibit reduced Cx expression as tumor grade progresses, and decreased proliferation is evident when gap junctional coupling is re-established (see *Gap Junctions and Cancer*). A role of Panxs in tumorigenicity was not investigated prior to these studies, but the tissue-specific expression of Panxs suggests a functional role in the CNS and conversely, aberrant expression under pathological conditions. I therefore set up to test the hypothesis that, *similar to Cxs, Panxs expression modulates the growth and tumorigenesis of gliomas by forming functional channels*.

The methodology used in the first two studies employed in vitro experiments with the rat C6 glioma cell line, primary cultured rat cortical astrocytes, human glioma cell lines, and in vivo tumorigenicity assay in immunodeficient mice. The use of cultured neoplastic cells allows relatively quick establishment of a study model that usually mimics prominent biochemical and biophysical properties of the neoplasm. This is especially the case with gliomas where biopsies from patients are difficult to obtain due to their unique location. The rat C6 glioma cell line is believed to be of astrocytic origin with low Cx expression, limited GJIC, and exhibits similar cellular and molecular characteristics to human gliomas (Naus et al., 1991; Naus et al., 1992b; Zhu et al., 1991). Therefore, the effect of an increase in gap junctional activities should be apparent in C6 cells. Together with its well-characterized properties, C6 cells served as a good glioma model in these studies. To minimize cell culture specificity, an array of human glioma cell lines were also used, including U118, A172, U138, LN229, U251 and T98G gliomas. Primary cultures yield the advantage in isolating specific cell types from different anatomical locations and time during normal and pathological development, and often represent cellular properties of the cells *in vivo*. In addition, primary cultures are incubated in a controlled environment which can be utilized for constructing various experimental conditions. In vivo models using immunodeficient mice provide a valuable means to study the response of treated cells under physiological conditions in the context of cell-cell interactions within the tissue.

The purpose of the third project in this thesis was to explore the role of Panxs in neuronal differentiation. Cx expression is spatiotemporally regulated and has been associated to the proper formation of neural network in the cortex during CNS development (Cina et al., 2009; Elias et al., 2007; Fushiki et al., 2003). Similarly, Panxs expression fluctuates during brain development (Vogt et al., 2005). Panx hemichannels have also been demonstrated to release ATP, which was reported responsible for Cx-induced limited autodifferentiation and enhanced neurite outgrowth (Belliveau et al., 2006), hence suggesting a role of Panxs in neuronal differentiation. I therefore tested the hypothesis that *Panx expression induces neuronal differentiation and enhances neurite outgrowth via functional hemichannels*.

Neural crest-derived pheochromocytoma cells (PC12) were employed in the third study as they showed no detectable endogenous Cx (Belliveau et al., 2006) and Panx protein expression in my pilot study. Therefore, an increase in Panx channel activity would be readily identifiable in PC12 cells. Moreover, PC12 cells can be induced by NGF to differentiate into a neuronal phenotype, making them an ideal model system to study the effect of Panx expression during the neuronal differentiation process.

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# **Chapter 2**

# Tumor-Suppressive Effects of Pannexin1 in C6 Glioma Cells

A version of this chapter has been published. Lai, C.P., Bechberger, J.F., Thompson, R.J., MacVicar, B.A., Bruzzone, R., Naus, C.C. (2007) Tumor-Suppressive Effects of Panx1 in C6 Glioma Cells. *Cancer Research*. 67:1545-1554.

#### **2.1 INTRODUCTION**

Since their identification, connexins (Cxs) have been considered to be the sole mammalian gap junctional constituent proteins. More recently, sequences with low similarity to the invertebrate innexins (Inxs) have been identified in chordates, leading to the emergence of a novel family of proteins, called pannexins (Panxs), which are also capable of forming functional hemichannels and intercellular channels (Bruzzone et al., 2003; Bao et al., 2004). To date, three Panx members have been identified in vertebrates: Panx1, Panx2 and Panx3 (Baranova et al., 2004), and several invertebrate innexins are also referred to as Panxs (Sasakura et al., 2003). Although protein expression of these Panxs has not been defined in mammals due to the lack of specific antibodies. Northern blot analysis and *in situ* hybridization experiments have identified location-specific Panx mRNA expression in rats: Panx1 is ubiquitously expressed, Panx2 is particularly abundant in the brain, and Panx3 is present in the skin (Bruzzone et al., 2003; Vogt et al., 2005). Previous studies on Inx mutants in Drosophila have demonstrated Inx-specific functions including synaptogenesis in the giant-fiber system, epithelial organization and morphogenesis, and germ cell differentiation processes (Bauer et al., 2005b). Although it remains to be seen whether Panxs can be regarded as vestigial Inxs that have survived in higher animals, this implies that, along with Cxs, Panxs may also play functional roles in chordates (Bauer et al., 2005b; Barbe et al., 2006).

Gap junctions are proteinous membrane channels that are located between the lateral surfaces of two adjacent mammalian cells, directly connecting their cytosols. A Cx-based gap junction is composed of two connexons, each composed of a hexamer of connexins. Each Cx has four membrane-spanning domains, two extracellular loops, a cytoplasmic loop, and cytoplasmic NH<sub>2</sub>- and COOH-termini (Simon and Goodenough, 1998). These channels allow the direct passage of small ions and molecules with a molecular weight less than 1 kDa such as  $Ca^{2+}$  ion and cyclic AMP, and function in maintaining intercellular communication and homeostasis

within and between tissues (Loewenstein, 1987; Naus et al., 2005). In accordance, gap junctions have been found to be down-regulated in many types of cancer, including gliomas, breast carcinoma, and prostate cancers (Mesnil, 2002; Naus, 2002). Moreover, using transfection of cDNAs encoding Cxs to restore gap junctional intercellular communication (GJIC) in cancer cell models reverted some of the transformed phenotype and reduced proliferation of the neoplasms (Naus et al., 2005). This suggests that Cxs function as tumor suppressors, and numerous studies have explored restoration of GJIC as a potential therapy against cancer (Trosko and Ruch, 2002).

Despite sequence dissimilarity between Cxs and Panxs, the two protein families share predicted structural resemblance (Panchin, 2005). Analogous to Cxs, formation of Panx-based hemichannels and intercellular channels are Panx-specific (Bruzzone et al., 2003). Using the *Xenopus* oocyte model system, Panx1 and Panx1/Panx2, but not Panx2 alone, were discovered to exhibit functionally different hemichannels in single oocytes (Bruzzone et al., 2003). Similarly, in paired oocytes, Panx1 and Panx1/Panx2 formed homomeric and heteromeric intercellular channels, respectively, with distinctive functional properties (Bruzzone et al., 2003). In addition, Panx-based channels demonstrated similar electrical channel properties and sensitivities to the same classes of pharmacological blockers as Cxs (*i.e.* carbenoxolone) (Bruzzone et al., 2003; Bruzzone et al., 2005). Collectively, the parallel characteristics between Cxs and Panxs raise the possibility that aberrant Panx expression may also occur in cancer cells, and restoration of Panx expression may revert the transformed phenotype.

Given that Panx1 alone is capable of forming functional channels and its mRNA is endogenously expressed in the brain, it raises a potential correlation between aberrant Panx expression and glioma tumorigenicity. In this study, we used C6 cells, a well characterized glioma cell line, as our model system. C6 cells are believed to be of astrocytic origin and have been shown to exhibit low Cx43 expression and gap junctional coupling (Barth, 1998; Naus et al., 1991). While Panx1, Panx2, and Panx3 transcripts were present in rat primary astrocytes, none were detected in C6 cells. Restoration of Panx1 expression by stable transfection with either fluorescent- or epitope-tagged Panx1 cDNAs induced a dramatic morphological change and reversed the neoplastic phenotype of C6 glioma cells. Together, these findings suggest a tumor-suppressive role of Panx1 in C6 glioma cells.

Cxs have been extensively studied in their putative role as tumor suppressors. Panxs, on the other hand, have just been recently identified and thus far their implication in cancers has not been examined. The present study is the first report on Panx1 as a negative growth regulator and provides insights into novel aspects of gap junctions in cancer research.

### 2.2 MATERIALS AND METHODS

**2.2.1 Cell Culture.** Primary culture of cortical rat astrocytes and human glioma cells lines (U87, U251, SF188, SF539) were prepared as previously described (Ozog et al., 2004; Naus et al., 1996). C6 glioma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's minimum essential medium (DMEM) (Invitrogen Corp., Burlington, ON, Canada) containing 10% fetal bovine serum, 10 units/ml penicillin, and 10  $\mu$ g/ml streptomycin at 37°C, 5% CO<sub>2</sub>. Stable transfectants were maintained in C6 medium supplemented with 3  $\mu$ g/ml puromycin (Sigma-Aldrich, St. Louis, MO). Prior to experimentation, stable transfectants were sub-cultured into puromycin-free medium.

**2.2.2 Total RNA Extraction, Reverse Transcription-PCR.** Total RNA of cultured cells was extracted using TRIzol reagent (Invitrogen Corp.) and human adult brain total RNA was acquired from Clontech Laboratories Inc. (Mountain View, CA). RT-PCR was performed as previously described (Ozog et al., 2002). The PCR profile for Panxs was 94°C for 45 s, 50°C (rat) or 58°C (human) for 1 min, and 72°C for 60 s for 35 cycles. Panx primer sequences and predicted sizes are listed in Table 2-1. GAPDH primer sequences and PCR profile were used as previously

described (Yang et al., 2001). 30 µl of PCR products and 1 Kb DNA ladder (Invitrogen Corp.) were simultaneously run on a 1.5% ethidium bromide-stained agarose gel. As a negative control, Superscript III was replaced with ultra-pure MilliQ water to discern false-positives of PCR products due to DNA-contaminated cDNA products.

Standard	Species	Predicated	Sense/Antisense
Name		Size (bp)	(Nucleotide Position (nt))
Panx1	Rat	185	5' - TTCTTCCCCTACATCCTGCT -3' (756 - 775)/
			5' – GGTCCATCTCTCAGGTCCAA -3' (940 – 921)
Panx2	Rat	258	5' – TGGACATCGTATTGCTCTGC -3' (1204 – 1223)/
			5' – CCACGTTGTCGTACATGAGG -3'(1461 – 1442)
Panx3	Rat	336	5' – GACCCCCTGAAACACTTTGA -3' (1891 –
			1910)/
			5' – TCTAACCCACTGGCCTTCAC -3' (2226 – 2207)
Panx1	Human	195	5' – GGGATCCTGAGAAACGACAG – 3' (1136 –
			1155)/
			5' – AAAAGTGGGGAGGATTTCGT – 3' (1330 –
			1311)

**Table 2.1. Panx primer sequences used for RT-PCR and their nucleotide positions.** Primer specificity was confirmed with nucleotide-nucleotide BLAST on the National Center for Biotechnology Information (NCBI) website.

**2.2.3 Plasmid Construction and Transfection.** Expression vector pRK 5 encoding *Rattus norvegicus Panx1* tagged with *c-myc* (Panx1-myc) or *enhanced green fluorescence protein* reporter gene (*EGFP*) (Panx1-EGFP) were prepared as previously described (Bruzzone et al., 2005). As a control, AP2 retroviral vector encoding *EGFP* was used (Mao et al., 2000). The day prior to transfection,  $1 \times 10^6$  cells were seeded per 35-mm dish in C6 medium and co-transfected with Murine Stem Cell Virus vector encoding a puromycin resistance gene (pMSCVpuro) (Clontech Laboratories Inc.) and the plasmid of interest (EGFP, Panx1-myc, or Panx1-EGFP)

using Lipofectamine<sup>TM</sup> 2000 (Invitrogen Corp.) as previously described (Ozog et al., 2004). Cotransfectants were selected with 3  $\mu$ g/ml puromycin, and clones were subsequently isolated using cloning rings.

2.2.4 **Protein** Isolation and Western Blot Analysis. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (complete, Mini; Roche Diagnostics Corp., Indianapolis, IN) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail 2; Sigma-Aldrich), and DNA sheared with a 22-gauge needle. Protein concentration was determined by using a BCA Protein Assay Kit (Pierce-BioLynx, Brockville, ON, Canada). Following boiling for 2 min in SDS sample buffer, 20 µg of total protein and molecular weight standard (Precision Plus Protein All Blue Standards; Bio-Rad Lab., Hercules, CA) were simultaneously subjected to 10% SDS-PAGE and the separated protein bands were transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in TBS containing 1% Tween-20 for 1 h and then incubated overnight at 4°C with anti-c-myc (mouse; 1/400 dilution; Roche Diagnostics Corp.) and anti-GFP antibodies (mouse; 1/1,000 dilution; Stressgen, Ann Arbor, MI). The membranes were immersed in secondary antibody (goat antimouse or anti-rabbit IgG-horseradish peroxidase; 1:5,000 dilution; CedarLane Lab. Ltd., Hornby, ON, Canada) for 1 h, incubated with Supersignal (Pierce-BioLynx), and exposed to Xray film for detection of antibody-bound proteins. As a loading control, the membranes were immunoblotted for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (mouse, 1:20,000 dilution; CedarLane Lab. Ltd.).

**2.2.5 Crude Membrane Fractionation.** Cells on a confluent 100-mm plate were harvested into a fractionation solution (10 mmol/L Tris-HCl, pH 7.5, 250 mmol/L sucrose, protease inhibitors (complete, Mini; Roche Diagnostics Corp.)). Samples were then sonicated for 15 s and

centrifuged at 30,000 x g for 30 min using a TLA120.1 rotor in an Optima <sup>TM</sup> TLX Ultracentrifuge (Beckman Coulter, Mississauga, ON, Canada). Supernatant (cytoplasmic fraction) was collected and pellet (membrane-enriched fraction) was resuspended in a RIPA buffer supplemented with protease inhibitors (complete, Mini; Roche Diagnostics Corp.) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail 2, Sigma-Aldrich) using 22-gauge needles. Protein concentration was determined using a BCA Protein Assay Kit (Pierce-BioLynx).

**2.2.6 Immunocytochemistry and Live-cell Imaging.** For live-cell imaging, cells grown on coverslips were washed twice in PBS containing Mg<sup>2+</sup> and Ca<sup>2+</sup> and immediately mounted onto glass slides. Samples were viewed under an epifluorescence microscope (Axioplan 2, Carl Zeiss, Toronto, ON, Canada) and images captured using Axiovision (Carl Zeiss). For immunocytochemistry, cells were grown on coverslips, fixed in 4% formaldehyde at room temperature for 10 min and permeablized with 0.3% Triton X-100. Plasma membrane and Golgi apparatus were stained with Alexa 594-conjugated wheat germ agglutinin (WGA) (1/200 dilution; Molecular Probe) using protocols outlined in the product manual. Nonspecific antibody binding was blocked using 10% bovine serum albumin (BSA) in PBS and immunolabeled with anti-c-myc (mouse; 1/100 dilution; Roche Diagnostics Corp.) antibodies for 1 h. Cells were then incubated in Alexa-Fluor-conjugated secondary antibodies (goat anti-mouse or anti-rabbit; 1:500 dilution; Molecular Probe, Eugene, OR) for 1 h and mounted in ProLong<sup>®</sup> Gold antifade with 4',6-Diamidino-2-phenylindole (Molecular Probes) onto glass slides. Samples were examined by confocal microscopy (Olympus IX81, Carsen Group Inc., Markham, ON, Canada).

**2.2.7 Whole-cell Patch-clamp Dye Loading Assay.** Coverslips with a confluent layer of cells were placed into artificial cerebrospinal fluid (aCSF) that contained (in mM) NaCl (120); NaHCO<sub>3</sub> (26); KCl (3); NaH<sub>2</sub>PO<sub>4</sub> (1.25); MgSO<sub>4</sub> (1.3); CaCl<sub>2</sub> (2), and glucose (10). The aCSF

was aerated with 95% O<sub>2</sub>/5% CO<sub>2</sub>, maintained at 32-34°C with an inline heater and perfused at a rate of 2 ml/min. Sulforhodamine 101 (SR101) loading was achieved with the whole-cell configuration of the patch clamp technique using pipettes of 5-7 M $\Omega$  resistance when loaded with intracellular solution containing (in mM) Kgluconate (130); KCl (10); HEPES (10); EGTA (10); CaCl<sub>2</sub> (1); MgATP (3); Na<sub>2</sub>GTP (0.6); and SR101 (0.01), at pH=7.3.

The membrane under the pipette was disrupted by gentle suction and SR101 loading of the cell was monitored with confocal microscopy using a Zeiss 510 confocal/multiphoton microscope. SR101 excitation was with a HeNe laser (543 nm). Fluorescence emission was detected by a photomultiplier tube and emitted light filtered (605-660 nm filter). The time constant of dye coupling ( $\tau$ ) was determined by fitting a single exponential to the SR101 fluorescence versus time plot using Clampfit software (Molecular Devices, Sunnyvale, CA). SR101 fluorescence was measured in a user-defined region of interest drawn over the loaded C6 cell, and over the first adjacent cell that showed coupling.

