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Regulation of Glioma Stem Cells by the Notch Signaling Pathway: Mechanisms and Therapeutic Implications

Hong-Yan Qin, Luo-An Fu and Hua Han
State Key Laboratory of Cancer Biology,
Department of Medical Genetics and Developmental Biology,
Fourth Military Medical University, Xian 710032,
China

1. Introduction

Glioma is the most common malignant tumor of the central nervous system (CNS) following with high fatality. A large body of evidence has been accumulated showing that a group of neural stem cell (NSC)-like tumor cells in glioma, or glioma stem cells (GSCs), play a critical role in the initiation and the progression of glioma. The Notch signaling pathway is a highly conserved pathway maintaining stem cell state and governing the differentiation of NSCs. Several research groups, including ours, have recently shown that Notch signaling is also critically involved in the maintenance of GSCs. Most impressively, blocking Notch signaling appears to result in the exhaustion of GSCs in animal models, leading to the proposition that the Notch signaling pathway might be a potential therapeutic target of glioma. In this review, we will briefly introduce the concepts of cancer stem cells (CSCs) and GSCs, followed by a summary of the cellular and molecular properties so far uncovered about GCSs. We will then discuss the major progresses about the roles and mechanisms of Notch signaling in the proliferation, differentiation, and apoptosis of GSCs, in addition to the function of Notch signaling in tumor microenvironment especially stem cell niches. Finally, we will prospect the application of Notch signaling as a therapeutic target of glioma, emphasizing its potential advantages and disadvantages.

2. Glioma stem cells (GSCs)

2.1 An overview of cancer stem cells

It is believed that the earliest hint of CSCs was proposed about 150 years ago by pathologists Rudolph Virchow and Julius Cohnheim, who discovered certain similarities between developing fetus and terotoma, such as that they both possess proliferation and differentiation abilities (Dick, 2009). However, solid evidence of CSCs has not been available until Dick and co-workers found that only rare cells in progressively diluted leukemia cells, which were harvested from patients, had the ability to reproduce leukemia in immunodeficiency mouse models. They named these cells as leukemia initiating cells (LICs)

that had greater differentiation potential and looked like stem cells in normal organs (Lapidot et al., 1994). Subsequently, CSCs have been isolated from many solid tumors, such as breast cancers (Al-Hajj et al., 2003), myelomas (Niederwieser et al., 2004), brain tumors (Singh et al., 2003, 2004), pancreatic (Meszoely et al., 2001) and prostate cancers (DL Hudson, 2004), among others. All these observations indicate that these stem-like cell populations may represent CSCs in cancer; they have strong potentials for self-renewal and multi-lineage differentiation and may responsible for tumor initiation and growth, invasion and metastasis, chemo- and radio-resistance. As a result, CSC theory was expected to bring promising innovations on finding new tumor therapeutic targets and improving therapeutic strategies.

2.2 Identification of GSCs

Glioma is believed to be derived from the glial tissue and is classified into astrocytoma (70%), oligodendroglioma (10% to 30%), mixed oligoastrocytoma and ependymoma (less than 10%). Low grade giloma, mostly astrocytomas (WHO grade II), are progressively transforming into malignant glioma, that is, anaplastic tumors (WHO grade III) and ultimately into glioblastoma multiforme (GBM; WHO grade IV). Because of their high heterogeneity and infiltrating nature, traditional surgery, unspecific and cytotoxic chemotherapy and radiotherapy can only impede glioma progression for a limited time. In addition, during treatment chemo- and radio-therapy resistance were often concomitant, followed by tumor relapse (Lino et al., 2010).

In 1920s, the famous neuropathologist Bailey postulated that tumor cells in the brain were mostly derived from the transformation of normal astrocytes, oligodendrocytes or neuronal precursors. However, this hypothesis was challenged following the isolation of NSCs by Reynolds and Weiss in 1992. Since then researchers started to speculate if malignant glioma results from the transformation of NSCs (Oliver& Wechsler-Reya, 2004).

