

# Ionic Channels in the Therapy of Malignant Glioma

Xia Ding<sup>1,2</sup>, Hua He<sup>3</sup>, Yicheng Lu<sup>3,\*</sup> and Yizheng Wang<sup>1,\*</sup>

<sup>1</sup>*Lab of Neural Signal Transduction, Institute of Neuroscience, Shanghai Institute for Biological Sciences, State Key Laboratory of Neuroscience,*

<sup>2</sup>*The Graduate School, Chinese Academy of Sciences, Shanghai,*

<sup>3</sup>*Department of Neurosurgery, Changzheng Hospital, Second Military Medical University; Shanghai Institute of Neurosurgery, Shanghai, China*

## 1. Introduction

Glioma is among the deadliest tumors worldwide. Despite its relative low onset incidence, glioma, especially malignant glioma, causes high mortality. Due to the utmost aggressiveness of tumor cells, malignant glioma is almost incurable by conventional therapeutic approaches. Finding new molecular targets, which are responsible for tumor progression, can amplify our understanding about malignant glioma and targeting these molecules combining with conventional approaches may ameliorate the therapeutic outcome for patients with malignant glioma.

Intracellular ions are fundamentally essential for cell behavior and ionic channels have been known to play versatile roles in numerous physiological and pathological processes. As for glioma biology is concerned, many types of ionic channels such as  $\text{Ca}^{2+}$ ,  $\text{K}^+$ ,  $\text{Na}^+$  and  $\text{Cl}^-$  channels are involved in glioma cell proliferation, survival, invasion and also glioma angiogenesis. In this chapter, we are going to discuss the implications of ionic channels in the therapy of malignant glioma. Our recent work has indicated the role of one type of  $\text{Ca}^{2+}$  channels, namely the transient receptor potential (TRP) channel in human glioma progression. We thus are going to discuss the roles of  $\text{Ca}^{2+}$  channels in glioma cell biology as well as the possibility of  $\text{Ca}^{2+}$  channels to be therapeutic targets in glioma treatment.

Calcium ( $\text{Ca}^{2+}$ ) is the second messenger for signal transduction to direct many cellular processes and  $\text{Ca}^{2+}$  channels play critical roles in controlling cell behavior, such as neurotransmitter release and muscle contraction. In recent years, the roles of  $\text{Ca}^{2+}$  channels in tumor cell biology have undergone intensive study. Many types of  $\text{Ca}^{2+}$  channels have abnormal expression in tumor cells compared to their corresponding normal cells and they also have specific functions in tumor cell proliferation, survival and invasion, making them appropriate candidate targets in tumor therapy. It has now become clear that TRP channels and voltage-gated  $\text{Ca}^{2+}$  channels participate in the progression of human glioma, some TRP

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\* Corresponding Authors

channel proteins are highly expressed in malignant glioma and function as essential regulators of glioma cell proliferation. The potential of these channels to be anti-glioma target will be highlighted in this chapter.

## 2. Difficulties in treating malignant glioma

Glioma is the most common form of brain tumor. It accounts for about half of all the brain tumors (Central Brain Tumor Registry of the United States [CBTRUS], 2008). According to the histological features, glioma has three major types: astrocytoma, oligodendroglioma and oligoastrocytoma (Huse & Holland, 2010). The World Health Organization classifies glioma as I to IV grade. As for astrocytoma, grade I is the pilocytic astrocytoma, grade II is the diffuse astrocytoma, grade III is anaplastic astrocytoma, and grade IV is glioblastoma multiforme (GBM) (Wen & Kesari, 2008). Grade I and II are low-grade glioma, high-grade glioma including grade III and IV are usually regarded as malignant glioma. GBM is the most common type of malignant glioma. It accounts for approximately 60 to 70% of all malignant glioma (Wen & Kesari, 2008). Histologically, GBM has several characteristics: nuclear atypia, enriched mitosis, necrosis and microvascular enrichment (Behin et al., 2003). GBM can be either original or secondary and secondary GBM develops from low-grade glioma.

GBM is extremely lethal, despite advances in therapy approaches, patients with GBM have very short survival time, averaging approximately 12 to 15 months (Wen & Kesari, 2008). Current therapeutic approaches for GBM include surgery resection, irradiation therapy and chemotherapy. However, all these approaches have very limited improvement on patients' survival, largely due to the intrinsic nature of GBM tumor cells, which are highly proliferative, invasive and often drug resistant. Finding new and specific drug targets for GBM challenges basic research. Current GBM drugs mainly targets DNA synthesis and DNA damage repair processes, for example, DNA alkylating agents (Temozolomide, 1,3-Bis(2-chloroethyl)-1-nitrosourea, BCNU, CCNU) and DNA topoisomerase inhibitors (Irinotecan, topotecan) (Brandes et al., 2001; Stupp et al., 2005). Accumulating evidences support the notion that intracellular ions, and especially ionic channels play important roles in the malignant behavior of glioma cells and it is possible that targeting the glioma-related ionic channels could suppress tumor cell growth. In the following, we are going to discuss the rationale and practice of this channel-targeting strategy.

## 3. Intracellular ions and ionic channels are fundamental for biological behavior of cells

Intracellular ions provide the basic environment for cellular activity and are required for maintaining enzyme activity, protein folding, cytoskeleton dynamics, cellular adhesion and cellular excitability (Berridge et al., 2003; Kunzelmann, 2005). Because of the important role of intracellular ions, ionic channels are of especial importance to cells. They play versatile roles in cellular activity, such as action potential generation, muscle contraction and neurotransmitter release. Among all the ionic channels,  $\text{Ca}^{2+}$ ,  $\text{K}^+$ ,  $\text{Na}^+$  and  $\text{Cl}^-$  channels are four types of channels that receive the most attention. Extensive studies have reported their roles in both physiological and pathological processes. For example,  $\text{Ca}^{2+}$  channels in neuronal plasticity and cell apoptosis (Burgoyne, 2007),  $\text{K}^+$  channels in regulating neuronal

excitability and epilepsy (Lee & Cui, 2010; Zhang et al., 2010), Na<sup>+</sup> channels in action potential initiation and pain sensory (Cregg et al., 2010), Cl<sup>-</sup> channels in regulating cell volume (Duran et al., 2010). More and more evidence have also shown these four types of ionic channels to be important for cell proliferation, migration and survival, suggesting that they might serve as potential targets in tumor therapy. Indeed, ionic channels play important roles in a wide variety of malignant tumors, including in the breast (S. Yang et al., 2009), colon (House et al., 2010), liver (Holzer, 2011), stomach (Holzer, 2011), oesophagus (Holzer, 2011), ovary (S.L. Yang et al., 2009), prostate (Flourakis et al., 2010), endometrium (Wang et al., 2007), lung (S.H. Jang, et al., 2010), skin (Bode et al., 2009) and brain (Ding et al., 2010).

The following parts of the chapter will discuss the above four types of ionic channels in glioma cell biology and implications of these channels in glioma therapy (Table 1). Schematic topology of each channel is summarized in Table 2.

#### 4. Involvement of Ca<sup>2+</sup> signaling and Ca<sup>2+</sup> channels in GBM progression

The seminal role of intracellular Ca<sup>2+</sup> in cell behavior has been well established. Ca<sup>2+</sup> is a critical second messenger for signal transduction and Ca<sup>2+</sup> signaling is required for gene expression, cell proliferation, cell migration, cell survival, cytoskeleton dynamics, fertilization, axonal growth cone turning and so on (Berridge, 2003). Intracellular Ca<sup>2+</sup> signaling consists of many Ca<sup>2+</sup> signaling apparatus, including receptors/channels, transducers, Ca<sup>2+</sup> effectors, Ca<sup>2+</sup>-sensitive enzymes, Ca<sup>2+</sup> pumps and Ca<sup>2+</sup> exchangers (Roderick & Cook, 2008). Many of these Ca<sup>2+</sup> signaling apparatus are involved in regulating glioma behavior. For example, the Ca<sup>2+</sup>-permeable  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)-type glutamate receptors are expressed in GBM cells and can be activated to mediate extracellular Ca<sup>2+</sup> entry (Ishiuchi et al., 2002). Overexpression of the AMPA receptors facilitates tumor cell proliferation and migration. One of the Ca<sup>2+</sup>-sensitive enzymes is the Ca<sup>2+</sup>-activated protease calpain, which is required for GBM cell invasion (H.S. Jang et al., 2010).