**2.2.8 Growth Curve Assay.** Cells were seeded in triplicates at 10,000 cells/well in 12-well plates. C6 medium was replaced every 48 hr throughout the experiment. Day 0 denotes the day of seeding and the number of cells/well were counted on Day 1, 3-4, 6, and 10 using Z1 Coulter Particle Counter (Beckman Coulter) with IsoFlow<sup>TM</sup> Sheath Fluid (Coulter Corporation, Miami, FL) as a diluent.

**2.2.9 Transwell Assay.** 75,000 cells were seeded in duplicate at both the top of a transwell insert (BD Biocoat<sup>TM</sup> 8.0  $\mu$ m polyethylene terephthalate (PET) inserts in 24-well plates; BD Biosciences, Missisagua, ON, Canada) and in a separate well without the transwell insert. Cells were incubated for 14 h and subsequently trypsinized from the bottom of the insert (# of traversed cells) and the separately seeded well (# of total cells) for cell counting using Z1 Coulter

Particle Counter (Beckman Coulter) with IsoFlow<sup>TM</sup> Sheath Fluid (Coulter Corporation) as a diluent. Cell motility was determined as # of traversed cells/# of total cells.

**2.2.10 Soft Agar Assay.** Two-layer soft agar system was used in this assay. Briefly, 2 ml of C6 medium containing 0.6% agar was cast as a bottom layer for each 60-mm plate. Cells were then seeded in triplicates at 10,000 cells/plate in 2 ml of 0.3% agar medium above the bottom layer. Following 14 days of incubation with fresh medium added every 7 days, samples were stained overnight with 0.01% crystal violet and photographed using AlphaImager 3400 (AlphaInnotech, San Leandro, CA). Colonies were viewed under a microscope (Axioplan 2, Carl Zeiss) and images captured using Axiovision (Carl Zeiss). The area of each colony was measured and colonies with a size greater than 20,000  $\mu$ m<sup>2</sup> were scored as positive. The average area of the colonies was calculated by summing the area of all measured colonies and dividing by the total number of colonies measured. Percentage of colony formation was determined as positive/(positive + negative).

**2.2.11 Tumorigenicity Assay.** Experiments with 6 - 8 week old female immunodeficient Hsd: athymic nude-*Foxn1*<sup>nu</sup> mice (Harlan Sprague Dawley, Indianapolis, IN) were conducted in the animal facility of the British Columbia Cancer Research Center (Vancouver, BC, Canada). Five mice were used for each sample, and each mouse was inoculated at two sites on the flank/upper hips (500,000 cells/site). Animals were monitored three times a week for tumor growth. Tumor volume was calculated according to the equation L x W<sup>2</sup>/2 with the length (mm) being the longer axis of the tumor. Mice were sacrificed after a combined tumor size of 1000 mm<sup>3</sup> per mouse was reached. All animals were handled under practices and operating procedures complying with the policies of the local animal care committee and the Canadian Council of Animal Care.

**2.2.12 Data Analysis.** Results are expressed as means  $\pm$  SE of the means. One-way ANOVA analysis was used for statistical comparisons with P < 0.05 considered significant. Each experiment with the exception of the tumorigenicity assay was repeated three or more times with similar results.

## **2.3 RESULTS**

**2.3.1** Panx1, Panx2 and Panx3 are Endogenously Expressed in Rat Primary Astrocytes, but Not in C6 Glioma Cells. To determine the endogenous Panx distribution in rat primary astrocytes, RT-PCR of Panx transcripts was performed on total RNA of the astrocytes and showed presence of Panx1 and Panx2 mRNAs while Panx3 mRNA was detected as a less intense band (Figure 2.1A). To ensure purity of primary astrocyte cultures, expression of glial fibrillary acidic protein (GFAP), a common astrocytic marker, was examined by immunocytochemistry; >99% of the cells exhibited GFAP signal. However, when we tested the tumorigenic counterpart, rat C6 gliomas, no mRNAs for any of the Panxs were detected (Figure 2.1A). Human adult brain and a panel of human gliomas (U87, U251, SF188 and SF539) were also studied and Panx1 mRNA was detected (Figure 2.1B). Since Panx1 transcript is present in rat primary astrocytes but not in C6 glioma cells, we hypothesized that Panx1 may act as a tumor suppressor once its expression is restored.

**2.3.2 Stable Panx1-Transfected C6 Cells Express Transfected Panx cDNAs.** To verify translational expression of transfected cDNAs in the stable C6 transfectants, Western blot analysis was performed. We generated several clones for each type of transfectant with varying expression levels (Figure 2.2A). From them, we selected the following clones for further

**Figure 2.1. Expression of Panxs is absent in C6 glioma cells.** (A) RT-PCR performed on total RNA extracted from C6 glioma cells and rat primary astrocytes. C6 cells exhibited no corresponding products for Panx transcripts. RT-PCR products for Panx1 (185 bp), Panx2 (258 bp) and a less intense band for Panx3 (336 bp) were found in primary astrocytes. (B) A panel of human gliomas (U87, U251, SF188 and SF539) and human adult brain showed RT-PCR product for Panx1 transcript (195 bp). As a positive control for RT, bands for GAPDH (515 bp) were detected in all samples. No product was detected when reverse transcriptase (+) was replaced with ultra-pure MilliQ water (-).







**Figure 2.2. Expression and altered cellular morphology in stable Panx1-transfected C6 glioma cells.** (A) Western blot analysis of several stable EGFP- and Panx1-transfected C6 clones. Protein expression in various clones was determined using corresponding antibodies against GFP and c-myc. (B) Sample immunoblots demonstrating protein expression in selected stable C6 transfectants at predicted sizes (EGFP: 25 kDa; Panx1-myc: 50 kDa; Panx1-EGFP: 73kDa). GAPDH was probed with anti-GAPDH antibody as a loading control. (C) Live-cell imaging revealing cellular morphology of parental (a-c), stable control (EGFP #5) (d-f) and Panx1-EGFP #2 (g-i) C6 transfectants. DIC images of Panx1-EGFP-transfected C6 cells (h) exhibited a flattened morphology when compared to the control (e). Morphology of parental C6 cells (b) appeared to be similar to that of stable EGFP transfectants (e). *Bar*, 10 μm.





experimentation: EGFP: #5 and #15; Panx1-myc: #6 and #19; Panx1-EGFP: #2 and #8. Using EGFP clone #5, Panx1-myc clone #19 and Panx1-EGFP clone #2, we demonstrated corresponding protein bands for each transfected cDNA at the predicted product sizes using either anti-GFP or anti-c-myc antibody, confirming the specificity of protein expression (Figure 2.2B).

**2.3.3 Panx1 Expression Induces Cellular Morphological Change.** Cellular morphology of stable Panx1-expressing C6 cells was studied by live-cell imaging (Figure 2.2C). This technique was used as it preserves the cellular morphology without the artifacts induced by conventional fixation processes. GFP signals positively identified expression of the stable transfectants (Figure 2.2Cd, g). Control (EGFP) (Figure 2.2Ce) showed a spindle-shaped morphology, which is typical of normal C6 cells (Figure 2.2Cb). By contrast, Panx1-EGFP (Figure 2.2Ch) displayed a flattened cell morphology, which resembles the flat and polygonal shape of cultured astrocytes (Lascola and Kraig, 1996; Mobley et al., 1986; Nicchia et al., 2005). Additionally, while typical EGFP expression was found throughout the cytoplasm in the control transfectants (Figure 2.2Cd, f), Panx1-EGFP was prominently observed in the perinuclear and peripheral regions of the cells (Figure 2.2Cg, i).

**2.3.4 Cellular Localization Pattern of Panx1.** To further investigate the subcellular localization of Panx1 and the resultant morphological change observed by live-cell imaging (Figure 2.2C), direct fluorescent labeling of Golgi apparatus and plasma membrane using Alexa 568-conjugated wheat germ agglutinin (WGA) was performed (Kovacs et al., 2004), and samples were examined using confocal microscopy. Panx1-EGFP was localized predominantly at the cell membrane, as well as to the perinuclear region (Figure 2.3Af) as anticipated, since Panx1 is a transmembrane protein. Conversely, control cells expressing only EGFP displayed fluorescence throughout the

Figure 2.3. Cellular localization of Panx1 and altered membrane morphology in stable Panx1-transfected C6 glioma cells. (A) Immunocytochemistry for stable control (EGFP #5) (ac) and Panx1-EGFP #2 (d-f) transfectants. Expression is shown by GFP fluorescence (a, d); Golgi apparatus and plasma membrane (b, e) were stained with WGA. Nuclei are visualized by DAPI in merged images (c, f). Panx1-EGFP appeared to localize to the perinuclear region (*arrowheads*) and cell membrane (*arrows*). *Bar*, 30  $\mu$ m. (B) Western blot analysis showing that Panx1-EGFP is predominantly associated with the membrane fraction. Whereas EGFP expression of the control transfectant was identified in the cytosol fraction (*C*), Panx1-EGFP was only detected in membrane-enriched fractions (*M*). Probing with anti-GAPDH antibody showed traces of GAPDH in the membrane-enriched fractions when compared to the cytosol fractions.



entire cytoplasm, a typical expression pattern found in GFP-transfectants (Figure 2.3Ac) (Falk, 2000). This correlates with our finding from the live-cell imaging experiment (Figure 2.2C); while the control cells retained the spindle-shaped characteristic of C6 cells, Panx1-EGFP cells displayed a flattened morphology, which is clearly revealed upon the plasma membrane staining (Figure 2.3A). To verify the observed Panx1-specific localization pattern, Western blot analysis on cytoplasmic and membrane-enriched fractionations was carried out (Figure 2.3B). As expected, EGFP expression was only detected in the cytosol fraction. By contrast, Panx1-EGFP expression was only found in the membrane-enriched fraction, which includes the Golgi apparatus and plasma membrane, concurring with the above findings. Using the anti-GAPDH antibody, we observed limited, but not appreciable amounts of this protein in the membrane-enriched fractions. To control for possible effects contributed by the EGFP reporter gene, the experiments were also conducted using stable C6 Panx1-myc transfectants, and similar results were found (data not shown).

**2.3.5 Panx1 Expression Increases Dye Coupling in C6 Cells.** As both Panx1-myc and Panx1-EGFP localize to the cell membrane, we hypothesize that functional Panx1-based channels might be present, yielding an increase in gap junctional coupling in the Panx1-expressing cells. Using the whole-cell patch-clamp loading assay with SR101, a fluorescent dye that labels astrocytic networks and passes through presumptive Panx1 neuronal hemichannels (Nimmerjahn et al., 2004; Thompson et al., 2006), the Panx1-transfectants exhibited a statistically significant, but limited, increase in dye transfer when compared to the control (EGFP only) (Figure 2.4A, B). Panx1-expressing transfectants were strongly coupled to at least one neighbor, but weaker coupling to additional neighbors was also observed (Figure 2.4A). In addition, the SR101 fluorescence increase in the control cells occurred with a slow time constant ( $\tau$ ),  $\tau = 1153 \pm$  Figure 2.4. Dye coupling in C6 Panx1-transfectants is observed following intracellular loading with a patch pipette. (A) Donor cells from the transfectants indicated were loaded with 10  $\mu$ M sulforhodamine 101 (red) via the whole-cell configuration of the patch-clamp technique. No significant dye coupling was observed in control cells (top row). By contrast, Panx1 transfectants showed dye transfer to neighboring cells (arrows) following loading of the donor cell (middle and bottom rows) (EGFP: 5/11 cells with weak coupling; Panx1-EGFP: 9/10 cells with stronger coupling; Panx1-myc: 7/9 cells with stronger coupling). (B) Time course of SR101 intensity inside dye loaded cells and neighboring cells. (C) The SR101 fluorescence increase in the control cells occurred with a significantly slower time constant when compared to the Panx1-transfectants (\* P < 0.05).


С

500

400

В





379.2 s (n=5), and increased to only <5% above the background (Figure 2.4C). The time constant of dye coupling for Panx1-expressing cells was significantly faster when compared to the control cells (Panx1-EGFP: 238.7  $\pm$  99.4 s (n=9); Panx1-myc: 230.5  $\pm$  41.3 s (n=7);  $\tau$  was not significantly different between Panx1-EGFP and Panx1-myc cells) (Figure 2.4C).

**2.3.6 Panx1 Expression Suppresses the Transformed Phenotype of C6 Cells.** Proliferation properties were first examined using the growth curve assay. A significant decrease in growth rate was evident in both stable tagged Panx1-transfectants when compared with the control cells starting from day 3 (Figure 2.5A). The reduced growth rate was continually observed up to day 6, which coincided with the confluence of the cultures. In addition, stable Panx1-transfectants displayed a significant decrease in saturation density, as revealed by counting cells post confluence (day 6-10). Furthermore, samples set up in parallel to the growth curve experiments showed no apparent increase in the percentage of DAPI-stained apoptotic nuclei in the stable Panx1 transfectants (data not shown).

Aberration in cellular morphology often associates with changes in motility and invasiveness of cancer cells (Etienne-Manneville and Hall, 2002). As Panx1 expression induces a distinctive morphological change from the typical spindle shape to the flattened morphology (Figure 2.2C), it suggests that Panx1 may also affect the motility of the glioma cells. To examine this possibility, transwell assays were carried out and a significant decrease in cell motility was observed in the Panx1-transfectants when compared to the control (Figure 2.5B). The assays were completed in 14 h, which is less than the doubling time of C6 cells (approximately 16 h) (Zhu et al., 1992).

Another hallmark of the transformed phenotype is anchorage-independent growth. To assess this, we performed soft agar assays and found a significant decrease in the percentage of colony formation in the Panx1-transfectants compared with the control (Figure 2.6B). Similarly,

Figure 2.5. Panx1 expression reduces C6 cell proliferation, saturation density and motility. (A) Growth curve assay of the control (EGFP) and stable tagged Panx1-transfectants (myc or EGFP). Cells were seeded at 10,000 cells/well in 12-well plates. Day 0 denotes the day of seeding and the number of cells/well were counted on Day 1, 3-4, 6, and 10 with C6 medium replaced every 48 h. A significant reduction in the growth rates of the Panx1-transfected C6 cells was observed when compared to the control (EGFP) beginning on day 3 to 4 (\*\* P < 0.001 for Panx1-transfectants compared with control). This reduced growth rate was consistently observed into day 6 (\* P < 0.01 for Panx1-transfectants compared with control). A significant decrease in saturation density of the Panx1-transfectants was also evident from day 6 to day 10. (B) Transwell assay exhibiting a significant reduction in cell motility of the Panx1-transfectants (Panx1-myc: 14.44  $\pm$  1.308 %; Panx1-EGFP: 12.54  $\pm$  1.106 %) when compared to the control (EGFP: 25.99  $\pm$  1.792 %) (\* P < 0.001).



Figure 2.6. Panx1 expression suppresses anchorage-independent growth and *in vivo* tumor formation in C6 glioma cells. (A) Above sample images showing appearance of individual colonies of each stable transfectant in the soft agar. Note that while large colonies were readily detected in the control (EGFP:  $54358 + 8144 \text{ }\mu\text{m}^2$ ), Panx1-transfectants exhibited fewer and smaller colonies (Panx1-myc: 3107.84 + 585.86 µm<sup>2</sup>; Panx1-EGFP: 14706.05 + 1226.0 µm<sup>2</sup>). (B) Above images display sample plates from soft agar assay showing an overall reduction of colony formation in stable Panx1-transfectants (Panx1-myc: 2.03 + 0.65 %; Panx1-EGFP: 15.70 + 1.13 %) when compared with the control (EGFP: 27.89 + 2.486 %). Upon quantification and statistical analysis, Panx1-transfectants demonstrated a significant decrease in both the average area of colonies (A) and efficiency of colony formation (B) (\* P < 0.001, compared with control). (C) Tumorigenicity assay of stable Panx1-transfectants. Day 0 denotes the day of injection. A significant reduction in the tumor size of the Panx1-transfected C6 cells was observed when compared to the control (EGFP) beginning on day 12 and consistently observed into day 16 (\* < P 0.05, \*\* P < 0.001). Mice injected with the Panx1-expressing cells only reached similar tumor size as the control on day 16 until an average of 6 days later (Panx1-myc: 5/5 mice on day 23; Panx1-EGFP: 3/5 mice on day 19, 2/5 mice on day 23).



numerous large colonies (>100,000  $\mu$ m<sup>2</sup>) were readily detected in the control samples, which is far greater than the average size of both Panx1-myc and Panx1-EGFP colonies (Figure 2.6A). Intriguingly, Panx1-EGFP samples consistently showed a greater average area of colonies and percentage of colony formation than that of Panx1-myc (*P* < 0.001) (Figure 2.6A).

Finally, tumorigenicity assays were performed to examine tumor-suppressive effects of Panx1 *in vivo*. All of the injected sites from the control and Panx1-transfectants developed tumors. When compared with the control, a significant reduction of average tumor size was found in both tagged Panx1-expressing cells starting from day 12 to 16. By day 16, the mice injected with the control cells were sacrificed as the tumor size per mouse had exceeded 1000 mm<sup>3</sup> (Figure 2.6C).