The first evidence of cells with stem-like characteristics in malignant was reported by Ignatova et al., who isolated clonogenic, neurosphere-forming precursors from post-surgery specimens of human GBM. These stem-like cells expressed both neuronal and astroglial markers on differentiation, together with several key determinants of NSC fate (Ignatova et al., 2002). Later in 2003, Singh et al. also found a part of cancer cells from medulloblastoma can form neurospheres in vitro culture conditions, and some surface markers for NSCs, such as Nestin, Musashi-1, Bmi-1 and CD133, were also identified on these neurosphere cells. These neurosphere cells even possessed stronger capacities of proliferation, self-renewal and differentiation than neurosphere cells derived from normal NSCs. Corresponding to NSCs, these cancer cells were named as GSCs. GSCs could differentiate in culture into tumor cells that phenotypically resembled tumor cells from the patient, and also these cells can further differentiate into neuron or astrocytes under differentiation condition. These studies suggested that GCSs might originate from the transformation of NSCs (Singh et al., 2003). The further study by Singh et al. showed that injection of as few as 100 $CD133^+$ GSCs into immuno-compromised hosts produced tumors that could be serially transplanted and phenotypically resembled the patients' original tumors. In contrast, the injection of as many as 10^5 $CD133^-$ cells did not grow a tumor (Singh et al., 2004). At the same time, Kondo et al. successfully isolated side population (SP) from glioma cell line C6 by Hoechst 33342 staining, which shared similar prosperities as GSCs (Kondo et al., 2004). All these studies indicate that a minority of stem-like cells can represent GSCs in glioma.

2.3 The roles of GSCs in glioma

GSCs share similarities to normal NSCs in the brain, therefore possess the potential for self-renewal and multi-lineage differentiation. These population cells may thus play important roles in glioma initiation and growth, metastasis, drug resistance and disease relapse.

In contrast to Singh et al. finding that $CD133^-$ neurosphere cells did not recapitulate tumors in SCID mouse, Zheng et al. found that both $CD133^-$ and $CD133^+$ cells isolated from C6 glioma cell line could form neurospheres and show GSC features by neurosphere assay. This result suggested that $CD133^-$ cells could de-differentiate into stem cells or progenitor cells to generate tumorigenic clones again (Zheng et al., 2007). The observation was supported by two other independent groups: Beier et al. and Zhang et al. showed that $CD133^-$ tumor cells possessed apparent stem cell-like properties, but had distinct molecular profiles and growth characteristics from $CD133^+$ tumors in vitro and in vivo (Beier et al., 2007; Zhang et al, 2006). However, no matter with these controversial findings, these stem-like cells indeed exist and are involved in glioma initiation and recurrence.

Although the relationship between CSCs and metastasis has not been elucidated clearly, it has been demonstrated that the number of metastasis cancer colonies is correlated with the frequency of CSCs in primary tumors, and cancer cells undergoing epithelium-mesenchymal transitions (EMT) displayed with some stem-like cells properties (Mani et al., 2008). It is known that on one hand EMT is a differentiation switch between polarized epithelial cells and motile mesenchymal cells, and facilitates cell movements and generation of new tissues during embryogenensis. On another hand, EMT has been found to contribute to tumor invasion and vascular intravasation during cancer metastasis (Yang & Weinberg, 2008). The recent recognition of mesenchymal change in glioblastoma and its association with more aggressive clinical phenotypes suggest that mechanisms promote EMT in carcinoma may be of great clinical relevance in GBM. Recently Mikheeva et al. demonstrated that the transcription factor TWIST1 plays an important role in glioma invasion through activation of mesenchymal change, and suggesting its potential as a therapeutic target (Mikheeva et al., 2010). However, whether TWIST1 is involved in GBM metastasis by EMT process through regulating GSCs still needs to be further investigated.

Except the hallmark of "stemness" discussed above, it has been postulated that tumor stem-like cells may also possess a number of other properties associated with normal stem cells, including a slow proliferation rate, active DNA damage repair and antiapoptotic pathways, and the expression of multidrug transporters on the plasma membrane (Dean et al., 2005). Indeed, it has been shown that glioma cells exhibiting the side-population phenotype, which is characterized by cellular exclusion of the dye Hoechst 33342 primarily attributed to the ABCG2 multidrug transporter, are enriched in GSCs (Kondo et al., 2004). Furthermore, $CD133^+$ GSCs have been found to resist doses of radiation lethal to surrounding non-stem glioma cells in the tumor by preferential activation of the DNA damage response (Bao et al., 2006). The retention of such properties by GSCs suggests that, like normal stem cells, GSCs may be inherently resistant to many traditional anticancer therapies that target rapidly dividing cells. This represents a daunting therapeutic challenge because the characterization of the GSCs as the proliferative driving force in the tumor suggests that GSCs must be eradicated to permanently or significantly arrest tumor growth. As such, there is growing interest in developing therapeutic strategies specifically aimed at eliminating or affecting the GSCs population.