In the intricate network of Ca<sup>2+</sup> signaling, Ca<sup>2+</sup> channels are essential contributors to Ca<sup>2+</sup> signaling transduction in response to different stimuli. Different types of Ca<sup>2+</sup> channels are activated to initiate specific Ca<sup>2+</sup> signaling pathways to allow cells to respond to stimuli. As for GBM cells are concerned, Ca<sup>2+</sup> channels are involved in cell survival, proliferation, invasion and tumor angiogenesis. These GBM-related Ca<sup>2+</sup> channels now include the transient receptor potential (TRP) channels and voltage-gated Ca<sup>2+</sup> channels (VGCC).

##### 4.1 TRP channels

TRP channels were first discovered in the fly visual system and participate in light sensing. TRP channel family is now known to be a large family containing 28 members in mammals (Montell, 2005; Ramsey et al., 2006). TRP channel family encompasses seven subfamilies with respect to channel structure similarity, these seven subfamilies include TRPC (Canonical), TRPV (Vanilloid), TRPM (Melastatin), TRPA (Ankyrin), TRPN (Nomp), TRPP (Polycystin) and TRPML (Mucolipdin) (Montell, 2005; Ramsey et al., 2006). All of the TRP family members have six transmembrane domains and the pore region is located between the fifth and sixth transmembrane domains. Both the N- and C-terminals are located

intracellularly. Functional TRP channels are formed as homotetramers or heterotetramers of different TRP members. They are non-selective cation channels and are primarily permeable to  $\text{Ca}^{2+}$  and  $\text{Na}^+$ , some are also permeable to  $\text{Mg}^{2+}$ . TRP channels were found to functionally express in diverse tissues. These channels participate in a variety of physiological and pathological processes, such as neuronal survival (Jia et al., 2007), axon guidance (Li et al., 2005), pain sensory (Cortright et al., 2007), endothelial permeability (Ahmed & Malik, 2005), pathogenesis of certain renal disease (Reiser et al., 2005; Winn et al., 2005; Heeringa et al., 2009), cardiovascular disease (Kuwahara et al., 2006; Onohara et al., 2006) and so on. The functions of many TRP channels still remain to be explored. The glioma-related TRP channels now include the TRPC, TRPV and TRPM channels.

#### 4.1.1 Implication of TRPC channels in glioma progression and therapy

TRPC channels are the first mammalian TRP subfamily to be discovered and share the highest homology with fly TRP (about 30-40% in protein sequence identity) (Montell, 2005). In mammalian cells, TRPC channels contain seven members from TRPC1 to TRPC7 (Vazquez et al., 2004). TRPC channels can be activated by receptor-operated pathway, store-operated pathway, mechanical stretch, membrane trafficking, oxidative stress and  $\text{Ca}^{2+}$ /Calmodulin (Boulay, 2002; Maroto et al., 2005; Miller, 2006; Montell, 2005; Singh.B et al., 2004; Tang et al., 2001; Vazquez et al., 2004; Zhang et al., 2001). The receptor-operated and store-operated pathways are the most intensively studied. In the receptor-operated pathway, when G-protein coupled receptor or receptor tyrosine kinase on the cell surface are activated by ligand binding, their corresponding downstream phospholipase C are activated to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) into inositol 1,4,5-triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). The DAG can directly bind to and activate TRPC channels (Montell, 2005). In the store-operated pathway, when intracellular  $\text{Ca}^{2+}$  store (mostly refer to the endoplasmic reticulum) are released, for example under thapsigargin (inhibitor of  $\text{Ca}^{2+}$ -ATPase on the ER) treatment, the IP<sub>3</sub> receptor or STIM1 on the ER can physically interact with TRPC channels on the plasma membrane and activate TRPC channels (Bolotina & Csutora, 2005; Ramsey et al., 2006; Varnai et al., 2009). It is worth mentioning that under different conditions, one single type of TRPC channels can have more than one activation pathways (Ding et al., 2010; Hofmann et al., 1999).

TRPC channels are found in a wide diversity of tissues and cells, including neurons, glial cells, smooth muscle cells, endothelial cells, kidney podocytes and tumor epithelial cells (Ahmed & Malik, 2005; Aydar et al., 2009; El Boustany et al., 2008; Golovina, 2005; Guilbert et al., 2008; Heeringa et al., 2009; Jia et al., 2007; Reiser et al., 2005; Winn et al., 2005; S.L. Yang et al., 2009; Yu et al., 2003; Yu et al., 2004). They form functional channels as homotetramer or heterotetramer, as has been revealed that TRPC1, 4 and 5 can interact with each other and TRPC3, 6 and 7 can interact with each other to form functional channels (Hofmann et al., 2002; Strubing et al., 2001; Strubing et al., 2003). TRPC channels regulate neuronal survival, neurite development, synapse formation, axon guidance, endothelial permeability, cell migration, differentiation and proliferation (Ahmed & Malik, 2005; Cai et al., 2006; Florio Pla et al., 2005; Jia et al., 2007; Li et al., 2005; Louis et al., 2008; Tai et al., 2008; Zhou et al., 2008). Among the seven TRPC members, TRPC1 and TRPC6 have been reported to play important roles in glioma cell proliferation, migration and invasion, TRPC6 channels are also involved in tumor angiogenesis (Bomben et al., 2010; Bomben & Sontheimer, 2010; Chigurupati et al., 2010; Ding et al., 2010; Ge et al., 2009; Hamdollah Zadeh et al., 2008).

Channel	Cell type	Functions in glioma cells	Abnormal expression in glioma	Pharmacological or molecular antagonists	Ways of activation	Animal experiments or clinical trial	Distribution in normal tissues and cells
TRPC1	Cell line (D54MG)	Proliferation, cytokinesis, EGF-induced chemotaxis	Not known	SKF96365, RNAi		No	Heart, brain, testis, ovary,
TRPC6	Cell lines (U251, T98G, U87) and patient samples	Cell cycle progression,	High expression	SKF96365, DN-TRPC6, RNAi	PDGF	Yes, intracranially implanted glioma in nude mice	Neuronal cells, cardiac myocytes, smooth muscle cells, vascular endothelial cells, kidney podocytes
	Cell line (U373MG) and patient samples	Notch-induced invasion	High expression	SKF96365, RNAi	OAG	No	
TRPM2	Cell line (A172)	H <sub>2</sub> O <sub>2</sub> -induced cell death	Not known		Overexpression of wild type TRPM2	No	Brain
TRPM8	Cell line (DBTRG)	Menthol-induced cell migration	Not known		Menthol	No	Prostate, Trigeminal (TG), dorsal root ganglion (DRG)
TRPV1	Cell line (U373, U87) and patient samples	Capsaicin-induced cell death in TRPV1-high cells	Inversely correlated with glioma grade		Capsaicin	No	TG, DRG, urinary bladder
TRPV2	Cell line (U87) and patient samples	Negatively regulated proliferation	Inversely correlated with glioma grade	RNAi	Overexpression of wild type TRPV2	No	DRG, spinal cord (SC), brain, spleen, small and large intestine, vascular myocytes
Cav3.1	Cell lines (U87)	Promote proliferation	Not known	Mibefradil, NNC55-0396	Overexpression of wild type Cav3.1 $\alpha$ 1 subunit	No	Vascular smooth muscle, fibroblasts, myocytes
	Cell lines (U87, U563, U251) and patient samples		Specific splicing form expressed in glioma cells			No	
BK	Cell lines (U251, U87)	Do not affect proliferation	A specific isoform highly expressed	Iberiotoxin, paxilline, penitrem A	NS1619	No	Neurons, smooth muscle cells

Channel	Cell type	Functions in glioma cells	Abnormal expression in glioma	Pharmacological or molecular antagonists	Ways of activation	Animal experiments or clinical trial	Distribution in normal tissues and cells
	Animal model	Increase the permeability of BTB		Iberitoxin	NS1619	Yes, intracranial RG2 cell implantation in Wistar rat	
IK	Cell lines (U251, U87)	Do not affect proliferation, but promote cell migration	Not known	Clotrimazole and TRAM-34		No	Neurons, smooth muscle cells
	HUVEC, HMVEC	Promote angiogenesis		TRAM-34		Yes, in vivo matrigel plug assay in nude mice	
K <sub>ATP</sub>	Cell lines (U251, U87)	Promote proliferation, cell cycle progression through G0/G1 phase	High expression	Tolbutamide	Diazoxide Minoxidil sulfate	Yes, subcutaneous coinjection of drugs with glioma cells in nude mice	Heart, skeletal muscle cells, pancreatic islet cells, vascular smooth muscle cells,
	Animal model	Increase permeability of BTB			Minoxidil sulfate	Yes, intracranial implanted GBM in nude mice	
TASK3		Negatively regulate cell survival	Not known	Bupivacaine, spermine	Isoflurane	No	Brain, kidney, liver, lung, colon, stomach, spleen, testis, skeletal muscle
hERG1	Cell lines (U138, A172) and patient samples	Modulate VEGF secretion	High expression	WAY		No	Heart, pancreas, colon
ASIC1	Cell line (D54MG)	Promote cell migration	Not known	Amiloride, psalmotoxin1 (PcTX-1)		No	CNS, PNS
CIC2	Cell line (D54MG)	Mediated Cl <sup>-</sup> current	High expression			No	
CIC3	Cell line (D54MG)	Mediate Cl <sup>-</sup> current required for M phase progression	High expression	Chlorotoxin		Yes, phase I clinical trial	Neurons
	Cell lines (STIG1, U251)	Cell invasion	High expression			No	

Table 1. Glioma-related ionic channels. The glioma-related ioninc channels are summarized in this table. Detailed information can be retrieved from the body text.