## **2.4 DISCUSSION**

In the past, Cxs have been considered the sole mammalian gap junctional proteins. Using the *Xenopus* oocyte system, studies have shown formation of functional hemichannels and intercellular channels by Panx1 and Panx1/2, implicating that Panx-based channels may play important physiological roles in mammalian cells (Bruzzone et al., 2003). In view of the functional similarity with connexins, for which an inverse relationship between gap junctions and tumor progression has long been suggested (Naus, 2002), we sought to examine the possible correlation between aberrant Panx expression and tumorigenicity in gliomas by using the C6 glioma cell model. We have shown that Panx1, Panx2, and Panx3 transcripts are expressed in primary astrocytes but not in its tumorigenic counterpart, C6 glioma cells. Induction of Panx1 expression by gene transfection revealed a unique Panx1 localization pattern and elicited a dramatic morphological change in Panx1-expressing cells. Using the whole-cell patch-clamp loading assay with SR101, a significant increase in dye coupling was discovered in Panx1-expressing cells. Moreover, stable Panx1 expression consistently suppressed proliferation both *in* 

*vitro* and *in vivo*. These findings provide strong evidence that Panx1 mediates tumor suppressive effects in C6 glioma cells, possibly via functional Panx1-based intercellular channels.

To date, protein expression of Panxs has not been fully defined in mammals due to the lack of specific antibodies. Nevertheless, through Northern blot analysis and in situ hybridization experiments in rats. Panx mRNA expression was reported to be location-specific (Bruzzone et al., 2003; Ray et al., 2005; Vogt et al., 2005). As Panx1 and Panx2 mRNAs are present in rat brain at P1, we anticipated that primary astrocytes would possess both transcripts (Ray et al., 2005; Vogt et al., 2005). Albeit Panx3 distribution has not been extensively studied, previous reports have suggested low Panx3 mRNA expression in the brain, as well as in osteoblasts and synovial fibroblasts as shown by EST data (Baranova et al., 2004; Bruzzone et al., 2003; Panchin et al., 2000). While a previous study by Vogt et al. using in situ hybridization demonstrates Panx1 (in hippocampus) and Panx2 (in cerebral cortex) expression in neuronal but not astrocytic cells of postnatal rat brain, we positively identified both of these Panx mRNAs in primary astrocytes (Vogt et al., 2005). These differences could be attributed to the reactive-like nature of newly cultured astrocytes, a distinct population of astrocytes extracted from the neonatal cortex, and/or increased sensitivity of PCR analysis in detecting Panx mRNAs over the in situ hybridization technique (Wu and Schwartz, 1998). Additionally, we used cultures in which >99% of primary astrocytes were GFAP-positive, to avoid possible false-positive observations as a result of neuronal and/or fibroblastic cell contaminations. We also observed a less intense band for Panx3 in the primary astrocytes, which agrees with the previous study that Panx3 is present in the brain as a very low-level transcript (Baranova et al., 2004). The absence of Panx transcripts in C6 cells implicates the loss or down-regulation of Panx genes during tumor transformation. Similarly, down-regulation or absence of Cx expression, including Cx43 in C6 cells, has been reported in various types of neoplastic cells (Laird et al., 1999; Naus et al., 1991;

Tsai et al., 1996; Sawey et al., 1996). Since Panxs are gap junctional proteins, such a relationship could also hold true for the Panxs, suggesting their tumor-suppressive role.

Interestingly, Panx1 transcript was detected in a panel of human gliomas (U87, U251, SF188 and SF539) and human adult brain. Since Panx1 is ubiquitously expressed, it was expected to be observed in the brain and therefore used as a positive control. The presence of Panx1 mRNA in the human gliomas, in contrast to the C6 cells, could indicate that the loss of Panx1 transcript specifically apply to a subset of glioma cell lines. Nevertheless, aberrant Panx expression may also occur at the translational level, resulting in tumor transformation. Further examination of Panx1 protein expression in the human glioma cell lines is needed to test this supposition.

As Panx1 expression has been demonstrated in primary astrocytes and, unlike Panx2, has been reported to readily form functional channels, the present study focused on the possible tumor-suppressive roles of Panx1 in C6 cells (Bruzzone et al., 2003). Since a specific anti-Panx1 antibody was unavailable at the time of the experiment for the identification of positive Panx1-transfected cells, clones were generated from stable transfection with Panx1-myc and Panx1-EGFP cDNAs. Both types of stable transfectant were used in all experiments wherever possible to verify the findings, and similar properties induced by Panx1-expression were found throughout the study. Additionally, more than one clone from each type of stable transfectants was used to minimize clonal variation. Furthermore, Cx43 was examined to ensure similar expression levels between the selected clones in order to avoid Cx43-elicited effects in this study (data not shown).

In the stable tagged Panx1-transfectants, transfected cDNAs were successfully expressed and the protein prominently localized to the perinuclear Golgi apparatus and plasma membrane. Since Panx1 has a predicted topology resembling Cxs, as well as reported channel-forming ability, it is expected that Panx1 is localized to the plasma membrane (Bruzzone et al., 2003; Panchin, 2005). Moreover, the unique localization pattern of Panx1 to the perinuclear Golgi apparatus and plasma membrane appeared to be similar to that of Cx26 and Cx43 expression (Falk, 2000; Thomas et al., 2005). Previous studies have revealed a protein trafficking pathway shared by Cx26 and Cx43 where both proteins are transported through the Golgi apparatus before being translocated to the membrane (Laird et al., 1995; Musil and Goodenough, 1993; Thomas et al., 2005). While it is beyond the scope of this study, our current findings suggest that Panx1 may be trafficked in a similar pathway as the Cxs and thus warrants future investigation.

Coinciding with Panx1 expression at the plasma membrane, Panx1-transfectants displayed a flattened morphology similar to the flat and polygonal shape of cultured astrocytes (Lascola and Kraig, 1996; Mobley et al., 1986; Nicchia et al., 2005), as well as reduced cell motility. When Cx43 was overexpressed in C6 cells, actin stressed fibers were induced and a flattened morphology along with an increase in gap junctional coupling were observed (Naus et al., 1992; Zhu et al., 1991). Since then, various studies have demonstrated direct interactions between Cxs and other junctional proteins including zonula occludens-1 (tight junction) and cadherins (adherens junction), suggesting gap junctions as an anchorage site for the cytoskeleton on the plasma membrane (Giepmans, 2004). Furthermore, Inx2 and Inx3 have also been shown to co-localize with components of the adherens junction in Drosophila (Bauer et al., 2005a). Specifically, a recent study by Lehmann *et al.* has demonstrated that Inx2 directly interacts with DE-cadherin and  $\beta$ -catenin (Lehmann et al., 2006). Together with our current findings and previous literature, we hypothesize that Panx1 may interact with tight and/or adherens junctional proteins and hence contribute to the observed flattened morphology and reduced cell motility via cytoskeletal reorganization.

A significant reduction of cell proliferation and saturation density was clearly evident in the stable Panx1-transfectants. In addition, the reductions observed were not a result of cell death as samples set up in parallel to the growth curve experiment showed no apparent increase in the percentage of apoptotic nuclei (data not shown). These *in vitro* results are further strengthened by the *in vivo* tumorigenicity assay. As Panx1 expression was found at the plasma membrane, we were interested in determining whether functional Panx1 intercellular channels were present. Loading Panx1-expressing cells via dialysis from a patch-pipette with SR101, a fluorescent dye, demonstrated a significant increase in coupling when compared to the control. This finding suggests that Panx1 forms functional intercellular channels when stably expressed in C6 cells to mediate tumor-suppressive effects. However, it should also be noted that the extent of dye coupling observed in Panx1-expressing cells was limited to immediate neighboring cells, whereas Cx43-mediated dye passage in C6 cells can reach cells up to five orders away (Zhu et al., 1991). The difference in dye passage order implies that Panx1 does not facilitate GJIC as efficiently as Cxs, and alternative pathways such as hemichannel and/or protein-protein interaction may be employed by Panx1 to elicit its functions.

Nevertheless, the effects induced by Panx1 expression may also be dependent on electrical as well as biochemical coupling (Bao et al., 2004; Bruzzone et al., 2003). Like Cxs, Panx1 may exert its effects via intracellular signaling pathways without direct communication with neighboring cells (Naus, 2002). Increasing evidence indicates that the tumor-suppressive effects of Cxs may be unrelated to functional gap junctions and are instead a result of interplay with intracellular signaling pathways (Naus, 2002). The present study shows that Panx1 expression induces a dramatic morphological change, implying that molecular targets other than gap junctional coupling are affected. Another piece of evidence supporting this notion is derived from the soft agar assays. In the assay, single cells are seeded into soft agar and are therefore devoid of cell-cell communication in the initial stage of colony development. Consistently, the Panx1-transfectants exhibited a significant decrease in the percentage of colony formation, suggesting a gap junction-independent effect of Panx1. From the same assay, we also found a significant reduction in the average size of colonies. In agreement with the dye coupling data,

this could implicate a gap junction-dependent role of Panx-based channels during the later stages of colony development as multiple cells become available for cell-cell communication. Collectively, our findings suggest that Panx1 may act both gap junction-dependently and – independently to elicit tumor suppression.

Albeit not statistically significant in all experiments, Panx1-myc consistently exhibited a stronger tumor-suppressive phenotype than Panx1-EGFP both *in vitro and in vivo*. As tagged Panx1 forms hemichannels less efficiently than untagged Panx1 (Bruzzone et al., 2003), we speculate that the difference could be attributed to the different tags; since EGFP yields a greater sized protein, it may hinder the normal conformation of Panx1 protein and in turn affect Panx1 functionality more readily than the c-myc tag, resulting in the more prominent tumor-suppressive phenotype seen in stable Panx1-myc transfectants.

Although Cxs have been shown to modulate glioma cell proliferation and tumorigenesis, tumor-suppressive implications of Panxs have not been investigated. To our knowledge, this is the first study which demonstrates a direct correlation between aberrant Panx1 expression and tumorigenicity. In the present study using C6 glioma cells, we clearly demonstrated that restoration of Panx1 expression induces tumor suppression. It would be of great interest to examine if a reduction in Panx expression occurs in other types of cancer. Similar to Cxs, a recent report has also shown K(v)ss3 to be a potential regulator of Panx1, implicating that Panxs can be regulated through protein interactions (Bunse et al., 2005). While many agents have been reported to suppress tumors by up-regulating Cxs and GJIC, it is possible that such agents could also regulate Panx expression (Dhein, 1998). The model cell system described here can be used for further studies on the molecular mechanisms underlying the phenotypes and effects elicited by Panx1 overexpression.

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**Chapter 3** 

Pannexin2 as a Novel Growth Regulator in C6 Glioma Cells

## **3.1 INTRODUCTION**

Gap junctions are membrane channels that connect the cytosols of adjacent cells, allowing the direct passage of small molecules less than 1 kDa (Loewenstein, 1987). A lack of cell-cell communication has been demonstrated in neoplasms including gliomas (Mesnil, 2002; Naus, 2002), and restoration of gap junctional intercellular communication in cancer cell models has been found to reverse the transformed phenotypes and to reduce proliferation (Naus et al., 2005). Later studies have also demonstrated a tumor-suppressive role of connexins (Cxs), the canonical gap junction proteins, via protein-protein interactions, further supporting its function as a tumor suppressor (Duffy et al., 2007; Fu et al., 2004).

Pannexins (Panxs) are a novel family of gap junction proteins recently identified as the mammalian homologues of the invertebrate gap junction proteins, innexins. To date, three Panx members have been identified in vertebrates: Panx1, Panx2, and Panx3. A functional role of Panx1 has been demonstrated in ER Ca<sup>2+</sup>-leak, ATP release from erythrocytes, inflammatory response, ischemic cell death and taste cell signaling, advocating the importance of Panx1 under both normal and pathological conditions (Huang et al., 2007; Vanden Abeele et al., 2006; Locovei et al., 2006; Pelegrin and Surprenant, 2006; Pelegrin and Surprenant, 2007; Romanov et al., 2007; Thompson et al., 2006). In addition, we demonstrated a reversion of the transformed phenotype of C6 gliomas when Panx1 was overexpressed (Lai et al., 2007), suggesting a tumor-suppressive role of Panxs in neoplasms. However, Panx2's function remains largely undiscovered, and its role in neoplasms has not been examined.

Panx2 transcripts, unlike Panx1, are particularly abundant in the brain in rodents and presumably brain-specific in humans (Baranova et al., 2004; Bruzzone et al., 2003; Ray et al., 2006; Vogt et al., 2005). In parallel to the loss of Panx2 transcript in C6 gliomas when compared to rat primary astrocytes (Lai et al., 2007), a high-throughput cDNA microarray analysis of human brain tumor samples has shown an overall reduction of Panx2 gene expression in gliomas

(Litvin et al., 2006). A direct correlation was also found between Panx2 up-regulation and post diagnosis survival in patients with glial tumors using the brain cancer gene expression database REMBRANDT (Repository of Molecular Brain Neoplasia Data, <u>http://rembrandt.nci.nih.gov/rembrandt</u>) (Litvin et al., 2006). Moreover, human *Panx2* is located within chromosomal region 22q13.3, where deletions are often found in human astrocytomas and ependymomas (Hu et al., 2004; Ino et al., 1999; Oskam et al., 2000; Ray et al., 2006). Thus, *Panx2* expression may be commonly altered during glioma development. Altogether, the findings suggest a role of aberrant Panx2 in gliomagenesis and oncogenicity.

## **3.2 RESULTS AND DISCUSSION**

**3.2.1 Reduced Panx2 Expression in Human Glioma Cells.** To investigate if aberrant Panx2 expression occurs in gliomas, a panel of human glioma cell lines was examined using Western blot analysis and showed a reduction or absence of Panx2 protein when compared to human astrocytes (Figure 3.1A). The observation is in agreement with the result from gene array analysis of human brain tumor samples where gliomas have an overall decrease of Panx2 expression (Litvin et al., 2006). A notable level of Panx2 expression was detected in human brain, advocating a role of Panx2 in the brain (Ray et al., 2006; Vogt et al., 2005; Zappala et al., 2007). An appreciable amount of Panx2 protein was also found in human astrocytes. This is in contrast to the observation made in rats where Panx2 was only detected in neurons and reactive astrocytes but not in normal astrocytes (Vogt et al., 2005; Zappala et al., 2007). The disparity could be a result of species differences and warrants further investigation to determine the type(s) of astrocytes expressing Panx2 in humans. Overall, the present finding suggests an inverse correlation between Panx2 expression and glioma oncogenicity.

Figure 3.1. Panx2 expression is reduced in human glioma cell lines, and alters cell morphology in stable Panx2-transfected C6 cells. (A) Using anti-Panx2 antibody (Aviva Systems Biology, San Diego, CA). Western blotting of total protein extracted from a panel of human gliomas (American Type Culture Collection, Manassas, VA), human brain (Zyagen, San Diego, CA), astrocytes (ScienCell Research Lab, Carlsbad, CA), and stable Panx2-transfected C6 cells showed very limited or negligible Panx2 signal in the human glioma cells. GAPDH served as a loading control (CedarLane Lab. Ltd., Horby, ON, Canada). The 50 kDa band observed in Panx2-expressing C6 cells and some other glioma lines is nonspecific (Appendix A). (B) C6 cells were co-transfected with Murine Stem Cell Virus vectors encoding a puromycin resistance gene (pMSCVpuro) (Clontech Laboratories Inc., Mountain View, CA) and the plasmid of interest (EGFP, Rattus norvegicus Panx2 tagged with human influenza hemagglutinin tag (Panx2-HA) or enhanced green fluorescence protein reporter gene (Panx2-EGFP) at carboxy-terminus) using Lipofectamine<sup>™</sup> 2000 (Invitrogen Corp., Burlington, ON, Canada) as previously described (Bruzzone et al., 2005; Lai et al., 2007). Co-transfectants were selected with 3 µg/ml puromycin, and clones were subsequently isolated. Cell lysates from selected clones were probed with anti-HA (Cedarlane Lab. Ltd., Hornby, ON, Canada) and anti-GFP antibodies (Stressgen, Ann Arbor, MI). The following clones were selected for further experimentation: Panx2-HA clone 3 and 8; Panx2-EGFP clone 1 and 13. (C) A representative immunoblot demonstrating specificity of exogenous protein expression using EGFP clone 5, Panx2-HA clone 8, Panx2-EGFP clone1 (EGFP: 25 kDa; Panx2-HA: 73 kDa; Panx2-EGFP: 98 kDa). (D) Live-cell imaging with differential interference contrast (DIC) shows Panx2-EGFP cells exhibiting a moderate flattened morphology and greater cell-cell contacts. By contrast, Panx1-EGFP displayed the most flattened morphology between the samples. *Bar*, 50 µm.



(E) Crude membrane fractionation assay (Lai et al., 2007) shows EGFP in the cytosol fraction (*C*) while Panx2-HA and Panx2-EGFP were only found in the membrane-enriched fraction (*M*). To verify fraction specificity, immunoblots were probed for Cx43 (Sigma-Aldrich, St. Louis, MO) and GAPDH (CedarLane Lab. Ltd.); Cx43 was only detected in the membrane-enriched fraction, whereas GAPDH was predominantly found in the cytosol-enriched fractions.