3. The Notch signaling pathway

3.1 Notch signaling

The *Notch* gene was first discovered by Thomas Morgan in 1914 in the fruit fly *Drosophila melanogaster*, with an adult phenotype consisting of "notches" at the wing margin. Genetic analysis of Notch loss-of-function mutations also revealed an embryonic phenotype with an expanded population of neuroblasts at the expense of epidermal cells (Artavanis-Tsakonas et al., 1995). In 1985, Artavanis-Tsakonas et al. successfully cloned the *Notch* gene, which encodes a large type I transmembrane receptor consisting of the extracellular domain (ECD) that containing 36 epidermal growth factor-like tandem repeats and 3 cysteine-rich LIN-12 repeats, a transmembrane domain (TMD), and the intracellular domain (NICD). Therein NICD is composed of a RAM (RBP-J association molecule) domain, nuclear localization signals (NLS), an ankyrin repeats (ANK), transactivation domain (TAD), and a praline/glutamate/serine/threonie-rich (PEST) region that is involved in protein degradation (Bray, 2006).

The Notch signaling pathway is evolutionarily highly conserved. The canonical Notch signaling pathway mainly comprises receptors, ligands, transcriptional complex components in the nucleus, and downstream genes, with a growing roster of regulatory molecules. In mammals, four Notch receptors (Notch1-4) are activated by five type I transmembrane ligands, three Delta-like (Dll1, Dll3 and Dll4) and two Serrate/Jagged (Jag1 and Jag2) receptors. When Notch is triggered by direct interaction with its ligands, the Dll/Jagged family proteins expressed on neighboring cells, NICD is released from the membrane after receptor cleavage executed by a γ-secretase-like protease. NICD translocates into nucleus and associates with RBP-J through its N-terminal RAM domain, and transactivates promoters harboring RBP-J-binding sites, which leads to expression of genes associated cell differentiation, such as the hairy/enhancer of Split (HES) family of basic helix-loop-helix (bHLH) transcription factors. Inhibitors of γ-secretase (GSIs) are widely used to block Notch signaling in vitro and in vivo. Moreover, in the absence of transactivators such as NICD, RBP-J suppresses transcription of promoters recognized by RBP-J (Bray, 2006). Although RBP-J has been generally accepted as the major effector of Notch pathway, a non-canonical RBP-J-independent and Deltex-dependent alternative pathway has also been reported in human and in *Drosophila* (Martinez Arias et al., 2002).

3.2 Modulators of Notch signaling

During Notch signal activation, NICD translocates into nucleus and transactivates downstream gene promoters harboring RBP-J-binding sites through recruiting co-activators such as GCN5, p300/CBP and the Mastermind-like (MAML) proteins (Moellering et al., 2009). In addition to NICD, RBP-J also mediates transactivation of Epstein Barr (EB) virus nuclear antigen (EBNA) 2 and therefore may play a role in the immortalization of cells infected by EB virus (Waltzer, 1994; Henkel, 1994). On another hand, in the absence of transactivators such as NICD or EBNA2, RBP-J suppresses transcription of promoters recognized by RBP-J (Dou et al., 1994). This is mostly attributable to multiple co-suppressors and/or adaptor molecules recruited by RBP-J. Kao et al. and Zhou & Hayward reported that SMRT/N-CoR interacts with RBP-J and suppresses the RBP-J-mediated transcription by competing with NICD and by recruiting histone deacetylases (HDACs), which renders the chromatin into an architecture that is inaccessible to the general transcriptional machinery (Kao, 1998; Zhou & Hayward, 2001). In addition, CIR (CBF1 interacting co-repressor),

another RBP-J-interacting protein, also suppresses the RBP-J-mediated transactivation by associating with HDAC2 and SAP30 (Hsieh et al., 1999). Recently, Oswald et al. and Kuroda et al. demonstrated that MINT (MSX2-interacting nuclear target protein), a nuclear matrix protein interacting with SMRT/N-CoR, also interacts with RBP-J and suppresses the RBP-J-mediated transactivation by competing for binding sites and by recruiting co-repressors (Oswald, 2002; Kuroda, 2003). Taniguchi et al. and Qin et al. subsequently demonstrated that a LIM domain protein KyoT2 can inhibit RBP-J-mediated Notch signal transactivation by competing with NICD, and by recruiting Polycomb suppression complex containing HPC2 and RING1 protein through the LIM domains (Taniguchi, 1998; Qin, 2004, 2005) (Fig.1).