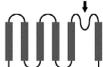
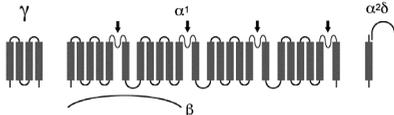
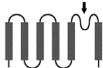
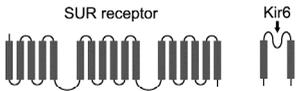
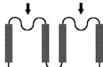
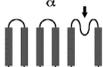
Channel	Subunit	Subunit assembly
TRP		Tetramer
VGCC		$\alpha_1, \beta, \alpha_2\delta, \gamma$
BK		$\alpha \times 4, \beta \times 4$
IK		Tetramer
K <sub>ATP</sub>		SUR receptor x 4, Kir6 x 4
TASK		Dimer
hERG		Tetramer
ASIC		Tetramer
CIC	Complex structure, with 17 intramembrane domains	Dimer

Table 2. Schematic topology of subunit and subunit assembly of glioma-related ion channels. Transmembrane domains are represented as grey bars and pore-forming regions are indicated by the short arrows.

TRPC1 is the first TRPC member to be cloned (Wes et al., 1995). TRPC1 channels function in the regulation of neural stem cell proliferation, skeletal myoblast migration and differentiation, cell apoptosis and so on (Florio Pla et al., 2005; Louis et al., 2008; Bollimuntha et al., 2005). TRPC1 channels can be gated by receptor-operated pathway, store-operated pathway or even by mechanical stretch, depending on the cell types examined (Kim et al.,

2003; Maroto et al., 2005; Saleh et al., 2008). Glioma-related TRPC1 channels are involved in glioma cell proliferation and cell migration. In D54MG glioma cells, TRPC1 channels were gated by store-operated pathway. Pharmacological inhibition or shRNA-mediated suppression of TRPC1 channels inhibited glioma cell cytokinesis and resulted in multinucleated cells and eventually slowed glioma cell proliferation (Bomben & Sontheimer, 2010). Although  $Ca^{2+}$  signaling is important for cytokinesis in cell division, the channel through which the  $Ca^{2+}$  enters cells remains unknown. It is possible that TRPC1-mediated  $Ca^{2+}$  signaling is indispensable for cytokinesis in glioma cells, though the detailed molecular mechanism needs further exploration. Besides cytokinesis and proliferation, TRPC1 is also required for glioma cell migration. In response to the epidermal growth factor (EGF), TRPC1 protein was enriched in the leading edge of D54MG glioma cells and co-localized with lipid raft proteins. Inhibition of TRPC1 channels pharmacologically or by shRNA knockdown retarded EGF-induced cell migration, but did not affect the motility of un-stimulated cells. These results suggest that TRPC1 channels contribute to glioma chemotaxis in response to specific stimuli (Bomben et al., 2010).

Another TRPC channel member, TRPC6 channel is also essential for glioma progression. The TRPC6 channels are known to regulate axon growth cone turning (Li et al., 2005), survival of cerebellum granule neuron (Jia et al., 2007), dendrite development (Tai et al., 2008), synapse formation (Zhou et al., 2008), proliferation of pulmonary artery smooth muscle cells (Yu et al., 2004), cardiac myocytes (Kuwahara et al., 2006), vascular endothelial cells (Ge et al., 2009; Hamdollah Zadeh et al., 2008) and tumor cells (Cai et al., 2009; El Boustany et al., 2008; Thebault et al., 2006; Shi et al., 2009). Furthermore, TRPC6 functional mutations also contribute to the pathogenesis of a familiar renal disease named focal segmental glomerulosclerosis (Heeringa et al., 2009; Reiser et al., 2005; Winn et al., 2005). TRPC6 can be activated by receptor-operated pathway or by store-operated pathway as determined by different cell types. For example, in tumor cells, TRPC6 channels in most cases are store-operated and can be activated by thapsigargin or other ER  $Ca^{2+}$ -ATPase inhibitors (Ding et al., 2010; El Boustany et al., 2008), and in neuronal cells, TRPC6 channels are often receptor-operated and can be activated by neurotrophic factors or growth factors, such as brain-derived neurotrophic factor (BDNF) (Jia et al., 2007; Li et al., 2005).

The expression of TRPC6 was elevated in glioma tissues compared to normal brain tissues. By using neuronal marker, NeuN to distinguish normal neurons and normal glial cells in normal brain tissues, it was found that normal neurons expressed a high level of TRPC6, which was comparable to that in glioma cells, however in normal glial cells, the level of TRPC6 was barely detectable, suggesting that TRPC6 was specifically up-regulated in glioma cells, but not in neurons or in normal glial cells. Moreover, compared to low-grade glioma, TRPC6 expression level was even higher in GBM, suggesting that TRPC6 expression level was associated with glioma grade. TRPC3 is a closely related homolog to TRPC6, but unlike TRPC6, its expression level in glioma tissues was not significantly different from that of normal brain tissues. The selective up-regulation of TRPC6 channels in GBM implies the reliance of GBM tumor cell behavior on TRPC6 channels.

SKF96365 is a putative, but non-specific inhibitor for TRPC channels, treatment of glioma cells with SKF96365 could dramatically inhibit glioma cell proliferation. Specific inhibition of TRPC6 channels by a dominant-negative mutant channel (DN-TRPC6) (Hofmann et al.,

2002) or by RNA interference (RNAi) could also significantly inhibit glioma cell proliferation *in vitro* and in nude mice subcutaneous xenograft model. In nude mice intracranial xenograft model, DN-TRPC6 slowed the growth of tumors and significantly prolonged survival of tumor-bearing animals. Flowcytometry assay revealed that this inhibition of glioma cell proliferation was through arresting cell cycle in G2/M phase, not through induction of cell death, suggesting that TRPC6 channels are important for G2/M phase progression of glioma cells. Further analysis revealed that inhibition of TRPC6 channels down-regulated the expression of central cell cycle regulators, such as CDC25C, a phosphatase in activating CDC2/Cyclin B complex, which can drive cell cycle through G2/M phase (Boutros et al., 2007; Grana & Reddy, 1995). As has been known that Ca<sup>2+</sup> signaling is essential for gene transcription (Greer & Greenberg, 2008), it is possible that TRPC6-mediated Ca<sup>2+</sup> signaling contributes to the transcription of many cell cycle proteins in order to regulate glioma cell cycle progression.

As a Ca<sup>2+</sup>-permeable channel in glioma cells, TRPC6 is functionally expressed. In U87-MG glioma cells, PDGF triggered a transient wave of intracellular Ca<sup>2+</sup> elevation as reflected by Fura 2-AM Ca<sup>2+</sup> image. This Ca<sup>2+</sup> elevation was dramatically attenuated by SKF96365 perfusion, or by DN-TRPC6, or by TRPC6 RNAi, suggesting the contribution of TRPC6 channels to this induced Ca<sup>2+</sup> wave. In Ca<sup>2+</sup>-free medium, PDGF could only trigger a much smaller wave, but when Ca<sup>2+</sup> was re-applied, the Ca<sup>2+</sup> wave became much larger. When 2-APB (an IP<sub>3</sub> receptor inhibitor blocking Ca<sup>2+</sup> release from ER) (Maruyama et al., 1997) was present in the bath, PDGF-induced Ca<sup>2+</sup> elevation was completely abolished. These results implied that PDGF might first trigger Ca<sup>2+</sup> release from the ER and then through the store-operated pathway activate extracellular entry, which might through TRPC6 channels. It is known that when using cyclopiazonic acid (CPA, another ER Ca<sup>2+</sup>-ATPase inhibitor as thapsigargin) (Demaurex et al., 1992) to deplete ER Ca<sup>2+</sup> store under Ca<sup>2+</sup>-free condition, Ca<sup>2+</sup> re-application could induce the classical store-operated Ca<sup>2+</sup> entry. Further experiments revealed that DN-TRPC6 could decrease the CPA-induced store-operated Ca<sup>2+</sup> entry. This result clearly indicates that TRPC6 in glioma cells can be activated by PDGF and can mediate Ca<sup>2+</sup> entry via the store-operated pathway. Since it has been well established that PDGF is a critical regulator for glioma tumorigenesis and development, these results indicated that TRPC6-mediated Ca<sup>2+</sup> signaling might contribute to PDGF-induced glioma pathogenesis.