3.2.2 Panx2 Expression Alters Cellular Morphology. We next investigated whether restoring Panx2 expression could suppress glioma growth. Using a well established glioma cell line, rat C6 glioma cells, we investigated the effect of Panx2 expression on the transformed phenotype. C6 cells are considered to be of astrocytic origin and poorly coupled by gap junctions due to their limited Cx43 expression (Barth, 1998; Naus et al., 1991). In addition, a recent study demonstrated most of C6 cells as cancer stem cells with clonogenic, self-renewal, and tumorigenic capacities (Zheng et al., 2007). Furthermore, our recent report determined C6 gliomas to be devoid of Panx transcripts (Lai et al., 2007), which together makes it an ideal model system to study the effect of Panx2 expression on glioma oncogenicity. Several clones with varying expression levels were successfully generated for Panx2-HA and Panx2-EGFP stable transfectants to study the potential correlation between Panx2 and oncogenicity (Figure 3.1B, C). Clones for the control transfectants, EGFP, were previously created at the same time with tagged Panx2 transfectants (Lai et al., 2007). ER stress is an artifact commonly observed in the overexpression system, perturbing cell growth and survival (Lee, 2005). To address this possibility, stable control- and Panx-transfectants were examined for glucose regulated protein 78 (GRP78/BiP), an ER stress marker, and showed no evident increase in expression (Lee, 2005) (Figure 3.2A). Upon stable transfection, a notable flattened morphology was observed in Panx2expressing cells, which is similar to the flat, polygonal shape of cultured astorcytes (Figure 3.1D) (Lascola and Kraig, 1996; Mobley et al., 1986; Nicchia et al., 2005). Interestingly, the flattened morphology was moderate when compared to that of Panx1-expressing cells (Figure 3.1D). Moreover, Panx2-EGFP signal was observed in the cytoplasmic compartment, which is distinctly different from largely plasma membrane-located Panx1 (Lai et al., 2007).

**3.2.3 Panx2 is Not Predominately Detected at the Plasma Membrane.** To further examine the intracellular location of Panx2, a crude membrane fractionation assay identified Panx2-HA and

Figure 3.2. Panx2 does not form protein aggregates at the ER and Golgi apparatus, and is not actively degraded by endolysosomal pathway. (A) Western blot analysis of GRP78/BiP expression (Sigma-Aldrich) in parental, stable EGFP and Panx-transfected C6 cells. GRP78/BiP expression level was similar between samples and showed no increase in both Panx1- and Panx2-expressing cells. (B) Subcellular localization of Panx2. Top, live-cell imaging of ERlabeled Panx2-EGFP stable transfectants. Exogenous expression was visualized by GFP fluorescence and ER was stained with ER-Tracker<sup>TM</sup> Blue-White DPX (Invitrogen Corp.). Panx2-EGFP was detected throughout the cytoplasm in a vesicle-like granular pattern (arrowheads) with limited co-localization to ER (arrows). Bottom, confocal images of Panx2-EGFP immunolabeled with GM130, a Golgi apparatus marker (BD Transduction Lab, San Jose, CA). Panx2-EGFP signal was detected within the cytoplasm with no apparent co-localization to GM130. Bar, 50 µm. (C) Panx2 is not actively degraded by the endolysosomal system. Top, confocal images of Panx2-EGFP immunolabeled with EEA1, an early endosome marker (BD Transduction lab). The selected area (square) of merged images (b) was enlarged for clearer visualization (c). No significant signal overlap was observed between Panx2-EGFP and EEA1. Bar, 50 µm. Bottom, live-cell imaging of lysosome-labeled Panx2-EGFP (d, e). Lysosomes were stained with LysoTracker<sup>TM</sup> (d) and no considerable co-localization was found between Panx2-EGFP and lysosomes (e). Bar, 10 µm.



Panx2-EGFP only in the membrane-enriched fraction, suggesting that Panx2 is in membranebound vesicles and/or organelles (Figure 3.1E). Using specific markers, Panx2 showed a prominent signal overlap with the endoplasmic reticulum (ER) but only limited with the Golgi apparatus (Figure 3.2B). We then stained the cell membranes with wheat germ agglutinin (WGA), and detected Panx2-EGFP predominantly around the perinuclear region in a vesicle-like pattern but not at the plasma membrane nor at areas of cell-cell contact (Figure 3.3A). Importantly, Panx2 did not appear to aggregate in either organelle, indicating that its trafficking was not encumbered as a result of overexpression; previous studies have shown an aberration in Cx trafficking due to its overexpression (Das et al., 2005; Skerrett et al., 2004; Thomas et al., 2005). One possible explanation for Panx2's distinctive intracellular localization is that it was actively trafficked to the plasma membrane but promptly internalized for degradation, possibly via the endolvsosomal pathway previously shown for Cx43 (Berthoud et al., 2004; Laing et al., 1997; Oin et al., 2003). However, Panx2's did not appear co-localized to early endosomes and lysosomes, showing that Panx2 was not being actively degraded in the cytoplasm (Figure 3.2C). It was also unlikely that Panx2 was being actively degraded by the proteasomal degradation pathway since it was not readily detected in the cytosol-enriched fraction (Fig 3.1E). Several recent studies have reported Cx40 and Cx43 located in the mitochondria of cardiomyocytes, resembling Panx2 subcellular pattern (Boengler et al., 2005; Li et al., 2002; Schulz et al., 2007). However, co-labeling of mitochondria and Panx2 showed no apparent co-localization (Figure 3.4). Importantly, recent studies have shown a similar expression pattern of endogenous Panx2 in neuronal cells, reactive astrocytes following hypoxia, as well as in spiral ganglion neurons of the cochlea, where Panx2 was detected in the cytoplasmic compartments and especially prominent at the perinuclear region (Zappala et al., 2007; Wang et al., 2008). Together, the current finding indicates Panx2 is primarily located within the cytoplasmic compartments and not predominately detected at the plasma membrane in C6 cells.

Figure 3.3. Panx2 does not predominately associate with the plasma membrane. (A) Confocal images showing Panx2-EGFP signal in the cytoplasm and especially at the perinuclear region (*arrows*). Cell membranes were permeabilized and stained with Alexa 594-conjugated WGA (Invitrogen Corp.). *Bar*, 30  $\mu$ m. (B) Co-expression of Panx2 and Panx1 by transiently transfecting stable Panx2-HA transfectants with Panx1-myc (*top*) or Panx1-EGFP (*bottom*) using Lipofectamine 2000<sup>TM</sup> demonstrated no apparent co-localization between the two proteins at the plasma membrane or in the cytoplasm. Exogenous expression was immunolabeled with mouse anti-HA (Cedarland Lab Ltd.) or rabbit anti-myc (Abcam Inc., Cambridge, MA) antibodies followed by Alexa 568- or 488-conjugated anti-mouse or anti-rabbit IgG (Invitrogen Corp.), respectively. Nuclei were visualized by DAPI. *Bar*, 10  $\mu$ m.







Figure 3.4. Panx2 does not co-localize with mitochondria. Stable EGFP and Panx2-EGFP-transfected C6 cells were immunolabeled with OxPhos Complex V subunit  $\alpha$ , a mitochondria-specific marker (Invitrogen Corp.). Exogenous expression was detected by GFP fluorescence (a, d). No significant co-localization was observed between Panx2-EGFP and OxPhos Complex V subunit  $\alpha$  (f). *Bar*, 10 µm.



**3.2.4 Panx2 Does Not Co-Localize with Panx1 at Plasma Membrane.** Panx2's subcellular localization pattern is consistent with the finding that Panx2 alone was not able to form either hemichannels or intercellular channels in the *Xenopus* oocyte system on the cell surface (Bruzzone et al., 2003). Thus, we speculated that Panx2 can be recruited to the plasma membrane by Panx1 as previously suggested to elicit its function (Bruzzone et al., 2003; Bruzzone et al., 2005). Yet, when the two Panxs (tagged or untagged) were co-expressed, while Panx1 was found at the plasma membrane as previously reported (Lai et al., 2007), Panx2 localization remained unchanged (Figure 3.3B). The difference in findings suggests that Panx1/2 channel formation is not prevalent in C6 gliomas. Collectively, our findings suggest Panx2 exists in an individual entity, likely in the form of membrane-bound vesicles.

**3.2.5 Panx2 Reduces Oncogenicity of C6 Gliomas Cells.** Next, we set to determine the effect of restoring Panx2 expression on the oncogenicity parameters in C6 gliomas. Using growth curve assay, a significant retardation in proliferative rate was discovered in the stable Panx2 transfectants (Figure 3.5A). Since no significant increase in apoptotic nuclei was observed in cells seeded on coverslips in parallel to the growth curve assay (data not shown), the growth reduction by Panx2 is proposed to occur via suppression of cell cycle, but not cell death. In addition, Panx2 elicited a consistent reduction in saturation density (Figure 3.5A), which coincides with the flattened morphology of Panx2 transfectants; fewer cells of increased size are required to reach confluence. A similar growth suppression with human glioma cells was also observed (Figure 3.6). As Panx2 was only found within the cells but not at the plasma membrane like Panx1, and given that further examinations also showed that Panx2 does not affect expression of Panx1, Cx43, as well as gap junctional intercellular communication (Figure 3.7), Panx2 may induce its phenotype via a pathway distinct from Panx1. The notion is supported by the transwell assay where Panx2, in contrast to Panx1, showed no clear effect on cell motility

Figure 3.5. Panx2 reduces cell proliferation and saturation density but not motility. (A) Growth curve assay shows a dramatic reduction in saturation density of Panx2-HA and Panx2-EGFP from Day 6 to 10. The Panx2 transfectants also exhibited a reduced proliferative rate than the control from Day 3 to 4. 10 000 cells/well were seeded in 12-well plates, and the number of cells/well were counted on Days 1, 3-4, 6, and 10 using a Z1 Coulter Particle Counter (Beckman Coulter, Mississauga, ON). Day 0 denotes the day of seeding (\* P < 0.01, \*\* P < 0.01, \*\*\* 0.001 compared with control). Cells seeded on coverslips in parallel to the growth curve assay were stained with DAPI, and no significant increase in apoptotic nuclei was observed between the samples (data not shown). (B) Transwell assay reveals no decrease in cell motility of the Panx2 transfectants, whereas the Panx1 transfectants exhibited a significant retardation. 75 000 cells were seeded and incubated for 14 hr in duplicates in both the top of a transwell insert (BD Biocoat<sup>TM</sup> 8.0 µm polyethylene terephthalate (PET) inserts in 24-well plates; BD Biosciences, Mississauga, ON, Canada) and a separate well without the transwell insert. Cell motility was determined as # of traversed cells (bottom of the insert)/# of total cells (separately seeded well) (\* P < 0.05, \*\* P < 0.01 compared with control).







Figure 3.6. Panx2 exhibits intracellular expression pattern and reduces saturation density in human glioma cell lines. (A) Immunocytochemistry of stable Panx2-transfected T98G human glioma cells showing prominent Panx2 signal at the perinuclear region. Panx2 was immunolabeled with rabbit anti-Panx2 (Aviva Systems Biology) antibodies followed by Alexa 568-conjugated anti-rabbit IgG (Invitrogen Corp.). Nuclei were visualized by DAPI. *Bar*, 50 µm. (B) Growth curve assay of T98G and U251 human glioma cells stably expressing EGFP (control) or Panx2. A significant decrease in saturation density was detected in the Panx2 transfectants from Day 12 to 14. T98G and U251 cells were co-transfected with pMSCVpuro (Clontech Laboratories Inc.) and the plasmid of interest (*EGFP*, *Rattus norvegicus Panx2*) as described in Figure 3.1. Co-transfectants were selected with puromycin (2 µg/ml and 1 µg/ml for T98G and U251 cells, respectively). 20 000 cells/well were seeded in 12-well plates, and the number of cells/well were counted on Days 1, 3, 5, 8, 10, 12, and 14 using a Z1 Coulter Particle Counter (Beckman Coulter). Day 0 denotes the day of seeding (\* P < 0.05 compared with control).





**Figure 3.7. Panx2 does not drastically affect expression of Panx1 and Cx43, as well as gap junctional intercellular communication (GJIC).** Total protein extracted from stable EGFP, Panx1-myc, Panx1-EGFP, Panx2-HA and Panx2-EGFP-transfected C6 cells were subjected to Western blot analysis and immunoprobed with anti-Panx1 (a generous gift from Dr. Dale Laird, University of Western Ontario) and anti-Cx43 (Sigma-Aldrich) antibodies. (A) While Panx1 were readily detected in Panx1-myc and Panx1-EGFP cells, none were detected in EGFP and Panx2-expressing cells. (B) A representative Western blot showing no obvious elevation in Cx43 expression in Panx2- and Panx1-expressing cells when compared to EGFP. (C) Dye preloading assay (Goldberg et al., 1995) using DiI and calcein-AM (Invitrogen Corp.) demonstrated no obvious increase in GJIC in Panx2-expressing cells when compared to EGFP.


Figure 3.8. Panx2 suppresses anchorage-independent growth and *in vivo* tumor formation. (A, B) Panx2 dramatically retards anchorage-independent growth. (A) top, sample images of individual colonies in the soft agar. Bottom, Large colonies were consistently observed in the control (EGFP, 30 576.78 + 2 671.3  $\mu$ m<sup>2</sup>), whereas only small colonies were found in the Panx2 transfectants (Panx2-HA, 3 895.91 + 385.51 µm<sup>2</sup>; Panx2-EGFP, 8 812.03 + 400.61 µm<sup>2</sup>). Bar, 200 µm. (B) top, sample plates from the soft agar assay. Bottom, a significant reduction in the efficiency of colony formation of the stable Panx2 transfectants (Panx2-HA, 4.72 + 0.81%, Panx2-EGFP, 3.69 + 1.68%) when compared to the control (EGFP, 38.67 + 1.50%) was observed. Soft agar assay was performed as previously described (Lai et al., 2007) (\* P < 0.001compared with control). (C) Tumorigenicity assay demonstrates a significant reduction in the tumor size of the stable Panx2-transfected C6 cells beginning on Day 14 and consistently observed into Day 21 (\* P < 0.001). Nude mice injected with the controls (EGFP) were sacrificed on Day 21 as the tumor size had reached 1,000 mm<sup>3</sup>. In contrast, subjects injected with Panx2-expressing cells did not reach similar tumor size until an average of 12 days later (Panx2-HA: one of five mice on Day 28, two of five mice on Day 30, one of five mice on Day 32 and 35; Panx2-EGFP: one of five mice on Day 30, 32, and 35, two of five on Day 37). Day 0 denotes the day of injection. Five 6-8 week old female immunodeficient CrTac: NCr-Foxn1<sup>nu</sup> mice (Taconic, Hudson, NY) were injected at two sites on the flank/upper hips (500 000 cells/site) for each sample, and monitored for tumor growth as previously described (Lai et al., 2007).



(Figure 3.5B). Anchorage-independent growth is another hallmark of cancer and can be assessed by the formation of colonies in soft agar. Notably, both Panx2-HA and Panx2-EGFP transfectants drastically suppressed colony formation efficiency and anchorage-independent growth (Figure 3.8A, B). This suggests that Panx2 does not fully rely on channel activity to elicit its function. The proposition is strengthened by the soft agar assay since no cell-cell communication was present in the initial phase of the experiment when single cells were seeded in the soft agar. To further examine whether Panx2 elicits tumor-suppressive effects in vivo, a tumorigenicity assay was conducted and both Panx2-expressing cells exhibited a strikingly reduced average tumor size when compared to the control (Figure 3.8C). Anchorage independence on tethering to the extracellular matrix (ECM) is another hallmark of transformed phenotype. Findings reported here suggest Panx2 expression restores anchorage dependence of C6 gliomas. Whether this dependence is via an alteration of the expression of the cell surface receptors, integrins, glioma-derived ECM (i.e. vitronectin, tenascin-C), and/or the constitutive activity of survival pathways (i.e. PI3K/Akt, MEK/ERK, NF-kß), remains to be elucidated (Chiarugi and Giannoni, 2008; D'Abaco and Kaye, 2007).

In summary, the unique subcellular location of Panx2 suggests its functions via pathway(s) independent from gap junction channels. Since Panx2 has a 358 a.a. C-terminal tail which is more than twice the size of that of Cx43 (151 a.a), we envision that the long Panx2 C-terminal tail may be advantageous in providing potential sites for intracellular protein-protein interaction to mediate Panx2's functions. The present study provides the first report on the tumor-suppressive effects of Panx2 and implies Panx2 also functions in glioma stem cells as a recent study demonstrated that C6 cell line was mainly composed of cancer stem cells with perpetual self-renewal ability (Zheng et al., 2007). In conclusion, Panx2 may serve as a prospective therapeutic target against glial tumors and warrants further investigation.