Besides cell proliferation and cell cycle, TRPC6 is also essential for hypoxia-induced glioma invasion and migration. Under hypoxia condition, Notch signaling pathway was activated and TRPC6 expression level increased in a Notch-dependent manner. Hypoxia treatment (CoCl<sub>2</sub> treatment) could activate TRPC6 channels and boost the ability of glioma proliferation and invasion. Inhibition of TRPC6 channels reversed the hypoxia-induced proliferation and invasion (Chigurupati et al., 2010). It is known that Notch signaling pathway is important for development and for maintaining cells in an undifferentiated state by regulating the transcription of many critical proteins (Artavanis-Tsakonas et al., 1999), these results suggest that Notch-induced TRPC6 expression may enhance undifferentiated state of glioma cells and therefore enhance the aggressiveness of glioma cells.

TRPC6 channels are also essential for angiogenesis, which is another important feature of malignant glioma (Wong & Brem, 2010). Human microvascular endothelial cell (HMVEC) is a good experimental model to study angiogenesis. In HMVECs, VEGF could trigger

intracellular  $\text{Ca}^{2+}$  elevation and inhibition of TRPC6 channels by DN-TRPC6 alleviated VEGF-induced  $\text{Ca}^{2+}$  elevation. Meanwhile, DN-TRPC6 also inhibited the migration, sprouting and proliferation of HMVECs. On the contrary, overexpression of TRPC6 increased the migration and proliferation of HMVECs (Hamdollah Zadeh et al., 2008). In Human umbilical vein endothelial cells (HUVEC), similar phenomenon was observed. Inhibition of TRPC6 channels by SKF96365 or DN-TRPC6 arrested HUVEC cell cycle in G2/M phase and suppressed VEGF-induced cell proliferation and tube formation. Furthermore, inhibition of TRPCs abolished VEGF-, but not FGF-induced angiogenesis in the chick embryo chorioallantoic membrane (Ge et al., 2009). These results suggest that TRPC6 channels play an important role in VEGF-induced angiogenesis. Targeting TRPC6 in microvascular endothelial cells may inhibit the neo-angiogenesis of malignant glioma and eventually suppress tumor progression.

Based on the above basic findings, TRPC1 and TRPC6 channels could be potential drug targets in the therapy of malignant glioma. However, one major problem for TRPC channels as targets is that there is a severe lack of specific TRPC channel blockers. SKF96365 is a putative TRPC channel inhibitor, it can inhibit both TRPC1 and TRPC6 channels, but it can also inhibit many other types of channels and result in strong non-specific effect (Clapham, 2007; Fiorio Pla et al., 2005; Kim et al., 2003; Malkia et al., 2007; Mason et al., 1993; Merritt et al., 1990; Vazquez et al., 2004). Based on this situation, the currently available and efficient way of specifically inhibiting TRPC channels is to transfect cells with dominant-negative mutant form of specific channel proteins or with specific siRNA sequence to inhibit channel activity or knockdown gene expression. The DN-TRPC6 is a pore region-mutated channel, in which Leu678, Phe679 and Trp680 are mutated to Ala (Hofmann et al., 2002). DN-TRPC6 channel is impermeable, thus when overexpressed in glioma cell, DN-TRPC6 can chelate endogenous TRPC6 channels to form impermeable channel tetramers and achieve channel-specific blockade. Because TRPC6 can form functional tetramers with other TRPC channels, such as TRPC3, DN-TRPC6 also has certain side effects by inhibiting the activity of these TRPC6 binding channels. Besides DN-TRPC6, siRNA targeting TRPC6 is the most specific way of inhibiting TRPC6 channels without affecting other channel expression. Although channel dominant-negative and siRNA knockdown approaches are highly selective and have little side effects, the way of *in vivo* delivery of these nucleotide molecules will hinder their clinical use, because their inhibition effect largely relies on transfection efficiency. In order to get high transfection efficiency in cultured glioma cells, viral vectors have to be employed. In our publication, we used adenoviral vectors to deliver DN-TRPC6 and lentiviral vectors to deliver siRNA targeting TRPC6. Both these two types of vectors have high affinity to glioma cells and enable sufficient expression of DN-TRPC6 or siRNA to inhibit endogenous glioma TRPC6 channels (Ding et al., 2010). However, when systemically applied, the toxicities of virus will greatly restrict their usage, since adenovirus has high immunogenicity and lentivirus is genome integrative. Specific monoclonal antibody raised against the pore region of TRPC channels is another blockade approach. Such blockade antibody for TRPC5 channels has been reported. Monoclonal antibody against the third extracellular domain of TRPC5 was generated, by utilizing the specific recognition of antibody and antigen, this antibody can specifically bind to and inhibit TRPC5 channel activity (Xu et al., 2005). But such antibodies for TRPC1 or TRPC6 channels have not yet been reported. Therefore, in order to facilitate the clinical significance of TRPC channels in glioma therapy, developing specific blockers, especially small-molecule agents, to target TRPC1 and TRPC6 channels is an urgent need.

Besides the development of specific inhibitors, side effects of targeting TRPC channels also need a serious consideration. Since TRPC1 and TRPC6 channels have expression in many normal tissues and cells, especially in neuronal cells, cardiac myocytes, smooth muscle cells and vascular endothelial cells, side effects to these normal tissues and cells must be paid great attention to.

#### 4.1.2 Implication of TRPM channels in glioma progression and therapy

The TRPM subfamily is composed of eight mammalian members, TRPM1 to TRPM8. Besides  $\text{Ca}^{2+}$  and  $\text{Na}^+$ , TRPM channels, such as TRPM6 and 7 channels are also permeable to  $\text{Mg}^{2+}$ . Different from other TRP channels, some TRPM members (TRPM2, 6 and 7) have enzyme activity in their C-terminal domain. TRPM2 has a ADP-ribose pyrophosphatase domain and TRPM6/7 have protein kinase domains. These TRPM channels are the so-called chanzymes (Montell, 2005). TRPM channels can be activated by menthol, cold temperature, osmolarity alteration and so on. TRPM channels function in temperature sensing, redox sensing, taste sensing, ischemia, neuronal cell survival and regulation of  $\text{Mg}^{2+}$  ion homeostasis (Aarts et al., 2003; Montell, 2005; Wei et al., 2007). TRPM2 and TRPM8 channels have been reported to be involved in glioma cell survival and cell migration.

TRPM2 channels can be activated by reactive oxygen species and mediate cell death in several types of cells (Kaneko et al., 2006; Miller, 2006). In A172 glioblastoma cells, TRPM2 channels could be targeted to the plasma membrane and mediate the  $\text{Ca}^{2+}$  influx induced by  $\text{H}_2\text{O}_2$  treatment. This  $\text{Ca}^{2+}$  influx is important for  $\text{H}_2\text{O}_2$ -induced glioma cell death. However, overexpression of TRPM2 did not affect glioma cell proliferation, migration or invasion (Ishii et al., 2007). These results suggested that activation of TRPM2 channels can promote glioma cell death and that TRPM2 can be a candidate for glioblastoma therapy.

TRPM8 channels are also implicated in glioma migration. In DBTRG glioblastoma cells, menthol could activate  $\text{Ca}^{2+}$  entry and promote cell migration, and TRPM8 channels were found to mediate menthol-induced intracellular  $\text{Ca}^{2+}$  elevation and cell migration, suggesting that  $\text{Ca}^{2+}$  influx via TRPM8 is necessary for glioma cell migration in response to menthol stimuli (Wondergem et al., 2008).

#### 4.1.3 Implication of TRPV channels in glioma progression and therapy

Mammalian cells have six TRPV subfamily members, TRPV1 to TRPV6. The TRPV channels can be activated by heat ( $>43^\circ\text{C}$ ) or warm temperature ( $30\text{-}39^\circ\text{C}$ ), membrane stretch, osmolarity alteration etc. Therefore, TRPV channels mainly function in sensing hot pain or warm temperature and osmolarity (Montell, 2005). In glioma cells, TRPV channels are also functionally expressed and TRPV1 and TRPV2 channels are involved in glioma cell death and proliferation.

In glioma cells, TRPV1 regulates capsaicin-induced cell death. TRPV1 expression level inversely correlated with glioma grade and in a majority of Grade IV glioblastoma, TRPV1 was markedly lost. Concordantly, capsaicin could only induce cell death in TRPV1 high expression cells, such as U373 cells, but not in TRPV1 low expression cells, such as U87 cells (Amantini et al., 2007). These results suggest that TRPV1 activation can promote glioma cell death and TRPV1 may be a good target for low-grade glioma, but not necessarily good for

malignant glioma. The glioma-related TRPV2 channels are very much alike, its expression level was found to negatively correlate with glioma grade. Down-regulation of TRPV2 by RNA interference actually promoted U87MG glioma cell proliferation and rescued Fas-induced cell apoptosis. On the contrary, overexpression of TRPV2 in MZC glioma cells resulted in reduced cell viability and increased spontaneous and Fas-induced apoptosis (Nabissi et al., 2010).

The studies on glioma-related TRPM and TRPV channels suggest that activating these channels could inhibit glioma progression and further imply that agonists of these channels may serve as potential drugs for glioma therapy. TRPM8 channels are found to negatively regulate cell survival of prostate cancer and melanoma (Yamamura et al., 2008; Zhang & Barritt, 2004). Menthol, an activator of TRPM8 channels, can inhibit the growth of prostate cancer cells and melanoma cells and it seems to be a candidate drug also in glioma therapy. Since menthol is also an activator for many other pathways (Galeotti et al., 2002) and TRPM8 channels are functionally expressed in dorsal root ganglia (DRG) neurons (Montell, 2005), side effects of menthol in treating glioma have to be considered. Capsaicin is an ingredient of red chili peppers and an activator of TRPV1 channels. Capsaicin has been reported to possess anti-tumor activity, for example in prostate cancer and breast cancer, also in glioma (Sanchez et al., 2006; Mori et al., 2006; Thoennissen et al., 2010; Kim et al., 2010). Although the anti-tumor activity of capsaicin may not necessarily be through activation of TRPV1 channels (Ziglioli et al., 2009), capsaicin might be another potential anti-glioma drug and side effects to the DRG neurons should be considered, where TRPV1 channels are highly expressed.

#### **4.2 Implication of voltage-gated $\text{Ca}^{2+}$ channels (VGCC) in glioma progression and therapy**

The VGCC are also a channel family including ten members. Each VGCC member is assembled through interaction of four subunits ( $\text{Cav}\alpha_1$ ,  $\text{Cav}\beta$ ,  $\text{Cav}\alpha_2\delta$  and  $\text{Cav}\gamma$ ) and each VGCC member is distinguished by their channel forming subunit, the  $\text{Cav}\alpha_1$  subunit. The  $\text{Cav}\alpha_1$  subunit consist of four transmembrane regions, each region contains six transmembrane domains. VGCC can be activated by membrane depolarization and based on physiological and pharmacological properties, VGCC members can be categorized as low-voltage activated VGCC including T-type VGCC ( $\text{Cav}3.1$ ,  $\text{Cav}3.2$  and  $\text{Cav}3.3$ ) and high-voltage activated VGCC including, L-type ( $\text{Cav}1.1$ ,  $\text{Cav}1.2$ ,  $\text{Cav}1.3$  and  $\text{Cav}1.4$ ), N-type ( $\text{Cav}2.2$ ), P/Q-type ( $\text{Cav}2.1$ ) and R-type VGCC ( $\text{Cav}2.3$ ) (Catterall, 2000). Functions of VGCC are involved in neuronal plasticity (e.g. long-term potentiation), exocytosis (e.g.  $\text{Ca}^{2+}$ -dependent release of neurotransmitters) and in many pathological processes such as pain (Bauer et al., 2002; Wang et al., 2004; Zamponi et al., 2009).

It has been known that T-type VGCC ( $\text{Cav}3.1$ ) is involved in glioma cell proliferation. The  $\text{Cav}3.1$  was found to express in both patient glioma tissues and in cultured glioma cell lines (U87, U563 and U251) and could promote glioma proliferation. Inhibition of  $\text{Cav}3.1$  by its selective antagonist, mibefradil, could decrease its expression and suppressed glioma cell proliferation. Meanwhile, overexpression of  $\text{Cav}3.1$   $\text{Cav}\alpha_1$  subunit resulted in an increased cell proliferation (Panner et al., 2005), suggesting that  $\text{Cav}3.1$  could actually promote glioma cell proliferation. Furthermore, our work showed that inhibition of  $\text{Cav}3.1$  channels led to

glioma cell cycle arrest in S phase (Ding et al., 2010), suggesting that this channel could be important for DNA synthesis or DNA damage repair. Inhibition of Cav3.1 may also sensitize glioma cells to irradiation. Interestingly, it has been found that besides previous known Cav3.1  $\text{Cav}\alpha_1$  splicing alternatives, glioma tissues seemed to express a novel splicing variant of  $\text{Cav}\alpha_1$  subunit of Cav3.1 that was distinguished from normal brain tissues or fetal astrocytes (Latour et al., 2004). This finding implies that glioma-specific form of Cav3.1 might contribute to glioma pathogenesis and might be a unique target in glioma therapy.

Inhibition of T-type VGCC can be achieved by mibefradil, which is a synthetic small-molecule agent. Mibefradil is a widely used  $\text{Ca}^{2+}$  channel blocker and was once a drug for the treatment of hypertension (Ertel & Clozel, 1997; SoRelle, 1998). However, the potential use of mibefradil as therapeutic drug is greatly restricted by its lack of selectivity and its inhibition of other types of VGCCs, such as L-type VGCC (Mehrke et al., 1994; Bezprozvanny & Tsien, 1995). Since L-type VGCCs play important roles in many types of excitable cells (mainly myocytes and neurons) (Striessnig, 1999 & Greenberg, 1997), normal functions of skeletal/cardiac myocytes and the learning/memory abilities might be affected if T-type VGCC blockers can also interrupt the normal functions of L-type VGCC. Therefore, when targeting T-type VGCCs to treat glioma, these aspects must be seriously considered. In recent years, NNC55-0396 is synthesized as another inhibitor that is much more selective for T-type VGCC than mibefradil (Huang et al., 2004). In tumor research field, NNC55-0396 has been used to suppress human breast cancer cell proliferation *in vitro* (Taylor et al., 2008), but no studies on its use in glioma have yet been reported.

## 5. $\text{K}^+$ , $\text{Na}^+$ channels and glioma

The  $\text{K}^+$  channel family has 78 members and can be classified into four categories based on their activation mechanism and the number of transmembrane domains: inward-rectifying  $\text{K}^+$  channels, two-pore  $\text{K}^+$  channels,  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels and voltage-gated  $\text{K}^+$  channels (Wulff et al., 2009). The  $\text{K}^+$  channels play critical roles in cellular behavior and are involved in numerous biological processes, such as regulation of membrane potential and neuronal excitability and regulation of cell volume and cell proliferation (Bielanska et al., 2009; Grunnet et al., 2003; Jentsch, 2000; Trimarchi et al., 2002; Wang et al., 2007). The glioma-related  $\text{K}^+$  channels include the BK and IK1 channels ( $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels), ATP-sensitive  $\text{K}^+$  channels (inward-rectifying  $\text{K}^+$  channels), TASK3 (two-pore  $\text{K}^+$  channels) and hERG1 (voltage-gated  $\text{K}^+$  channels).

$\text{Na}^+$  channels are mostly voltage-gated, with a few ligand-activated  $\text{Na}^+$  channels. Their primary function is to generate action potential in the nervous system and they are often involved in epilepsy and pain (Kohling, 2002; Lampert et al., 2010; Naundorf et al., 2006). In glioma cells, one type of ligand-activated  $\text{Na}^+$  channels, the acid-sensing ion channels (ASIC, one type of the amiloride-sensitive  $\text{Na}^+$  channel) is known to participate in glioma cell migration.