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# **Chapter 4**

# Pannexin1 and Pannexin3 Enhance Neurite Outgrowth in PC12 Cells

#### **4.1 INTRODUCTION**

Gap junctions are intercellular membrane channels located between opposing surfaces of adjacent cells, directly connecting their cytosol. These channels allow the direct passage of small ions and molecules less than 1 kDa and function in maintaining intercellular communication and homeostasis within tissues (Loewenstein, 1987). Until quite recently, connexins (Cxs) were identified as the only mammalian family of gap junction proteins, which consists of more than 20 and 21 members in rodents and humans, respectively (Sohl and Willecke, 2004; Willecke et al., 2002). A novel family of gap junction proteins, pannexins (Panxs), was identified as the mammalian homolog to innexins, the invertebrate gap junction proteins. Three members of the Panx family have been identified in vertebrates: Panx1, Panx2, and Panx3, and under specific conditions these new proteins have been shown to form functional hemichannels and intercellular channels (Scemes et al., 2007).

In the developing central nervous system (CNS) of rodents, many Cxs including Cx26, Cx36, Cx37, Cx43 and Cx45 were found to be highly expressed in the embryonic cerebral cortex with distinct temporal and spatial patterns (Cina et al., 2007; Nadarajah et al., 1997). Together with each Cx channel's distinct permeability and regulation properties, Cxs have been suggested to play an important role in the proper formation of central nervous system (Elias and Kriegstein, 2008; Harris, 2007). Specifically, several studies have demonstrated a role of Cxs in neuronal differentiation (Bani-Yaghoub et al., 1999a; Santiago et al., 2010; Bani-Yaghoub et al., 1999b; Belliveau et al., 2006). Using neurospheres derived from Cx43-null mice and subsequently transfected with Cx43 mutant constructs, a recent study by Santiago *et al.* showed that the carboxyl-terminal of Cx43 is crucial in preventing premature neuronal differentiation (Santiago et al., 2010). In addition, when Cx43 channels were blocked with 18  $\alpha$ -glycyrrhetinic acid or carbenoxolone treatment, a great reduction in the number of mature neuronal differentiation (Bani-Y19 cells following retinoic acid-induced neuronal differentiation (Bani-Y19) cells following retinoic acid-in

Yaghoub et al., 1999a; Bani-Yaghoub et al., 1999b). Belliveau *et al.* further demonstrated that Cx32 and Cx43 overexpression resulted in limited autodifferentiation and enhanced neurite outgrowth in PC12 (pheochromocytoma) cells following nerve growth factor (NGF)-induced differentiation (Belliveau et al., 2006). However, different from the conventional cell-cell communication facilitated by Cx gap junctions, the enhanced neurite outgrowth was found to be a consequence of functional Cx hemichannel formation; the opening of hemichannels allows the release of ATP into the extracellular space, which then activates purinergic receptors to elicit enhanced neurite outgrowth (Belliveau et al., 2006).

A functional aspect of Panxs in CNS development, however, has not yet been discovered. Several studies have identified location-specific Panx expression in rodents: Panx1 is ubiquitously expressed, Panx2 is particularly abundant in the brain, and Panx3 is present in the skin (Bruzzone et al., 2003; Ray et al., 2005; Vogt et al., 2005; Weickert et al., 2005). Moreover, both Panx1 and Panx2 are abundantly expressed in the CNS, especially in neurons of different brain structures including cortex, cerebellum, hippocampus, and olfactory bulb (Bruzzone et al., 2003; Ray et al., 2005; Vogt et al., 2005; Zappala et al., 2006; Zappala et al., 2007; Zoidl et al., 2007). By contrast, Panx3 expression was also detected in the brain but to a lesser extent (Penuela et al., 2007). Interestingly, Panx1 and Panx2 are inversely expressed, and this expression resembles that of Cx43 and Cx32 during rodent neocortical development (Dermietzel et al., 1989; Nedergaard et al., 2003); Panx1 transcript was detected highest in embryonic stages and steadily decreases as the animal reaches adulthood, whereas Panx2 was low in prenatal brain but increased dramatically in postnatal developmental stages starting from P7 (Ray et al., 2005; Vogt et al., 2005). In addition, Panx1 was identified at postsynaptic regions in rodent hippocampal and cortical synapses, suggesting it functions as a hemichannel due to its asymmetric synaptic distribution in the neurons (Zoidl et al., 2007). Similar to Cx32 and Cx43 hemichannels which facilitate enhanced neurite outgrowth in PC12 cells via ATP release

(Belliveau et al., 2006), Panx1 hemichannels have also been shown to be an ATP conduit (Bao et al., 2004; Locovei et al., 2007). Altogether, these findings advocate a functional role of Panxs in neuronal differentiation.

In this study, we explored the potential function of Panxs in neuronal differentiation using PC12 cells. PC12 cells are neural-crest derived cells that can be induced by NGF to differentiate into a neuronal phenotype, and therefore serve as an excellent model to study neuronal differentiation (Greene and Tischler, 1976). Here, we characterized PC12 cells and showed no detectable endogenous Panx protein expression, thus providing an ideal model system to examine the effect of Panxs on neuronal differentiation. Using both transfection and lentiviral infection methods, Panx1 and Panx3 were detected at the perinuclear and plasma membrane regions, whereas Panx2 was only found in a vesicle-like pattern within the cytosol. Remarkably, Panx1 and Panx3, but not Panx2, induced a flattened morphology, as well as increased number of neurites prior to NGF induction. Following NGF treatment, both Panx1 and Panx3 induced a significant increase in the number of primary neurites and neurite branches, as well as neurite elongation. Notably, Panx1 and Panx3 expression was localized to the plasma membrane and appeared to aggregate at the protruding ends of the neurites. Efforts are currently underway to examine whether the enhanced neurite outgrowth is Panx-channel-dependent, and to determine its underlying mechanism(s). To pursue this investigation, stable Panx1 and Panx3 expressing PC12 cells were generated for long-term functional studies. Furthermore, Panx1 and Panx3 mutants with C-terminus truncation and mutated C-terminal predicted motif sites were created in retrovectors, and retrovirus has been produced for efficient expression of Panxs in upcoming This is the first study demonstrating a functional role of Panx1 and Panx3 during studies. neuronal differentiation, and ongoing studies will explore novel aspects of signaling mechanism(s) by which Panxs mediate their functions.

#### **4.2 MATERIAL AND METHODS**

4.2.1 Cell Culture. PC12 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in PC12 medium (Dulbecco's modified minimal essential medium (DMEM) (Invitrogen Corp., Burlington, Ontario, Canada) containing 10% heat-inactivated horse serum (Invitrogen Corp.) and 5% fetal bovine serum (FBS) (Thermo Scientific Hyclone, Waltham, MA) in a humidified, 37°C incubator containing 5% CO<sub>2</sub>. For all characterization and differentiation studies, plates and coverslips were coated with 10 µg/mL Type I collagen (Millipore, Billerica, MA). For Panx characterization studies, PC12 cells were seeded at  $2 \times 10^6$ cells/60-mm dish and 2 x  $10^4$  cells/35-mm dish for total lysate collection and immunocytochemistry, respectively. In studies involving PC12 differentiation, cells were treated with or without NGF (50 ng/ml) in DMEM containing N-2 supplement (Invitrogen Corp.); treatment media was replaced every 48 h. C6 glioma cells were cultured as previously described (Lai et al., 2007). A 293 GPG retroviral packaging cell line was maintained in 293 medium (DMEM (Invitrogen Corp.) containing 10% FBS, 10 units/mL penicillin, 10 µg/mL streptomycin, 0.3 µg/mL G418, 2 µg/mL puromycin, and 1 µg/mL tetracycline in a 37°C humidified incubator containing 5% CO<sub>2</sub>).

**4.2.2 Total RNA Extraction and Reverse-transcription PCR.** Total RNA extraction and RT-PCR of Panx transcripts were performed as previously described (Lai et al., 2007). For undifferentiated and differentiated PC12 cells, cells plated for 24 h were treated with or without NGF in N-2-supplemented DMEM for 48 h followed by sample collection.

**4.2.3 Plasmid Construction and Transfection.** pRK 5 expression vectors encoding *Rattus norvegicus Panx1, Panx2, and Panx3* were prepared as previously described (Bruzzone et al., 2003). A day prior to transfection, PC12 cells were plated at a density of 1.25 x 10<sup>6</sup> cells/35-mm

dish in PC12 medium and subsequently transfected with plasmids of interest (mock, Panx1, Panx2, or Panx3) using LipofectAMINE 2000 (Invitrogen Corp.) as described previously (Lai et al., 2007).

4.2.4 Protein Isolation and Western Blot Analysis. Cells grown to confluence were lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (complete, Mini, Roche Diagnostics Corp., Indianapolis, IN) and Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich). DNA was sheared using pulse sonication for 10 s in ice. For consistent loading between samples, protein concentration was determined using a BCA Protein Assay Kit (Pierce-BioLynx, Brockville, Ontario, Canada). Samples were boiled for 2 min in SDS sample buffer and 30 µg of total protein along with molecular weight standard (Precision Plus All Blue Standards, Bio-Rad Laboratories, Hercules, CA) were subjected to 10% SDS-PAGE followed by protein band transfer onto nitrocellulose membranes. To minimize non-specific antibody binding, the membranes were blocked with 5% nonfat milk in TBS containing 1% Tween 20 prior to overnight incubation at 4°C with anti-Panx1 (rabbit; 1:5,000 dilution), anti-Panx3 (rabbit: 1:5,000 dilution), and anti-Cx43 (rabbit: 1:8,000 dilution; Sigma-Aldrich); anti-Panx1 and anti-Panx3 antibodies were a generous gift from Dr. Dale Laird (University of Western Ontario, Canada). The membranes were next incubated in secondary antibody (goat anti-rabbit IgG-horseradish peroxidase; 1:5000 dilution; Cedarlane Laboratories Ltd., Ontario, Canada) for 1 h, immersed in Supersignal West Femto Substrate (Pierce-BioLynx), and exposed to X-ray films for detection of antibody-bound proteins. GAPDH (mouse; 1:10,000 dilution; Cedarlane Laboratories Ltd.) was immunoprobed as a loading control.

**4.2.5 Immunocytochemistry.** Samples were fixed in 10% buffered formalin phosphate at room temperature and permeabilized with 0.3% Triton X-100 for immunolabeling with anti-Panx1 and

anti-Panx3 (rabbit, 1:500 dilution) for 1 h. Alexa Fluor 488-conjugated secondary antibodies (goat anti-rabbit; 1:500 dilution; Molecular Probes, Eugene, OR) were then applied for 1 h, and samples were mounted in ProLong Gold Antifade with 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes) onto glass slides for examination under an epifluorescence microscope (Axioplan 2, Carl Zeiss, Ontario, Canada). F-actin was stained using Alexa Fluor 568-conjugated phalloidin (Molecular Probes).

**4.2.6 Lentivector Construction and Generation of Lentivirus.** Lentivector encoding either *Rattus norvegicus Panx1* or *Panx3* was constructed using Gateway<sup>®</sup> recombination cloning system (Invitrogen Corp.). Briefly, cDNA encoding *Rattus norvegicus Panx1* or *Panx3* were amplified with custom designed primers containing restriction enzyme sites followed by respective restriction enzyme digestion for ligation into Gateway<sup>®</sup> pENTR 2B entry vector (Invitrogen Corp.). Genes of interest were then recombined from the pENTR 2B vectors into pLenti6/UbC/V5-DEST (destination) vectors (Invitrogen Corp.) for subsequent lentiviral production. Orientation and sequence of all constructs were validated by bidirectional sequencing. In keeping with biosafety regulations, lentivirus was prepared via services provided by Viral Vector Facility (Brain Research Centre, the University of British Columbia). The use of lentivirus was later discontinued following the closure of Viral Vector Facility.

**4.2.7 Lentiviral Infection and Neurite Outgrowth Analysis of PC12 cells.** Cells were seeded onto Type I collagen-coated coverslips a day prior to infection at a density of  $1.5 \times 10^3$  cells/35-mm dish, and exposed to EGFP, Panx1, or Panx3 lentiviral-containing PC12 medium (time 0) for 48 h. The infected cells were treated with or without NGF for 96 h and samples were collected at 48, 96, and 144 h post-infection for immunocytochemistry and Western blot analyses. For neurite outgrowth analysis, samples were fixed in 10% buffered formalin

phosphate at room temperature for 10 min and immersed in phosphate-buffered saline (PBS) for image acquisition of the neurites under Axioplan 2 (Carl Zeiss); this method best prevented the loss of neurites from sample manipulation. Neurites were traced and measured from > 25 randomly captured fields using Axiovision software (Carl Zeiss, Ontario, Canada). The length and number of primary neurites per cell (extensions > 10  $\mu$ m directly from the cell body) and neurite branches per cell (second and higher order extensions > 5  $\mu$ m in length) (Sin et al., 2009) were determined for quantification. Statistical comparison was done by using one-way ANOVA analysis followed by Tukey-Kramer's Multiple Comparison test with *P* < 0.05 considered significant. Results are expressed as mean <u>+</u> SEM. Each experiment was repeated three or more times with similar results.

**4.2.8 Generation of Stable Panx-infected PC12 Cells.** Cells were plated at a density of  $1 \times 10^4$  cells/35 mm a day prior to transduction and infected with mock, Panx1, or Panx3 for a week with fresh lentivirus-containing PC12 medium replaced every 3 days. Samples were collected from 5<sup>th</sup> passage post-infection for verification of exogenous Panx expression by Western blot analysis and immunocytochemistry.

#### 4.3 RESULTS

**4.3.1** Panx1, Panx2, and Panx3 Proteins are Not Endogenously Expressed in PC12 Cells. To examine endogenous Panx expression in PC12 cells, RT-PCR analysis of Panx transcripts was first carried out and showed the presence of Panx1 and Panx2, but not Panx3, mRNAs in both undifferentiated and differentiated PC12 cells (Figure 4.1A). Given that Panx1 and Panx2 are temporally regulated during rat brain development (Vogt et al., 2005), it was expected that Panx expression would change following NGF-induced differentiation in PC12 cells. Yet, no apparent change in the occurrence of Panx1 and Panx2 transcripts was observed between undifferentiated

and differentiated PC12 cells. To verify Panx translational expression, Western blot analysis was done on PC12 total lysates (Figure 4.1B, C). Interestingly, while Panx3 protein expression was not present in accordance with its lack of mRNAs, Panx1 expression was also not detected in both undifferentiated and differentiated PC12 cells.

**4.3.2 Panx1 and Panx3**, **but Not Panx2**, **Enhance Neurite Outgrowth.** In our pilot studies, we set out to investigate the potential involvement of Panxs during neurite outgrowth. To do this, PC12 cells were transiently transfected with Panxs and treated with NGF to induce neuronal differentiation (Figure 4.2). Strikingly, both Panx1- and Panx3-, but not Panx2-expressing cells, exhibited a drastic increase in neurite outgrowth when compared to the mock transfectants under NGF treatment (Figure 4.2). In addition, Panx1 and Panx3 were predominantly observed at the perinuclear and plasma membrane regions of the cell, whereas Panx2 was mostly found in the cytosol in a vesicle-like pattern.

**4.3.3 Panx1 and Panx3 Do Not Affect Expression of the Other Panx nor Cx43.** Given that both Panx1 and Panx3 enhance neurite outgrowth, we questioned whether either Panx could affect the expression of another gap junction protein including Panxs and Cx43, which has been reported to increase neurite outgrowth in PC12 cells (Belliveau et al., 2006). To examine this possibility, Panx lentivirus was created to achieve high infection efficiency and minimize the cell death that often results from transfection methods. As shown in Figure 4.3, an appreciable amount of Panx1 and Panx3 expression was achieved following lentiviral infection. Intriguingly, Panx3 expression appeared to reach its prime expression at 96 h post-infection, which is slower than that of Panx1 (48 h post-infection) (Figure 4.3A, B). Furthermore, infection of Panx1 or Panx3 did not result in a change of expression in the other Panx (Figure 4.3A, B), as well as Cx43 (Figure 4.3C) in differentiated PC12 cells.

**Figure 4.1. PC12 cells do not significantly express Panx1 and Panx3 proteins.** (A) RT-PCR analysis of PC12 shows Panx1 (Px1; 185 bp) and Panx2 (Px2; 258 bp), but not Panx3 (Px3; 336 bp), transcripts were expressed in both undifferentiated and differentiated PC12 cells. GAPDH product bands were used as a positive control for the reverse transcription. No product was detected when the reverse transcriptase (+) was replaced with ultrapure MilliQ water (-), ruling out contamination by genomic DNA. (B, C) Western blot analysis demonstrating that Panx1 (B; 48 kDa) and Panx3 (C; 45 kDa) protein expression was not detected in undifferentiated and differentiated PC12 cells incubated in N-2 supplemented serum free medium with or without NGF for 0 to 96 h. Panx1-EGFP (75 kDa) from stable Panx1-EGFP-transfected C6 glioma cells was immunoprobed as a positive control for the anti-Panx1 antibody; stable Panx2-EGFP C6 cells was used as a negative control. The two Panx-expressing C6 cells also served as a negative control for the anti-Panx3 antibody. Protein bands detected above the predicted Panx3 size represent nonspecific binding of the anti-Panx3 antibodies. GAPDH was used as a loading control.