### 5.1 Implication of BK, IK1 channels in glioma cell proliferation and glioma therapy

The  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels include the big conductance channels (BK), intermediate conductance channels (IK) and small conductance channels (SK). BK channels are composed

of four  $\alpha$  subunits and four  $\beta$  subunits, IK and SK channels are composed of four pore-forming subunits and four calmodulin (Ledoux et al., 2006). BK channels and IK channels have been verified to express in glioma cell lines and primary glioma cells and can be properly activated to mediate  $K^+$  current. Moreover, a specific BK channel isoform was found to be highly expressed in human glioma and was positively correlated with glioma grades (Liu et al., 2002). Inhibition of BK channels by its blocker iberiotoxin or paxilline suppressed U251 glioma cell migration. It was found that other BK channel blockers, paxilline and penitrem A, could also inhibit U251 and U87 cell proliferation (Abdullaev et al., 2010; Weaver et al., 2004; Weaver et al., 2006). However, in gene knockdown experiments, specific siRNA targeting BK channels failed to affect glioma cell proliferation, despite the siRNA could well down-regulate protein expression and inhibit channel current (Abdullaev et al., 2010). The inconsistency between pharmacological and molecular results suggests that BK channel pharmacological blockers might have some side effects or that BK channels do not to regulate glioma cell proliferation. As for IK1 channel, its blocker clotrimazole and TRAM-34 suppressed U251 and U87 cell proliferation, but the anti-proliferation effect failed to be repeated in siRNA knockdown experiments (Abdullaev et al., 2010). In another study, TRAM-34 or IK1 specific siRNA knockdown abolished CXCL12-induced glioma cell migration (Sciacaluga et al., 2010). All these studies suggest that BK and IK1 channels do not participate in glioma cell proliferation, but IK1 channels indeed play a role in glioma cell migration. Moreover besides cell proliferation and migration, IK1 channels are found to regulate angiogenesis (Grgic et al., 2005). IK1 channels were expressed in HUVEC and HMVEC cells and could be stimulated by bFGF or VEGF to mediate  $K_{Ca}$  current. Blockade of IK1 channels by TRAM-34 suppressed bFGF- and VEGF-induced HUVEC or HMVEC cell proliferation. And in mice matrigel plug assay, administration of TRAM-34 could inhibit angiogenesis. This aspect concerning the *in vivo* use of TRAM-34 will be further discussed in the following section. Although BK channels do not seem to regulate cell proliferation, many studies have reported its role in regulating the permeability of blood-brain tumor barrier (BTB), which limits the chemotherapy agent delivery for glioma. This aspect will also be discussed in the following section.

Because BK and IK channels are essential for the regulation of smooth muscle contraction and neuronal excitability (McCarron et al., 2002; Vergara et al., 1998), side effects to smooth muscle cells and neurons must be considered.

## 5.2 Implication of ATP-sensitive $K^+$ channels ( $K_{ATP}$ ) in glioma cell proliferation and glioma therapy

The  $K_{ATP}$  channels are consisted of two different types of subunits, the inward-rectifying  $K^+$  channel member  $K_{ir6}$  and sulfonylurea receptor (SUR) subunit (Akrouh et al., 2009).  $K_{ATP}$  channels are found to be important for glioma cell proliferation and cell cycle progression (Huang et al., 2009). Compared to normal glial cells,  $K_{ATP}$  channels were highly expressed in glioma cell lines and glioma tissue samples and inhibiting  $K_{ATP}$  channels by its blocker tolbutamide or by siRNA targeting  $K_{ir6.2}$  subunit could decrease U251 and U87 glioma cell proliferation. Moreover, enhancing  $K_{ATP}$  channel activity by its opener diazoxide or by overexpressing  $K_{ir6.2}$  or SUR1 subunit could increase glioma cell proliferation. The regulation of proliferation was through regulation of cell cycle progression because inhibition of  $K_{ATP}$  channels led to cell cycle arrest in G0/G1 phase. In animal experiments,

subcutaneous co-injection of glioma cells with tolbutamide or with diazoxide could decrease or increase the growth of xenograft tumor, respectively. These results indicate  $K_{ATP}$  channels to be a potential target in glioma therapy.

$K_{ATP}$  channels are mainly present in heart (Snyders, 1999), pancreatic cells (Bokvist et al., 1999) and smooth muscle cells (Quayle et al., 1997), side effects to these tissues and cells have to be considered.

### **5.3 Implication of two-pore domain $K^+$ channel TASK3 in glioma cell death and glioma therapy**

The TASK3 (TWIK-related acid-sensitive  $K^+$  channel, KCNK9) channel belongs to the two-pore domain  $K^+$  channels (Enyedi & Czirjak, 2010). It is involved in regulating glioma cell death (Meuth et al., 2008). In high  $[K^+]_{ex}$  medium, activation of TASK3 channel by its opener isoflurane resulted in a reduction of glioma cell survival and inhibition of TASK3 channel by its blocker bupivacaine or spermine could reverse isoflurane-induced cell death. These results suggest that under high  $K^+$  environment, TASK3 channel activation actually promotes glioma cell death.

As a newly discovered gene, many normal functions of TASK3 remain to be discovered. But since TASK3 has been found to express in many organs, including brain, kidney, liver, lung, colon, stomach, spleen, testis and skeletal muscle (Kim et al., 2000), the side effect of targeting TASK3 channels has also to be considered.

### **5.4 Implication of hERG1 in glioma angiogenesis and glioma therapy**

The hERG1 (human *ether a go-go* related) channels (KCNH2 or Kv11.1) belong to the voltage-gated  $K^+$  channel family and are composed of four  $\alpha$  subunits (Asher et al., 2010). hERG1 is overexpressed in many types of human cancers (Arcangeli, 2005). hERG1 is also overexpressed in human glioblastoma and is important for VEGF secretion in glioma cells (Masi et al., 2005). hERG1 current was recorded in primary glioma cells and by immunohistochemistry analysis, hERG1 was found to be highly expressed in glioblastoma multiforme. It is well known that secretion of angiogenic factors by glioma cells can promote angiogenesis and tumor malignancy. In U138 glioma cells which expressed functional hERG1 channels, channel blocker WAY could inhibit cellular VEGF secretion and this inhibition was not observed in A172 glioma cells, which did not express functional hERG1 channels. These results suggest that hERG1 channels may boost glioma malignancy by promoting angiogenic factor secretion and this channel is a possible target for anti-glioma therapy.

Side effects to heart, pancreas and colon should be considered, where hERG1 is abundantly expressed (Luo et al., 2008).

### **5.5 Implication of acid-sensing ion channels (ASIC) in glioma cell migration and glioma therapy**

The ASIC channels are a group of amiloride-sensitive, voltage-independent  $Na^+$  channels and can be activated by decreased pH. The ASIC channels are homotetrameric, which are assembled by the known subunits ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3 and ASIC4.

ASIC subunits have two transmembrane domains. Functions of ASIC channels involve perception of pain, ischaemic stroke, mechanosensation and so on (Krishtal et al., 2003; Wemmie et al., 2006).

ASIC channels are functionally expressed in glioma cells and contribute to glioma cell migration (Kapoor et al., 2009). In D54-MG glioma cells, ASIC1 was found to express higher than in primary human astrocytes. D54-MG glioma cells showed amiloride and psalmotoxin (ASIC inhibitors)-sensitive whole cell current under basal condition, indicating that glioma cells expressed functional ASICs. ASIC1 dominant-negative mutant transfection could decrease the whole cell current, and meanwhile, it also inhibited D54-MG cell migration as indicated by transwell assay. These results suggest that targeting ASIC1 channels might be another anti-glioma approach by disrupting glioma cell migration.

ASICs are widely expressed throughout the central nervous system and peripheral nervous system, targeting ASIC channels therefore should avoid side effects to the nervous system (Krishtal et al., 2003; Wemmie et al., 2006).

## 6. Cl<sup>-</sup> channels and glioma

The Cl<sup>-</sup> channel is a superfamily of ionic channels that are relatively poorly understood. They are either voltage-gated or ligand-gated. Three Cl<sup>-</sup> channel families have been identified, the ClC, CFTR and ligand-gated GABA and glycine receptors. The ClC channels are dimerized from subunits, which might have 17 intra- or trans-membrane domains (Duran et al., 2010). Cl<sup>-</sup> channels take function in the regulation of cell resting membrane potential, cell volume, cell migration, proliferation and differentiation. Two types of voltage-gated Cl<sup>-</sup> channel family members 2 and 3 (ClC2 and 3) were found to functionally express in D54MG glioma cells (Olsen et al., 2003). ClC3 has been reported to be involved in glioma cell invasion and cell cycle progression. In D54MG glioma cells, ClC3 channels mediated the Cl<sup>-</sup> current, which was required for pre-mitotic condensation (PMC) (Habela et al., 2008). PMC refers to obligatory cytoplasmic condensation process happened before mitotic phase and it is required for M phase progression. Besides cell cycle progression, ClC3 was also involved in STTG1 and U251 glioma cell invasion (Lui et al., 2010).

Chlorotoxin (CTX), a peptide from scorpion venom, is a small-conductance Cl<sup>-</sup> channel blocker. CTX was found to specifically bind to the cell surface of glioma cell both in vitro and in vivo (Soroceanu et al., 1998), although the mechanism is still not clear. In vitro and in vivo delivery of CTX could well inhibit glioma invasion. This is because besides Cl<sup>-</sup> channels, CTX has many other targets, for example matrix metalloproteinase 2 (MMP2), and it has been reported that specifically up-regulation of MMP2 in glioma cells accounted for the anti-invasive effect of CTX to glioma cells (Deshane et al., 2003). Iodine-131 labeled synthetic CTX (131I-TM-601) has been used for phase I clinical trial of treating recurrent malignant glioma (Mamelak et al., 2006). Intracavitary administration of 131I-TM-601 (0.25mg to 1 mg) was well tolerated with no observed toxicity. 131I-TM-601 could specifically bind to tumor tissues and was minimally taken by any other organ system. Furthermore, 131I-TM-601 treatment was proved to improve patient outcome to certain extent. Based upon these studies, CTX seems to be a potential drug for glioma targeting and therapy, although the working mechanism may not necessarily be through inhibiting Cl<sup>-</sup> channels.

## 7. Ionic channels in brain tumor stem cells

In recent years, the concept of cancer stem cell (CSC) stands on the research focus (Gupta et al., 2009; Maitland & Collins, 2010; Takebe et al., 2010). CSCs are a population of cancer cells found in the tumor mass or hematological tumors. Unlike other cancer cells, CSCs possess the ability of reconstituting an entire tumor by giving rise to all cell types within the tumor, because CSCs have the characteristics of normal stem cells, which include the ability of self-renew, differentiation and proliferation. CSCs were firstly identified in leukemia (Bonnet & Dick, 1997), and were subsequently identified in many types of solid tumors, including brain (Singh. S et al., 2004), breast (Al-Hajj et al., 2003), ovarian (Zhang et al., 2008), colon (O'Brien et al., 2007), pancreatic (Li et al., 2007), prostate tumor (Maitland & Collins, 2008) and melanoma (Schatton et al., 2008) etc. CSCs have a completely different gene expression profile to other tumor cells, are extremely tumorigenic and are usually radiochemo-resistant. Although traditional therapy can kill most of the tumor cells, CSCs are considered to be mainly responsible for the relapse of tumor. The identification of brain tumor stem cells was first reported in 2004 (Singh. S et al., 2004). By dissecting primary surgical GBM or medulloblastoma samples, the authors have found that only the CD133<sup>+</sup> tumor cells within the tumor mass were capable of tumor initiation in SCID (severe combined immunodeficient) mouse brains. Injection of 100 CD133<sup>+</sup> cells was sufficient for xenograft tumor formation, whereas injection of 10<sup>5</sup> CD133<sup>-</sup> cells did not cause tumor formation. Importantly, the xenograft tumor histologically resembled the original tumor from patients. Further studies have revealed that the CD133<sup>+</sup> glioma cells promote glioma radioresistance and chemoresistance (Bao et al., 2006; Liu et al., 2006). Finding ways of targeting glioma stem cells are of great significance for therapy of malignant glioma.

As for targeting ionic channels, the implications of ionic channels in brain tumor stem cells have just begun to be understood. Many types of ionic channels seem to be highly expressed in brain tumor stem cells. In neuroblastoma cells, SH-SY5Y, CD133<sup>+</sup> cells (cell population in which CD133<sup>+</sup> cells% > 60%) were isolated as potential tumor stem cells, because CD133 is widely used as a cancer stem cell marker. In these CD133<sup>+</sup> cells, electrophysiological evidence indicated higher current density of large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels (BK) and tetrodotoxin (TTX)-sensitive voltage-gated Na<sup>+</sup> channels than in CD133<sup>-</sup> cells. Furthermore, RT-PCR analysis showed that mRNA expression of BK and Nav1.7 was higher in CD133<sup>+</sup> cells than in CD133<sup>-</sup> cell (Park et al., 2010).

BCNU is a commonly used chemotherapeutic agent for glioblastoma therapy, but in primary glioma tumor mass, there is a subpopulation of BCNU-resistant glioma cells, which are stem-like cells, because the authors found that this subpopulations expressed CD133, CD117, CD90, CD71, and CD45 cell-surface markers, and had the capacity for multipotency (Kang & Kang, 2007). In the dissociated BCNU-resistant glioma stem cells, there was a high expression of several types of ionic channels, the chloride intracellular channels 1 (CLIC1) was one of these high expression channels. When using the Cl<sup>-</sup> channel blocker, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) in combination with BCNU, DIDS increased the apoptosis of BCNU-resistant glioma stem cells *in vitro* and augmented BCNU sensitivity *ex vivo* (Kang & Kang, 2008). These studies suggest that CLIC1 channel may contribute to the BCNU-resistance of glioma stem cells and blockade of this channel may enhance the BCNU-sensitivity of glioblastoma.

Although the relevance of ionic channels with glioma stem cells is still obscure, the present studies imply that the expression of some channels are abnormal in glioma stem cells and may contribute to the malignant feature of glioma stem cells. Blocking of these channels may facilitate chemo- or radio-therapy of glioblastoma.

## 8. Targeting ionic channels in animal models

As discussed above, many types of ionic channels regulate glioma cell behavior and control glioma progression. However, a large number of these studies are restricted to *in vitro* experiments, which mainly rely on the results obtained from cultured glioma cell lines. Although they shed lights on the concept that ionic channels play important roles in glioma progression, they only provide limited information as to whether these ionic channels can actually be targeted *in vivo* and whether these channel blockers exert side effects in systemic use. In this section, the *in vivo* targeting of ionic channels in animal tumor models will be discussed.

In the studies of TRPC6 and glioma cell proliferation and cell cycle progression, the anti-glioma effect of adenovirus-mediated DN-TRPC6 was tested in intracranial glioma xenograft model. U87MG glioma cells were infected by DN-TRPC6 before implantation. In this *in vivo* experiment, the animal bearing DN-TRPC6-infected glioma cells survived longer than the animals bearing GFP-infected glioma cells and suggested the potent anti-glioma effect of DN-TRPC6 (Ding et al., 2010). Nevertheless, from the clinical aspect, the most convincing way for delivering adenoviral DN-TRPC6 would be tail vein or *in situ* injection after the implanted tumor has reached certain size.

SKF96365 is a small-molecule blocker for TRPC channels. SKF96365 was developed in the early 1990s as a blocker for receptor-mediated Ca<sup>2+</sup> entry, later it was found to block many types of TRP channels, including TRPC1, 3, 6 and 7. Additionally, it could block other types of TRP channels, such as TRPV2, TRPM8 and TRPP1 (Clapham, 2007; Fiorio Pla et al., 2005; Kim et al., 2003; Malkia et al., 2007; Mason et al., 1993; Merritt et al., 1990; Vazquez et al., 2004). Concerning glioma studies, SKF96365 has not been systemically used in animal models, but in the study of the implication of TRPC6 channels in gastric cancer progression, this drug has been applied intraperitoneally to suppress the subcutaneously implanted human gastric cancer cells in nude mice (6 weeks of age). SKF96365 was applied at the dose of 20 mg/kg daily for successive 5 days after 7 days of implantation and could apparently slow down the growth of xenograft. On the 51 day of implantation, the tumor volume in SKF96365-treated mice was approximately 20-30% smaller than in control mice. Meanwhile, physical conditions of the animals were not visibly deteriorating as compared to the animals receiving saline injection (Cai et al., 2009). The study suggested that SKF96365 at the above dose could be well tolerated by nude mice. However, the non-specificity of SKF96365 largely restricts the *in vivo* usage of SKF96365. New and specific TRPC6 channel blockers would be potential drugs for glioma therapy and the drug delivery approaches for treatment of glioma needs to be carefully designed. Because of the wide tissue distribution of TRPC6 channels, local rather than systemic delivery methods would be much desired.