**Figure 4.2. Exogenous expression of Panx1 and Panx3, but not Panx2, via transfection increase neurite outgrowth in PC12 cells following NGF treatment.** PC12 cells were transfected with Panx1, Panx2, or Panx3, and treated with NGF for 48 h in N-2 supplemented serum free medium. Enhanced neurite outgrowth was evident in both Panx1- and Panx3-, but not Panx2-, expressing PC12 cells when compared to mock-transfected samples under both differential interference contrast (DIC) and immunofluorescence images. Panxs were immunolabelled with respective Panx-specific antibodies and Alex Fluor 488conjugated secondary antibodies, and F-actin was stained using Alexa Fluor 568 phalloidin. Nuclei were revealed by DAPI staining. *Bar*, 10 μm.



**4.3.4 Panx1 and Panx3 Induce Cellular Morphological Change in PC12 Cells.** While Panx1 and Panx3 appeared to enhance neurite outgrowth in differentiated PC12 cells following NGF treatment, we were also interested in its effect on undifferentiated cells. Remarkably, we observed a dramatic change in morphology from the typical, small and round form of undifferentiated PC12 to a flattened shape (Figure 4.4). Similar morphological alteration was also consistently observed for the cell bodies of differentiated PC12 cells in addition to the enhanced neurite outgrowth effect following Panx expression (Figure 4.5). Moreover, Panx1 and Panx3 location did not appear to be affected by the NGF treatment, and were mostly detected at the plasma membrane and perinuclear regions (Figure 4.2, 4.4, 4.5). Noticeably, both Panxs showed limited signal overlap with F-actin, especially at protruding ends of the neurites (Figure 4.4, 4.5)

**4.3.5 Panx1 and Panx3 Increase Neurite Numbers and Enhance Elongation.** To further investigate the effect of Panx expression on neurite differentiation, neurite number and length were measured and quantified. Upon statistical analysis, Panx1 and Panx3 showed a significant increase in number of neurites (prior to NGF treatment) when compared to the control cells (EGFP) (Figure 4.6A). In addition, both Panxs significantly increased the number of primary neurites (Figure 4.6A; up to 48 h NGF treatment) and neurite branches (throughout NGF treatment) (Figure 4.6B). No significant difference was found between Panx1 and Panx3 in its enhancing effect on the number of neurites throughout the course of the experiment. Overall, both Panxs greatly increased the total number of neurites in PC12 cells during differentiation (Figure 4.6C).

As expected from the earlier observation using the transfection method (Figure 4.2), both Panxs also significantly enhanced elongation of primary neurites (Figure 4.7A) and neurite branches (Figure 4.7B) throughout the NGF treatment. The drop in the average length of neurite Figure 4.3. Lentiviral infection of Panx1 does not affect Panx3 nor Cx43 expression, and vice-versa. (A) Panx1 was readily detected at 48 h post-infection. Expression of Panx1 was most prominent at 96 h and followed by a slightly reduced signal at 144 h. Panx3-infected cells showed no detectable Panx1 expression. Stable Panx1- and Panx3-transfected C6 cells were used as positive and negative controls for the anti-Panx1 antibody, respectively. (B) A weak signal for Panx3 was found at 48 h followed by strong signals at 96 and 144 h post-infection. Stable Panx3- and Panx1-transfected C6 cells were used as positive and negative C6 cells were used as positive and negative (C) No Cx43 band was detected in both Panx1 and Panx3-infected PC12 cells. As a positive control, endogenous Cx43 (phosphorylated (P-Cx43) and non-phosphorylated (Cx43)) was immunoprobed in stable Panx1- and Panx3-transfected C6 cells. GAPDH was used as a loading control.



**Figure 4.4. Panx1 and Panx3 expression induces increased neurite numbers and flattened morphology in PC12 cells without NGF treatment.** PC12 cells were infected with EGFP, Panx1, or Panx3, and incubated in N-2 supplemented serum free medium for 96 hr. Panxs were immunolabelled with respective Panx-specific antibodies and Alex Fluor 488-conjugated secondary antibodies, and F-actin was stained using Alexa Fluor 568 phalloidin. Typical diffused cytoplasmic EGFP signal was detected in EGFP-infected cells. By contrast, Panx signals were detected predominantly at the plasma membrane and perinuclear regions of the cells. Both Panx1- and Panx3- expressing cells exhibited neurite processes and flattened cell bodies, whereas EGFP-infected cells presented no apparent neurites and change in shape from the typical small and round morphology of PC12 cells. Panx1 and Panx3 also showed limited signal overlaps with F-actin at the protruding end of the processes. Nuclei were revealed by DAPI staining. *Bar*, 50 µm.



**Figure 4.5. Panx1 and Panx3 expression induces enhanced neurite outgrowth in PC12 cells following NGF treatment.** PC12 cells were infected with Panx1 or Panx3 lentivirus and treated with NGF in N-2 supplemented serum free medium for 96 hr. Panxs were immunolabelled with their respective Pans-specific antibodies and Alex Fluor 488-conjugated secondary antibodies, and F-actin was stained using Alexa Fluor 568 phalloidin. Typical cytoplasmic EGFP expression was found in EGFP-infected cells, whereas Panx signals were predominantly detected at the plasma membrane and perinuclear regions of Panx-expressing cells. A drastic increase in neurite outgrowth was evident in both Panx1- and Panx3-expressing cells when compared to the control (EGFP). Panx1 and Panx3 signals also appear to overlap with F-actin, especially at protruding end of the neurite processes. Nuclei were revealed by DAPI staining. *Bar*, 50 μm.



**Figure 4.6. Panx1 and Panx3 increase number of neurites.** (A) A significant increase in the number of primary neurites was observed prior to NGF treatment (0 hr) in Panx1- and Panx3-infected PC12s. The elevated number of primary neurites was observed 48 h following NGF treatment. An increased number of neurite branches (B) and total neurites (C) were also observed throughout the experiment. (\*, P < 0.001, compared to EGFP.)



Figure 4.7. Panx1 and Panx3 enhance neurite elongation. A significant increase in the average length of primary neurites (A), branching neurites (B), and total neurite length (C) was observed in Panx1- and Panx3-expressing PC12 cells throughout the NGF treatment. (\*, P < 0.001, compared to EGFP.)



branches of Panx3-infected cells at 72 h NGF treatment was not statistically significant and was not consistently observed between experimental trials (Figure 4.7B). Altogether, the enhanced elongation effect of Panx1 and Panx3 was similar and observed in all neurites (primary neurites and neurite branches) (Figure 4.7C).

## **4.4 DISCUSSION**

While Panx1 and Panx2, but not Panx3, transcripts were detected by RT-PCR analysis, Western blot analysis revealed no detectable Panx protein expression. Although *Panx1* and *Panx2* are transcribed, they may not be actively translated at detectable levels in PC12 cells. Panx2 could not be examined due to the lack of specific antibodies at the time of this experiment. Since Panx-specific antibodies recently became available, each Panx antibody used in this study has been tested with its respective stable Panx-expressing C6 glioma cells for specificity (Figure 4.3) (Lai et al., 2007; Lai et al., 2009). Together with the fact that PC12 cells can be induced by NGF to differentiate into neuronal phenotype (Greene and Tischler, 1976), they served as a good cell model in this study to investigate a functional role of Panxs in neuronal differentiation. In addition, Panx transcripts and protein expression remained unchanged by NGF treatment, suggesting that Panxs are not downstream targets of NGF.

Introduction of Panx cDNAs into PC12 cells using both transfection and lentiviral transduction methods resulted in successful Panx protein expression. Panx1 and Panx3 were appropriately trafficked to the plasma membrane while some Panx was also observed at the perinuclear region. The localization pattern of Panxs resembles that of other cell types, including C6 gliomas (Figure 4.8; Panx3-transfected C6 cells) (Lai et al., 2007; Lai et al., 2009), normal rat kidney (NRK), rat breast tumor cells (BICR-M1R<sub>k</sub>), and human embryonic kidney cells (293T) (Penuela et al., 2007; Penuela et al., 2009). By contrast, Panx2 was predominantly detected within the cytosol in a vesicle-like pattern but not at the plasma membrane. A similar Panx2

expression pattern was also detected in C6 gliomas, U251, and T98G human gliomas, and 293T cells (Lai et al., 2009; Penuela et al., 2009), suggesting that this expression was not a result of aberrant trafficking from exogenous expression as previously found for Cxs (Das et al., 2005; Skerrett et al., 2004; Thomas et al., 2005). Panx1 and Panx3 did not influence the expression of one another without (data not shown) or with NGF treatment, indicating that expression of these Panxs are not associated with each other during the differentiation process of PC12 cells. Interestingly, Panx1 reached prime expression sooner than Panx3 following infection, suggesting a difference in protein synthesis and trafficking between the two proteins. A small difference in the protein product size of Panx1 between C6 and PC12 cells was also observed. As Panxs have been shown to be glycoproteins (Penuela et al., 2007), we speculate that the difference is a result of variation in Panx1 glycosylation status between different cell types.

A combination of Panx1 and Panx2 has been found to form functional hemichannels in *Xenopus* oocytes (Bruzzone et al., 2003). In accordance, a recent study by Penuela *et al.* demonstrated that Panx1, but not Panx3, facilitates cellular surface localization of Panx2 from its intracellular location, albeit not very pronounced (Penuela et al., 2009). The study also showed that Panx interactions are dependent on their glycosylation status, where Panx2 only interacts with the lower glycosylated form of Panx1 in 293T cells (Penuela et al., 2009). However, co-expression of Panx1 and Panx2 in PC12 cells did not result in an apparent recruitment of Panx2 to the plasma membrane (Figure 4.9). We speculate the difference in findings is cell type-dependent, possibly via a variation in the glycosylation status of Panxs. Conversely, co-expression of Panx1 and Panx3, or Panx2 and Panx3 did not result in a robust Panx-Panx interaction and formation of functional channels (Bruzzone et al., 2003; Penuela et al., 2009). Given that Panx2 and Panx3 are unlikely co-expressed in the same cell type due to their respective locations of expression (Penuela et al., 2007; Vogt et al., 2005), this aspect was not pursued in this investigation.

**Figure 4.8. Exogenous Panx3 expression in C6 glioma cells.** C6 cells were transiently infected with Panx3 lentivirus and immunolabeled for Panx3 and F-actin. Panx3 was detected predominantly at the plasma membrane and perinuclear regions. *Bar*, 50 µm.




Native and control-infected PC12 cells are typically round-shaped and small in size. Following Panx1 and Panx3 expression, a striking change in cellular morphology was observed in which the infected cells became considerably flatter and with more protruding ends. The flattening effect resembles that of C6 glioma cells where typically spindle-shaped C6 cells transformed into a flattened morphology with exogenous Panx1 expression (Lai et al., 2007).

This finding suggests that Panx1's morphological alteration effect is not cell type-specific and may be universal. We were surprised to observe a change in the cell shape of Panx3expressing cells. Endogenous Panx3 has been reported to be predominantly expressed in the skin, especially within the epidermis (Bruzzone et al., 2003; Penuela et al., 2007). Epidermis is composed of stratified squamous epithelial cells, which have the appearance of thin and flat plates. Although speculative, we question if Panx3 plays a role in the shape of squamous epithelial cells, and whether the flattened morphology elicited by Panx3 in PC12 cells shares a similar mechanism. A signal overlap was also found between the Panxs and F-actin, indicating a possible interaction of Panxs with F-actin to elicit this function. Panx2, on the other hand, did not induce an apparent change in cell shape of PC12 cells. Overall, these findings demonstrate that Panx1 and Panx3 induce morphological change in native PC12 cells.

Panx1 and Panx3-infected PC12 cells showed a significant increase in neurite numbers when compared to the control prior to NGF treatment. Increasing evidence confirmed that Panx1 and Panx3 function as hemichannels (Locovei et al., 2006a; Pelegrin and Surprenant, 2006; Pelegrin and Surprenant, 2007; Penuela et al., 2007; Penuela et al., 2009). Given the large pore formation and ATP permeability of Panxs, it was further suggested that Panxs are more suitable than Cxs as the molecular substrates of hemichannels (Scemes et al., 2007). In fact, ATP treatment alone on PC12 was shown sufficient to induce limited increase in neurite numbers, suggesting that activation of purinergic receptors is involved in enhancing neurite formation in PC12 cells (Belliveau et al., 2006; Behrsing and Vulliet, 2004). Using [ATP]-sensitive luciferin/

**Figure 4.9. Panx1 does not readily recruit Panx2 to the plasma membrane in PC12 cells.** Co-expression of tagged Panx1 and Panx2 demonstrate that Panx1 and Panx2 predominantly localized at the plasma membrane and perinuclear regions, respectively. *Bar*, 10 μm.



luciferase assay and purinergic receptor antagonists, including suramin and pyridoxal phosphate-6-azo (benzene-2,4-disulfonic acid) (PPADS), Belliveau *et al.* further demonstrated that exogenous Cx32 and Cx43 expressions formed hemichannels to facilitate ATP release into the extracellular space, which then activated purinergic receptors to enhance neurite outgrowth in PC12 cells (Belliveau et al., 2006). Coincidentally, Panx1 hemichannels have been shown as an ATP conduit following ATP-induced purinergic receptor activation (Locovei et al., 2006b; Locovei et al., 2007). Therefore, the increase in neurite numbers in the absence of NGF may be a result of purinergic activation facilitated by Panx1- and Panx3-mediated ATP release, and requires further investigation.

Following NGF treatment, Panx1- and Panx3-infected cells exhibited a significant increase in the number of primary neurites and neurite branches. Intriguingly, the increase in the number of primary neurites ceased by 72 h post-treatment (96 hr post-infection) and remained largely unchanged thereafter. Given that an appreciable amount of Panx expression was observed by Western blot analysis at 96 h post-treatment, the halted trend was unlikely a result of diminishing Panx expression. Instead, results from quantified data analysis suggested that Panxs potentiated neuronal differentiation by giving a "head start" in primary neurite formation from 0 to 48 h post-treatment, and as the maximum number of primary neurites was saturated at 48 h. the EGFP-infected cells finally caught up at 72 h post-treatment. A similar trend was also observed in the number of neurite branches. Yet, the number of neurite branches of the Panxexpressing cells was significantly greater while that of the EGFP-infected cells remained lower throughout the course of the experiment. Although the number of neurite branches appeared to fluctuate between Panx-infected cells over time, no distinct difference was observed between experimental trials. Overall, the variation in Panx-induced effect between primary and branching neurites imply that different pathway(s) were influenced by Panx expression.

Neurite elongation is another important process during neuronal differentiation and was examined in this study. Remarkably, Panx1- and Panx3-infected PC12 cells demonstrated a significant increase in the average length of both primary neurites and neurite branches throughout the course of the experiment. The enhanced neurite outgrowth was not a result of Panx intercellular channel function because cells were sparsely seeded as individual cells without cell-cell contacts, for accurate measurement of neurites. In addition, this especially holds true for cells treated with NGF since PC12 cells ceased to divide and differentiate. Therefore, the finding further supports the notion that Panxs induced their effects via hemichannels and/or protein-protein interactions. In summary, our findings indicate that Panx1 and Panx3 play a role in potentiating neuronal differentiation by enhancing neurite elongation and arborization.

While Panx expression induced a limited increase in neurite numbers and elongation without NGF, both traits were greatly enhanced following NGF application, indicating the necessity of NGF initiation, as well as a possible crosstalk of Panx- and NGF-mediated signaling pathways. NGF induces differentiation by primarily binding to and activating TrkA, a receptor tyrosine kinase, on the plasma membrane of PC12 cells (Meakin et al., 1992). This subsequently activates downstream effectors of TrkA, including mitogen-activated protein kinase (MAPK, ERK1/2), which is required for neurite outgrowth and maintenance (Sasagawa et al., 2005; Marshall, 1995; Xiao et al., 2006; Yasui et al., 2001; Qui and Green, 1992; York et al., 2000). The duration of MAPK signaling is also critical to NGF-mediated PC12 differentiation. Whereas epidermal growth factor (EGF)-stimulated Ras- and Rap1-dependent MAPK phosphorylation is rapid and transient (15 min), NGF-induced MAPK activation is rapid and sustained (> 1 hr) (Qui and Green, 1992; York et al., 2000). The sustained MAPK activation is Rap1-dependent and possibly a result of differential recruitment of PI3K to activated TrkA, but not stimulated EGFR complex (Qui and Green, 1992; Kao et al., 2001; York et al., 2000). Importantly, an elevation in MAPK activity was also demonstrated responsible for ATP-enhanced neurite outgrowth in NGF-

differentiated PC12 cells via activation of P2YR, but not P2XR;  $\alpha$ ,  $\beta$ -methylene ATP, a P2XRselective agonist, had no effect on enhancing NGF-initiated neuritogenesis (Behrsing and Vulliet, 2004; Arthur et al., 2005; Behrsing and Vulliet, 1999). Corresponding with the sustained MAPK activation by NGF (Kao et al., 2001), Behrsing and Vulliet further demonstrated that P2YR-mediated MAPK activation lasted up to 2 hr (Behrsing and Vulliet, 2004), suggesting that activated P2YR functions synergistically with TrkA by prolonging MAPK activity.

Notably, a recent study by Yamboliev and colleagues showed that ATP is spontaneously secreted by NGF-differentiated PC12 cells (Yamboliev et al., 2009). Given that Panx1, and perhaps Panx3, hemichannels are opened by ATP-induced activation of purinergic receptors, likely via P2YR-induced elevation in  $[Ca^{2+}]_i$  (Locovei et al., 2006a; Locovei et al., 2006b), Panxs may augment P2YR-enhanced neurite outgrowth in a positive feedback mechanism where 1) NGF induces ATP secretion, which then 2) activates P2YR in an autocrine and/or paracrine fashion, 3) resulting in increased  $[Ca^{2+}]_{i}$ , and 4) consequent opening of Panx hemichannels to release ATP. In addition, the release of ATP via Panx hemichannels may be more direct and efficient than spontaneous ATP secretion (Yamboliev et al., 2009), further suggesting that Panxs play an important role in this proposed pathway (Figure 4.10). Interestingly, slightly higher [ATP]<sub>o</sub> than that required for P2X<sub>7</sub> and P2Y<sub>2</sub> activation was demonstrated to inhibit Panx1 channel activity in erythrocytes (Qiu and Dahl, 2009), suggesting that ATP may also function in a negative feedback loop to regulate Panx1 channel activity. This negative regulatory mechanism could be critical as a "safety mechanism" since prolonged opening of the Panx1 hemichannel has been reported to induce apoptotic cell death (Locovei et al., 2007).

In future experiments, we will examine the effect of altering Panx expression on neurite outgrowth in primary neural progenitor cells and neurons by Panx-specific siRNA and/or transfection. To explore the mechanisms by which Panxs elicit their functions. Gap junction proteins are known to function via i) their functional channels and ii) interaction with other



Figure 4.10. Schematic diagram depicting proposed mechanism of Panx-enhanced neurite outgrowth in PC12 cells. 1) NGF activates TrkA receptor to initiate neuronal differentiation by increasing MAPK activity. 2) NGF-activated TrkA triggers spontaneous secretion of ATP into extraceulluar space, and thereby triggers P2YR. 3) Activated P2YR increases and sustains MAPK activity, enhacing NGF-induced neurite outgrowth. 4) Following P2YR activation,  $[Ca^{2+}]_i$  is released by activating IP<sub>3</sub> receptors at the ER. Elevation in  $[Ca^{2+}]_i$  subsequently triggers the opening of Panx hemichannels, resulting in ATP release into extracellular space. 5) Increased [ATP]<sub>o</sub> activates additional P2YR in an autocrine and/or paracrine fashion and consequently increases and maintains MAPK activity. 6) When  $[ATP]_o$  reaches concentration higher than those required for P2YR activation, the Panx hemichannel opening is inhibited to prevent further ATP release.

proteins (Naus et al., 2005). With the generation of stable Panx-expressing PC12 cells (Figure 4.11), we can now examine hemichannel activity using a dye uptake assay and the Panx1-specific hemichannel inhibitor, probenecid acid (Silverman et al., 2009); this experiment was previously confounded by a lack of infection efficiency of Panx-encoded lentivirus at high cell density. If significant Panx hemichannel activity is observed, we will next check for an increase in ATP release followed by treatments with apyrase (an ATP diphosphohydrolase), suramin, or PPADS to examine if any of them abolish the enhanced neurite outgrowth.

Little is known about Panx-interacting proteins other than Panx1's interaction with  $P2X_7R$ , and  $Kv\beta3$ , a potassium channel subunit recently shown to attenuate Panx1's sensitivity to changes in redox potential; Panx1 hemichannel current is reported to be reduced under treatment of reducing agent (Locovei et al., 2007; Bunse et al., 2009; Pelegrin and Surprenant, 2006). While numerous proteins are involved in the signaling pathways of PC12 cell differentiation (Vaudry et al., 2002) and hence their potential synergistic action with the Panxs to enhance neurite growth, deducing these candidates requires substantial time and resources for high throughput screening and the subsequent validation. Instead, two top-down approaches were taken here to investigate Panx mechanisms: 1) Panx C-terminal truncation; 2) point mutation of the predicted motif sites within the C-terminal. The C-termini of Cxs have been shown to contain important sites for Cx post-translational modification and regulation of channel properties (Lampe and Lau, 2004), as well as interaction with other proteins (Fu et al., 2004; Kojima et al., 2002; Maass et al., 2007; Xu et al., 2002). Given that Panxs and Cxs share similar predicted topology, we hypothesized that the C-terminus is crucial to Panx functions. Using MotifScan against Panx1 and Panx3 C-termini (Obenauer et al., 2003), we found predicted SH2 binding domains in both Panxs and an Akt kinase binding domain in Panx3 with high scores (Table 4.1). SH2 and Akt kinase domains bind to phosphorylated tyrosine and serine/threonine

**Figure 4.11. Expression of stable Panx1- and Panx3-infected PC12 cells.** (A) Western blot analysis demonstrating stable expression of Panx1 and Panx3. No Panx1 and Panx3 bands were detected in parental and mock-infected cells. (B) Immunocytochemistry of stable infected PC12 cells showing high efficiency and heterogeneity in Panx1 and Panx3 expression under normal passage condition. *Bar*, 50 μm.



Domain Name	Sequence (amino acid position)	Score
Panx1-SH2	(301 – KTDVLKV <u>¥</u> EILPTFD – 315)	<0.4
Panx3-SH2	(298 – DKRLLSI <u>Y</u> EMLPAFD – 312)	<0.45
Panx3-Akt kinase	(296 – RWDKRLL <u>S</u> IYEMLPA – 310)	0.5762

## Table 4.1. Predicted Panx C-terminus domains using MotifScan.

residues, respectively, and are involved in a wide variety of cellular responses. Wildtype and mutant retroviral constructs of Panx1 (Panx1, Panx1- $\Delta$ K302stop (C-terminus truncation), Panx1-Y308L (SH2) and Panx3 (Panx3, Panx3- $\Delta$ R296stop (C-terminus truncation), Panx3-Y305L (SH2), Panx3-S303L (Akt) were therefore created (Appendix B, C). By removing the C-terminus of each of the Panxs, we wish to abolish Panx channel function, if any, and examine its effect on neurite outgrowth in PC12 cells. If the truncation annuls Panxs' effects, it would then suggest Panxs function via 1) hemichannel function and/or 2) interaction with other proteins via the Cterminus. While it is yet early to speculate on the possible implication of these domains in Panx protein-protein interaction and function, observations made from these mutants may unearth new pathways in the mechanistic study of Panxs.

Altogether, these findings strongly advocate a functional role of Panx1 and Panx3 during neuronal differentiation, and efforts are currently underway to investigate their underlying mechanisms.

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Chapter 5

**General Discussion and Conclusions** 

Panxs are a novel family of gap junction proteins and their implication in cancers has not been examined prior to these studies. Comparison between the C6 glioma cell line and its normal counterpart, primary astrocytes, revealed that a loss of Panx expression may be associated with gliomagenesis. However, the inverse correlation between Panxs and glioma grades remains to be examined using Panx-specific antibodies and human glioma tissue samples (*i.e.* glioma tumor microarray). Following exogenous Panx1 expression in C6 cells, it was discovered that Panx1 shows a tumor-suppressive effect on monolayer growth, cell motility, anchorage-independence growth, and *in vivo* tumorigenesis. Unexpectedly, the tumor suppression was not accompanied by a strong increase in gap junctional coupling as shown in previous Cx-overexpression studies in gliomas (Figure 5.1) (Huang et al., 1998; Naus et al., 1992; Zhu et al., 1991; Zhu et al., 1992). This implies that Panx1 did not elicit the tumor-suppressive effects predominately via GJIC. The concept that Panx functions as a hemichannel was not prevalent until recently, and it is possible that that the observed tumor-suppressed phenotype was mediated through functional Panx1 hemichannels, but not intercellular channels (Figure 5.1). This supposition coincides with the discovery of Panx1's uniform distribution at the plasma membrane when compared to Cxs. Whereas Cxs were found as large, dense gap junction plaques at cell-cell contacts, Panx1 was observed outlining the plasma membrane without the plaque-like appearance. Although a functional role of Panx hemichannels in neoplasm has not been shown, it is hypothesized to play a part in tumor-suppression by acting as a direct passage of apoptotic signals and/or chemotherapeutic agents from the extracellular space into the cytosol (Goodenough and Paul, 2003). Given that Panx1 has been shown to function as a hemichannel in vitro in different systems (Bao et al., 2004; Pelegrin and Surprenant, 2006; Huang et al., 2007; Thompson et al., 2008; Reigada et al., 2008; Iglesias et al., 2009; Bruzzone et al., 2003; Locovei et al., 2006a; Penuela et al., 2007), it would be of interest to examine whether or not inhibition of Panx1 hemichannels, if present, perturbs the tumor-suppressive effects. Recent reports have discovered



**Figure 5.1. Schematic diagram of proposed Panx-mediated pathways.** 1) Panx forms gap junctions to facilitate dispersal of anti-mitotic factors. 2) Panx hemichannel allows the efflux and/or influx of secondary messenger molecules, such as ATP, to elicit its function (also see Figure 5.2). 3) Panx directly and/or indirectly regulates the intracellular signaling pathway via unidentified molecules. 4) Panx recruits ZO-1 and/or 5) cadherin- $\beta$ -catenin complex to the plasma membrane, and thereby mediates cytoskeletal reorganization and transcriptional regulation.

Panx1-specific channel inhibitors such as probenecid acid and the Panx1-mimetic peptide, <sup>10</sup>panx1, which would aid in examining this aspect (Silverman et al., 2008; Pelegrin and Surprenant, 2006; Wang et al., 2007).

Panx2, on the other hand, exhibited a unique intracellular localization distinctly different from Panx1 and Panx3. Importantly, later expression studies using untagged Panx2, as well as de *novo* expression in reactive astrocytes (Zappala et al., 2007), also showed a similar localization pattern to that observed in tagged constructs. Although Panx1 has been shown to "rescue" limited subcellular Panx2 expression to the plasma membrane (Bruzzone et al., 2003; Penuela et al., 2009), co-expression of Panx1 and Panx2 in C6 gliomas did not present an obvious alteration of subcellular localization of either protein. Panx2 expression, like Panx1, also significantly suppressed oncogenicity parameters in C6 glioma cells. The finding corroborates with a previous report by Litvin et al. where Panx2 was proposed as a tumor suppressor gene involved in gliomagenesis (Litvin et al., 2006). Panx2-elicited tumor suppression is not entirely identical to that of Panx1. While both Panx1 and Panx2 reduced in vitro monolayer saturation, anchorageindependent growth and *in vitro* tumorigenicity, Panx2 did not impede *in vitro* cell motility as did Panx1. Since Panx2 has an unusually long, proline-rich C-terminal tail (385 a.a.) when compared to that of Cx43 (151 a.a.), it may be advantageous in providing potential sites for intracellular protein-protein interaction to mediate Panx2's function (Figure 5.1). Together, findings from this study advocate that Panx2 functions via a channel-independent pathway uniquely different from Panx1. Moreover, knockdown experiments using Panx-specific siRNA/shRNA in astrocytes and glioma cell lines endogenously expressing Panxs is needed to further validate Panx-mediated tumor-suppressive effects.

In addition to the reduced motility, Panx1 expression induced a flattened morphology in C6 glioma cells, which are typically spindle-shaped. This finding suggests that Panxs, like Cxs, may also function via interaction with other proteins, such as zonula adherens junctional proteins,

to mediate cytoskeletal reorganization, morphological change, and consequently, alteration in glioma motility (Giepmans, 2004). In lieu of Cxs, Panxs generally possess a longer C-terminal tail than Cxs (Litvin et al., 2006), suggesting that the change in cell morphology is attributed to the association of Panx1 with other junctional proteins.

ZO-1, a peripheral tight junction protein belonging to the MAGUK family, interacts with Cxs via the second PDZ homology domain at the C-terminus (Giepmans and Moolenaar, 1998; Kausalya et al., 2001; Laing et al., 2001; Li et al., 2004; Toyofuku et al., 1998). Since ZO-1 links actin to tight and adherens junctions through its interaction with occludin and  $\alpha$ -catenin, respectively, Cxs were suggested to serve as a cytoskeletal anchorage site at the plasma membrane, hence modulating cell motility via its interaction with ZO-1 (Elias et al., 2007; Giepmans, 2004; Xu et al., 2006). In addition, C6 glioma cells were found to express a low level of ZO-1 protein when compared to primary astrocytes (Howarth et al., 1992), which may correlate with the invasiveness of C6 cells (Chicoine and Silbergeld, 1995; Bernstein et al., 1990). Although a putative PDZ domain was not found *in silico* in the C-terminus of rat Panx1 (data not shown), an interaction between Panx1 and ZO-1 still remains possible via non-canonical binding domain(s). Given that Panx1 expression reduced motility and flattened cell morphology in C6 cells, Panx1 may affect ZO-1 expression and/or subcellular localization to elicit its effect (Figure 5.1).

Adherens junctional proteins, including E-, N-cadherins, and  $\beta$ -catenin, were also shown to co-localize with Cxs (Jongen et al., 1991; Meyer et al., 1992; Fujimoto et al., 1997; Hertig et al., 1996; Ai et al., 2000). While cadherin mediates cell-cell adhesion, it also serves as a anchorage site via intermediary proteins for cytoskeletal componenents, such as actin and microtubules (Meng et al., 2008; Barami et al., 2006).  $\beta$ -catenin is critical in mediating cadherinassociated actin reorganization and therefore cell locomotion, by binding to both cadherin and  $\alpha$ catenin, which links to actin cytoskeleton (Barami et al., 2006). In fact, Perego *et al.*  demonstrated that instability and disorganization, but not reduced expression, of cadherin-catenin adhesion is associated with the invasiveness of T98G and U373 MG glioblastoma cell lines (Perego et al., 2002). Furthermore,  $\beta$ -catenin acts as a transcriptional co-factor downstream of the Wnt signaling pathway to regulate gene expression (Barami et al., 2006). By recruiting  $\beta$ catenin to the plasma membrane to form adherens junctions, cadherins have been shown to regulate Wnt-mediated gene transcription by sequestering cytoplasmic  $\beta$ -catenin and preventing it from translocating into the nucleus (Barami et al., 2006). Although the interacting domain in Cxs has not been identified, a similar interaction between Cxs and β-catenin has been shown by co-immunoprecipitation experiments (Ai et al., 2000), suggesting that Cxs, analogous to cadherins, may also influence gene expression modulated by the Wnt signaling pathway. Inx2, an invertebrate homologue of Panx, has recently been demonstrated to directly interact with DEcadherin and β-catenin in Drosophila (Lehmann et al., 2006). It is speculated that Panx1 may interact with adherens junctional proteins to act as an anchorage site for cytoskeletal components at the plasma membrane to modulate cell morphology and motility. Additionally, Panx1 may regulate gene transcription via the recruitment of  $\beta$ -catenin to the plasma membrane to retard the neoplastic phenotype (Figure 5.1).

To examine the possible effect of Panx1 on the subcellular localization of zonula adherens junctions, indirect immunocytochemistry was performed against ZO-1, N-cadherin, and  $\beta$ -catenin. Stronger signals of ZO-1 (Appendix D), N-cadherin (Appendix E), and  $\beta$ -catenin (Appendix F) were observed at the plasma membrane in Panx1-expressing cells when compared to the control, suggesting that Panx1 affects subcellular localization of these proteins. Quantification assays will be required to further examine this observation. A possible co-localization between Panx1 and the zonula adherens junction proteins was observed (Appendix D, E, F). To study this possibility, preliminary studies using co-immunoprecipitation assay revealed little, if any, direct and/or indirect interaction between Panxs and the zonula adherens

junction proteins (Appendix G), whereas  $\beta$ -catenin successfully pulled down N-cadherin as a positive control. These results demand further validation with varying solublization conditions for the junctional complex proteins. Chemical cross-linking may also aid in identifying proteins that weakly and/or transiently interact with Panxs. Additionally, proteomic analysis of Panx-expressing C6 glioma cells using SILAC (stable isotope labeling with amino acids in cell culture) technique may further reveal other candidate proteins that participated in the observed phenotype (Ong et al., 2002). Overall, the preliminary findings suggest that Panx1 expression alters the subcellular distribution of N-cadherin,  $\beta$ -catenin and ZO-1 via yet identified intermediary proteins and warrants future investigation.

Panx3, on the other hand, was not studied for its implication in glioma oncogenicity in this thesis since earlier studies suggest that Panx3 is mostly expressed in the skin and cartilage, but not in the CNS system (Bruzzone et al., 2003). However, a later study by Peneula *et al.* detected a very low level of Panx3 protein expression in the brain (Penuela et al., 2007), which is in agreement with the low signal for Panx3 transcript found in primary astrocytes (Lai et al., 2007). Given that Panx3, like Panx1, exhibited plasma membrane localization and formed hemichannels when overexpressed in NRK and 293T cells (Penuela et al., 2007; Penuela et al., 2009), it is tempting to speculate that Panx3 may also influence cell growth via similar proposed mechanisms for Panx1 in tissues with endogenous Panx3 expression, including skin and cartilage.