IK channels regulate glioma progression. Clotrimazole is a putative inhibitor of IK channels (Jensen et al., 1998). Besides, it is also an inhibitor of cytochrome P-450 and translation initiation (Aktas et al., 1998; Ritter & Franklin, 1987). Application of clotrimazole suppressed

proliferation of both human GBM cells and rat glioma cells (C6 and 9L). For *in vivo* experiments, either C6 or 9L cells were intracranially implanted into the brain of male Fischer-344 rats (between 250 and 300 g), and after 5 days, the animals were injected intraperitoneally daily with clotrimazole at the dose of 125mg/kg body weight for 8 consecutive days. This treatment caused a significant inhibition of intracranial tumor growth. Moreover, the survival of rats with 9L implantation were compared among clotrimazole, cisplatin (a commonly used chemotherapy agent for glioma) and combination of the two group and animals in the combination group survived longer than other groups (Khalid et al., 2005), suggesting that clotrimazole may enhance the glioma sensitivity to cisplatin, although conclusion has to be further verified and the mechanism remains to be revealed.

Although based on the current report, BK channels do not involve in glioma cell proliferation, it regulates the opening of blood-brain tumor barrier (BTB). NS1619 is the agonist of BK channels and iberiotoxin is a putative blocker of BK channels. The permeability of BTB was measured by rat glioma model, in which rat glioma cell line RG2 was intracranially implanted in female Wistar rat (180-200g). NS1619 (26.66  $\mu\text{g}/\text{kg}/\text{min}$ ) or iberiotoxin (0.26  $\mu\text{g}/\text{kg}/\text{min}$ ) was co-infused with the radiotracer [ $^{14}\text{C}$ ]- $\alpha$ -aminoisobutyric acid ([ $^{14}\text{C}$ ]-AIB) by intracarotid infusion. By using quantitative autoradiographic method to quantify the radioactivity in the tumor area, the BTB permeability for [ $^{14}\text{C}$ ]-AIB could be accurately measured. By using this animal model, NS1619 was found to increase BTB permeability and iberiotoxin could decrease BTB permeability (Ningaraj et al., 2002). It was also found that infusion NS1619 with bradykinin could selectively enhance BTB permeability in brain tumors, not in normal brain (Hu et al., 2007). Moreover, iberiotoxin could reverse nitric oxide donors-induced increase in BTB permeability (Yin et al., 2008). NO can increase the vascular endothelial permeability and NO donors, such as L-arginine and hydroxyurea, could increase BTB permeability. These studies on the regulation of BTB permeability by BK channels suggest that pharmacologically regulating BK channel activity could potentially be used to improve glioma chemotherapy. The effectiveness and side effect of NS1619 and iberiotoxin remain to be verified in future animal experiments.

Besides BK channels, the  $\text{K}_{\text{ATP}}$  channel activator, minoxidil sulfate (MS) could also be used *in vivo* and increase the delivery of anti-glioma drugs such as temozolomide and herceptin by increasing the permeability of BTB. In this experiment model, MS (100  $\mu\text{g}/\text{kg}/\text{min}$  for 15 min) was intravenously injected into nude rats with xenografted GBM. Temozolomide was labeled by [ $^{14}\text{C}$ ], and herceptin was labeled by fluorescein and when they were coinjected, the drug delivery to the tumor was significantly increased, suggesting temozolomide or herceptin could be used in combination with MS to improve the effectiveness of standard chemotherapy (Ningaraj et al., 2009). Based on the present studies, different  $\text{K}^+$  channel agonists can affect BTB permeability, including BK channel agonist and  $\text{K}_{\text{ATP}}$  agonists.

In a *in vivo* matrigel plug assay, which was used to examine angiogenesis *in vivo*, the IK channel blocker TRAM-34 was found to regulate angiogenesis (Grgic et al., 2005). In this experiment, standard matrigel supplemented with bFGF was implanted subcutaneously into the flank of C57/BL6 mice. Under control condition, the matrigel would get vascularized, but when the mice were treated daily with TRAM-34 (120mg/kg) intraperitoneally for two weeks, the vascularization would be decreased by approximately 85%, suggesting that TRAM-34 had anti-angiogenesis effect *in vivo*. Meanwhile, no visible side effects or macroscopic organ

damage was observed. These results imply that TRAM-34 might exert anti-glioma effect in vivo by suppressing glioma angiogenesis and also imply the limited side effect of systemic use of TRAM-34. However, since TRAM-34 was delivered intraperitoneally in this study, whether TRAM-34 can pass the BTB remains to be further investigated.

As seen from the current available studies, several types of ionic channels are indeed potentially drug targets in treating glioma based on the in vivo data. The results obtained from the in situ (intracranial) glioma model seem to be much more convincing than the subcutaneous model, although different brain tumor in situ animal models may affect the final readout of these experiments (Barth & Kaur, 2009).

## 9. Chapter summary (At a glance)

Ionic channels play essential roles in glioma cell behavior, several types of  $\text{Ca}^{2+}$ ,  $\text{K}^{+}$ ,  $\text{Na}^{+}$  and  $\text{Cl}^{-}$  channels are potential therapeutic targets for malignant glioma.

TRP channels are newly found anti-glioma targets, some TRP channels are overtly expressed in human malignant glioma and they take function in glioma cell proliferation, migration or invasion.

Targeting several ionic channels might facilitate outcome of conventional chemo- or radio-therapy for malignant glioma.

Targeting ionic channels to treat malignant glioma remains in preclinical stage. Small-molecule compounds against ionic channels are experimentally tested in animal models. Glioma-related channel biology has to be more carefully studied before the possible clinical usage of channel drugs.

## 10. Summary and perspective

Many types of  $\text{Ca}^{2+}$ ,  $\text{K}^{+}$ ,  $\text{Na}^{+}$  and  $\text{Cl}^{-}$  channels have been implicated in glioma progression and serve as potential targets for malignant glioma therapy, but the studies linking ionic channels and glioma are a relatively new area in glioma therapy and very limited knowledge has been provided as to how ionic channels contribute to the glioma progression. Therefore, although the relation between ionic channels and glioma are getting clearer, there is still a long way to go to use ionic channels as potential drug targets in treating glioma. There are several major obstacles in this direction. First of all is the possible side effects of targeting ionic channels. Because ionic channels are rather universally expressed in different types of normal tissues, possible side effects have to be considered when targeting ionic channels to treat glioma. The cardiovascular system is the tissue that has to be considered in priority, because many types of ionic channels play important roles in regulating the normal functions of cardiovascular system. The possible side effects to nervous system also need great attention, because of the critical involvement of ionic channels in regulating normal neuronal function. Another obstacle is the permeability of BTB of these channel drugs. How they can be efficiently delivered to the glioma tumor tissue needs serious attention.

Because glioma is a multi-gene disease, combinative inhibition of multiple signal pathways is a promising strategy in glioma therapy. For example, simultaneous inhibition of EGFR and mTOR (Rao et al., 2005), RAF and mTOR (Hjelmeland et al., 2007) have been

experimentally studied. However, the ionic channel-related signal pathways in glioma cells are poorly understood, and it is not known if there are certain pathways that are overtly activated to compensate the inhibition of specific channels. It would be ideal if we can target both the ionic channels and their compensatory pathways to maximize inhibition of glioma cells.

The ionic channels have several features as listed below, based on which the channel-targeting strategy could be theoretically justified. a). Ionic channels have membrane localization and are easily accessible to drugs, some types of channels have highly specific antagonists. b). Some types of channels have selective up-regulation in glioma cells. For example, TRPC6, KATP, hERG1 and CIC3 expression levels are very high in malignant glioma cells, but are low in normal glial cells or benign glioma cells. c). Channel blocker may boost the effect of standard glioma therapy. For example, TRPC6 blocker could be used as radiosensitizer for malignant glioma. Irradiation is a standard and effective therapy for malignant glioma and radiosensitizers could reduce the required irradiation dose and minimize damage to normal tissues. Inhibition of TRPC6 channels arrests glioma cell cycle in G2/M phase, which is an irradiation-sensitive phase, therefore, TRPC6 blocker may be a potential radiosensitizer for malignant glioma. d). Channel drugs can be used in combination with chemotherapy agents. Since several types of channel drugs can enhance the permeability of BTB, thus may facilitating the delivery of standard chemotherapy agents, such as temozolomide and BCNU.

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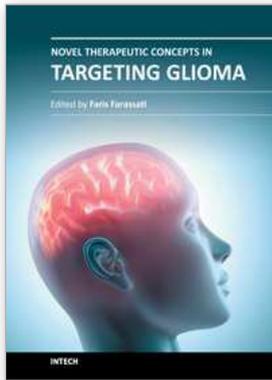
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Unit 405, Office Block, Hotel Equatorial Shanghai  
No.65, Yan An Road (West), Shanghai, 200040, China  
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Phone: +86-21-62489820  
Fax: +86-21-62489821

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