Panx1 and Panx3, but not Panx2, were found to increase neurite numbers and enhance neurite outgrowth during NGF-induced differentiation of PC12 cells. Similar to previous findings, Panx1 and Panx3 were detected at the plasma membrane, whereas Panx2 was observed within the cytosol of PC12 cells (Lai et al., 2007; Lai et al., 2009; Penuela et al., 2007; Penuela et al., 2007; Penuela et al., 2009). The increased number of neurites is based on Panx expression alone and occurred prior to NGF treatment. By contrast, the significant increase in neurite elongation required the induction of neuronal differentiation by NGF, suggesting that Panxs play a role in *preparing* and

*potentiating* neural progenitors for the upcoming differentiation events. The effects are believed to be independent of Panx intercellular channel function as most cells were not in close proximity to one another throughout the experiments, pointing towards hemichannel activity and/or intracellular signaling interplay as possible functional pathways. Since Panx1 and Panx3 can function as hemichannels, they may facilitate the release of ATP to activate purinergic receptors and consequently enhance neurite outgrowth (Figure 5.2), which is similar to the mechanism proposed for Cx43- and Cx32-enhanced neurite outgrowth (Belliveau et al., 1997).

Findings from the neurite outgrowth study in PC12 cells suggest that Panx1 and Panx3 function synergistically with the NGF-stimulated TrkA signaling pathway, likely by a sustained increase in MAPK (ERK1/2) activity via ATP-activated P2Y receptors. NGF induces neurite outgrowth in PC12 cells by binding to and activating TrkA, which subsequently phosphorylates and activates its downstream effectors, including Ras, Rap1, and subsequently MAPK (Meakin et al., 1992). Specifically, the sustained elevation in MAPK activity by NGF stimulation was shown to be critical for neurite outgrowth and maintenance (Sasagawa et al., 2005; Marshall, 1995; Xiao et al., 2006; Yasui et al., 2001; Qui and Green, 1992; York et al., 2000).

MAPK regulates transcriptional and posttranslational modifications of transcription factor, AP-1, by phosphorylation (Kayahara et al., 2005; Coronella-Wood et al., 2004; Musti et al., 1997; Oldenhof et al., 2002; Okazaki and Sagata, 1995; Kalra and Kumar, 2004). AP-1 is consisted of dimers of c-Fos and c-Jun proteins, which binds to DNA to direct gene transcription under various stimuli and thereby yields different cellular responses, including NGF-induced neuronal differentiation of PC12 cells (Gil et al., 2004). Upon NGF exposure, a sustained and heightened MAPK activity was found to associate with concomitant c-Fos and c-Jun expression and phosphorylation in PC12 cells (Eriksson et al., 2007). By using siRNA against c-Fos and c-Jun, Eriksson *et al.* then determined that NGF induces neuronal differentiation of PC12 cells via MAPK-dependent activation of both c-Fos and c-Jun (Eriksson et al., 2007).



Figure 5.2. Schematic diagram of proposed Panx hemichannel-mediated pathways. 1) NGF activates the TrkA receptor to initiate neuronal differentiation by increasing MAPK activity. 2) Activated TrkA triggers spontaneous secretion of ATP into the extraceulluar space, and thereby activates P2YR. 3) Activated P2YR increases and sustains MAPK activity, which may enhance NGF-induced neurite outgrowth and alter glioma oncogenicity. 4) Following P2YR activation,  $[Ca^{2+}]_i$  is released from the ER by activating IP<sub>3</sub> receptors. Elevation in  $[Ca^{2+}]_i$  subsequently triggers opening of Panx hemichannels, resulting in ATP release into the extracellular space. 5) Increased  $[ATP]_o$  activates additional P2YR in an autocrine and/or paracrine fashion and consequently increases and maintains MAPK activity, further augmenting Panx-induced phenotypes. 6) When  $[ATP]_o$  reaches concentration higher than those required for P2YR activation, Panx hemichannel opening is inhibited to prevent further ATP release.

While NGF induces neuronal differentiation, co-administration of NGF and ATP was found to significantly enhance neurite outgrowth in PC12 cells (Behrsing and Vulliet, 1999; Arthur et al., 2005; Behrsing and Vulliet, 2004). The enhancing effect of ATP was demonstrated as a result of ATP-induced P2YR activation, which led to a sustained increase of MAPK activity in parallel to that of NGF-stimulated MAPK response (Behrsing and Vulliet, 2004; Arthur et al., 2005). Therefore, activated P2YR may function synergistically with TrkA by prolonging MAPK activity. Given that Panx1 and Panx3 have been shown to form hemichannels and Panx1 mediates ATP release following ATP-induced activation of purinergic receptors (Locovei et al., 2006a; Penuela et al., 2007; Penuela et al., 2009; Locovei et al., 2006b), Panxs may further enhance P2YR-mediated neurite outgrowth in a positive feedback loop (Figure 5.2). Moreover, since Panx1, and perhaps Panx3, hemichannels close in response to slightly higher [ATP]<sub>0</sub> than that is required for P2X<sub>7</sub> and P2Y<sub>2</sub> activation (Qiu and Dahl, 2009), the proposed positive feedback mechanism may be regulated by high [ATP]<sub>0</sub> in a negative feedback mechanism to regulate Panx hemichannel activity (Figure 5.2).

Meanwhile, since PC12 cells were seeded at a very low density in the neurite outgrowth experiments, ATP released from individual cells alone may not be sufficient to constitute paracrine signaling. While autocrine signaling by self-released ATP is certainly plausible, it is hypothesized that Panxs, in addition to hemichannel activity, function by interacting with other molecules, including N-cadherin and  $\beta$ -catenin. NGF-induced neurite outgrowth in PC12 cells involves a dynamic interaction between adenomatous polyosis coli (APC) protein and  $\beta$ -catenin (Votin et al., 2005). APC is a multi-domain protein found to be enriched in the growth cone (Shi et al., 2004; Morrison et al., 1997), and it is required for NGF-induced neurite outgrowth in PC12 cells by associating with microtubules (Zhou et al., 2004; Kita et al., 2006). Through this interaction, APC promotes microtubule assembly and bundling at the growth cone upon NGF stimulation, and thereby facilitates neurite elongation (Zhou et al., 2004). Similar to the Wnt

signaling pathway, APC and  $\beta$ -catenin are phosphorylated by activated GSK3 $\beta$ , together forming a "B-catenin degradation complex" in the absence of NGF signaling, where the phosphorylated β-catenin is targeted for degradation (Dobashi et al., 2000; Zumbrunn et al., 2001; Ikeda et al., 1998; Ikeda et al., 2000; Rubinfeld et al., 1996; Dobashi et al., 1996). In addition, phosphorylated APC has a lower affinity for microtubules (Zumbrunn et al., 2001). Following NGF treatment, GSK3 $\beta$  at growth cones is phosphorylated and inactivated, resulting in unphosphorylated APC and stabilization of β-catenin (unphosphorylated form) (Dobashi et al., 2000; Zhou et al., 2004; Dobashi et al., 1996). Whereas unphosphorylated APC exhibit higher affinity to microtubules to allow APC-mediated microtubule bundling and consequent neurite outgrowth, stabilized  $\beta$ -catenin was suggested to act as a negative regulator in this pathway by binding to APC (Votin et al., 2005). Therefore, neurite outgrowth depends on balancing the ratio of free- versus  $\beta$ -catenin-bound APC, where more free APC results in neurite elongation, and vice-versa (Votin et al., 2005). A possible scenario for reduced β-catenin-bound APC (and hence neurite elongation) could occur when free  $\beta$ -catenin levels are reduced from the growth cone, such as when sequestered to the cytoplasmic side of the plasma membrane. Given that  $\beta$ -catenin appeared to be localized to the plasma membrane following exogenous Panx1 expression in C6 glioma cells, it raises the possibility that similar alteration in  $\beta$ -catenin subcellular localization also occurred in PC12 cells, perhaps via Panx/N-cadherin-mediated sequestration (Figure 5.1). Reciprocally, constituents of the proposed signaling pathways for Panx-enhanced neurite outgrowth in PC12 cells, including receptor tyrosine kinase (ie. Ras, Rap1, MAPK) and Wnt signaling pathways (ie. N-cadherin, β-catenin, APC), are closely associated with glioma tumorigenesis and oncogenicity (Figure 5.2) (reviewed in (Barami et al., 2006; Kapoor and O'Rourke, 2003). In summary, these proposed prospects require further detailed examination and may unearth novel Panx-mediated signaling pathways involved in both glioma oncogenicity and neuronal differentiation.



Figure 5.3. Schematic diagram of Panx1 and Panx3 mutant constructs. (A) Panx1- $\Delta$ K302stop (C-terminus truncation) and Panx1-Y308L (SH2) mutant retroviruses were created. (B) Panx3- $\Delta$ R296stop (C-terminus truncation), Panx3-Y305L (SH2), and Panx3-S303L (Akt) mutant retroviruses were generated.

While many protein candidates and pathways may be involved in Panx-induced effects described in this thesis, including tumor-suppression and neurite outgrowth, a top-down approach taken by generating Panx mutants and examining functional consequences of the mutants shall provide novel insights into elucidating Panx mechanisms (Figure 5.3, Appendices 6 and 7). In light of the suggested functional significance of the Panx C-terminal tail, mutated Panx C-terminus constructs of Panxs were generated and further cloned into retrovirus to substantially enhance expression efficiency previously limited by transfection methods. Through the aforementioned mutational analysis, several questions can be addressed: 1) Is the C-terminal tail required for Panx-induced effects? 2) Is the C-terminal tail required for Panx channel activity, and if so, does it regulate its opening? 3) Does the C-terminal tail interact with other molecules, and if so, at which amino acid location(s)? Furthermore, recent studies have suggested a correlation between the glycosylation status of Panx and channel activity (Penuela et al., 2007; Penuela et al., 2009). It would also be of interest to pursue whether the glycosylation status of Panx correlates with tumor oncogenicity and neurite outgrowth.

In conclusion, a loss of Panx expression is associated with gliomagenesis, and Panx1 and Panx2 act as novel negative growth regulators in gliomas. In addition, Panx1 and Panx3 promote neuronal differentiation by enhancing neurite outgrowth. Altogether, findings from here advocate that Panxs play a functional role in normal and pathological conditions of the CNS, and merit critical future investigations to explore their functional properties and possible therapeutic implications in disease.

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### **APPENDIX** A



**Appendix A. Panx2 antibody specificity.** Total protein extracted from parental, stable Panx2-EGFP-transfected C6 cells were subjected to Western blot analysis and immunoprobed with anti-Panx2 antibody (Aviva Systems Biology). Panx2-EGFP signal was detected at predicted size (98 kDa). The 50 kDa signal is detected in both the parental and the Panx2-EGFP samples, suggesting it as a result of nonspecific binding of the Panx2 antibody.

# **APPENDIX B**

Name	5'/3'	Primer Sequence
Panx1-	5'	5'- GGA CGT CCT CAA GGT A <u>TT A</u> GA GAT CCT GCC CAC CT-3'
ΔY308L		$\Delta$ Y308L
$(\Delta SH2)$		
	3'	5'- AGG TGG GCA GGA TCT C <u>TA A</u> TA CCT TGA GGA CGT CC -
		ΔY308L
		3'
Panx3-	5'	5'- AAA CGA CTC CTC TCC ATC <u>TTA</u> GAG ATG CTC CCA GCT
ΔY305L		ΔY305L
$(\Delta SH2)$		TTT G -3'
	3'	5'- CAA AAG CTG GGA GCA TCT C <u>TA A</u> GA TGG AGA GGA
		ΔY305L
		GTC GTT T -3'
Panx3-	5'	5'- GGG ACA AAC GAC TCC TC <u>T TA</u> A TCT ACG AGA TGC TCC
$\Delta$ S303L		$\Delta$ S303L
(ΔAkt		CA -3'
kinase)	3'	5'- TGG GAG CAT CTC GTA GAT <u>TAA</u> GAG GAG TCG TTT GTC
		$\Delta S303L$
		CC -3'

Appendix B. Primers for site-directed mutagenesis of predicted motif sites at Panx C-terminus.

# **APPENDIX C**

Panx1       5'       S'- AGG CGC CGG AAT TAG ATC T <u>CT CGA G</u> AC CAT GGC CAT CGC CCA Xhol       Start         2'       S'- CCC CTA CCC GGT AGA ATT CGT TAA C TT AGC AGG ATG AAT TCA Hpal       Stop         GAA GTC GC -3'       GAA GTC GC -3'         Panx1- CT       5'       S'- AGG CGC CGG AAT TAG ATC CT CGA GAC CAT GGC CAT CGC CCA Xhol         Start       CCT GGC CA-3'         3'       S'- CCC CTA CCC GGT AGA ATT CGT TAC TT ACT GCG GGA ACG GGA (GA AGA GC -3')         Panx1- ΔSH2       5'       S'- AGG CGC CGG AAT TAG ATC TC CGA GAC CAT GGC CAT CGC CCA Xhol         Start       CCT GGC CA-3'       Xhol         Panx1- ΔSH2       5'       S'- AGG CGC CGG AAT TAG ATC TC TAC TAG CAT GGC CAT CGC CCA Hpal       Stop         GAA GTC GC -3'       Xhol       Start       CCT GGC CA-3'         3'       S'- CCC CTA CCC GGT AGA ATT CGT TAC C TT AGC AGG ATG AAT TCA Hpal       Stop         GAA GTC GC -3'       Xhol       Start         CCT GGC CGA -3'       Xhol       Start         CAC TGC TG CG GG AAT TAG ATC CT CGA GAC CAT GTC AC ACA Xhol       Start         CAC TGC TG CG GG AAT TAG ATT CGT TAA C CT ATG GGT GTT CAT CAT Hpal       Stop         Panx3- CT       S'- AGG CGC CGG AAT TAG ATC CT CGA GAC CAT GTC GCT CGC ACA Xhol       Start         CAC TGC TG -3'       S'- CCC CTA CCC GGT AGA ATT CGT TAA C CT AGC CAC GCC GCA	Name	5'/3'	Primer Sequence
$\begin{array}{ c c c c c c c c c c c c c c c c c c c$	Panx1	5'	5'- AGG CGC CGG AAT TAG ATC T <u>CT CGA G</u> AC C <u>AT G</u> GC CAT CGC CCA
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3' 5' CUU CIA UUU GGI AGA ATI U <u>GT TAA U UT A</u> TG GGI GTI CAT CAT Hpal Stop			CAC TGC TG -3'
		3'	5- CUU UTA CUU GGT AGA ATT C <u>GT TAA C CT A</u> TG GGT GTT CAT CAT Hngl Ston
			ATG TAT GT -3'

Appendix C. Primers for directional subcloning of cDNAs of interest into pMSCVpuro expression vector.

### **APPENDIX D**



**Appendix D. Possible co-localization between overexpressed Panx1 and ZO-1 in C6 glioma cells at cell-cell contacts.** ZO-1 signal also appeared to be stronger and localized more prominently at cell-cell contacts in the Panx1-expressing C6 cells than the control (EGFP). Primary mouse astrocytes exhibited a clear ZO-1 signal at areas of cell-cell contact. Scale bar, 30 μm.

### **APPENDIX E**



Appendix E. Possible co-localization between overexpressed Panx1 and N-cadherin in C6 glioma cells. Both Panx1-myc and Panx1-EGFP were detected at the plasma membrane and in the cytoplasm. N-cadherin appeared to increase and locate predominately at cell-cell contacts in the tagged Panx1 transfectants, showing a possible co-localization between Panx1 and N-cadherin. Scale bar, 30µm.

### **APPENDIX F**



Appendix F. Possible co-localization between overexpressed tagged Panx1 and  $\beta$ -catenin in C6 glioma cells. Both Panx1-myc and Panx1-EGFP were detected at the plasma membrane and in the cytoplasm.  $\beta$ -catenin appeared to be more defined and locate predominately at cell-cell contacts in the Panx1 transfectants, showing a possible co-localization between Panx1 and  $\beta$ -catenin. Scale bar, 30 µm.

#### **APPENDIX G**



Appendix G. Panx1 and Panx2 do not show significant direct interaction with N-cadherin,  $\beta$ -catenin, and ZO-1. Panx1 and Panx2 total lysates were immunoprecipitated (IP +) with respective antibodies against N-cadherin (A),  $\beta$ -catenin (B), and ZO-1 (C) using Dynabeads magnetic system (Invitrogen), and subjected to Western blot analysis. Immunoblots were then immunoprobed with Panx1- or Panx2- specific antibodies. (D) To ensure method accuracy,  $\beta$ catenin was immunoprecipitated and successfully pulled down N-cadherin in both Panx1 and Panx2 total lysates. Antibodies were omitted in IP (-) samples as a negative control. Total lysates were used a positive control.