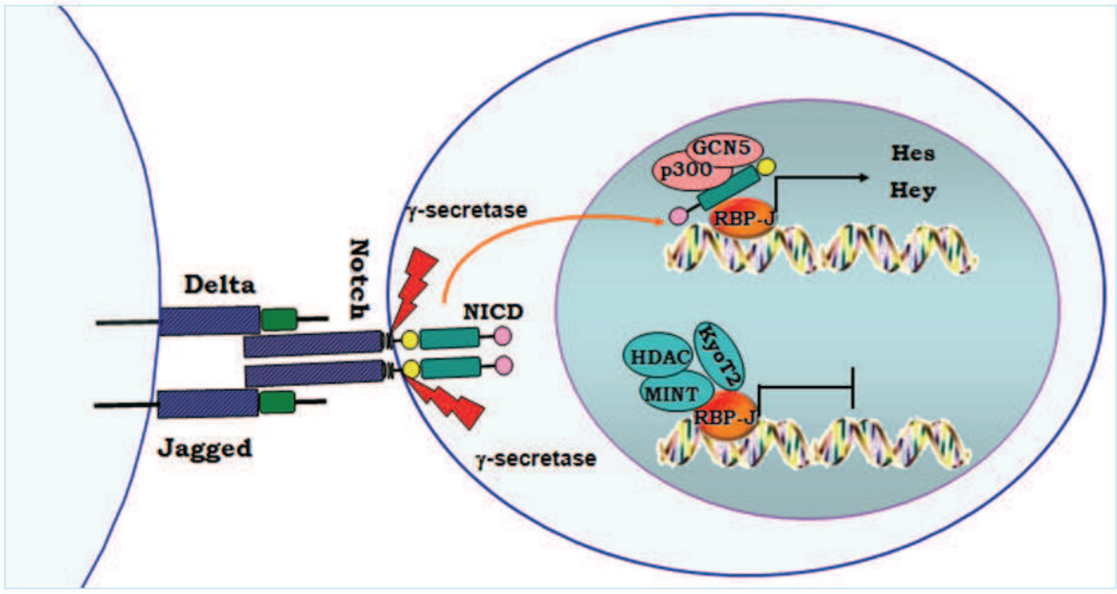

Fig. 1. The canonical Notch signaling pathway and its regulatory mechanisms

Productive Notch ligand-receptor binding depends on posttranslational modifications, such as glycosylation of receptors mediated by OFUT-1 and Fringe (Heines & Radtke, 2003). The half-life of Notch and DSL proteins on membrane is determined by the endocytosis of receptors and ligands, executed mainly by ubiquitin E3 ligase such as Deltex and Mindbomb, respectively (Kopan and Ilagan, 2009). In addition, the local distribution of Notch receptors on the cell membrane are controlled by some polarity proteins, for example, Numb and Crumbs, which result in region-specific Notch activity (Ilagan and Kopan, 2007). Taking all these observations together, Notch signal pathway should be regulated within a subtle and strict molecular network.

4. The roles of Notch signaling in the proliferation and differentiation of GSCs

4.1 Notch signaling in normal NSCs

Proliferation and differentiation of progenitor cells and stem cells during development are controlled by signals from other cells. The Notch signaling pathway plays a pivotal role in the regulation of the progenitor as well as stem cell differentiation by mediating cell-cell interaction, and is involved in multiple human diseases. During the development of

vertebrate CNS, NSCs in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampus undergo self-renew and give rise to neurons and glia in a spatially and temporally defined manner (Temple,2001; Doetsch, 2003). NSCs, which possess the characteristics of radial glial cells (RGCs), can proliferate and self-renew through symmetric cell division (Alvarez-Buylla et al., 2001; Merkle and Alvarez-Buylla, 2006). As the development proceeds, some NSCs generate certain types of early-born neurons (e.g., Cajal-Retzius cells), while other NSCs differentiate into intermediate neural progenitors (INPs) through asymmetric cell division (Lillien, 1998). It has been demonstrated that the RBP-J-mediated Notch signaling plays an important role in the maintenance and the differentiation of NSCs into INPs and neuron/glial cells.

During early neurogenesis, activated Notch signaling has been shown to promote RGC identity (Gaiano et al., 2000). This was supported by Mizutani et al., who recently reported that Notch signaling determines the differentiation of NSCs into INPs through differential activation of RBP-J (Mizutani et al., 2007). Notch1 mutant mice have fewer NSCs and INPs, as shown by that the frequency of neurosphere formation is reduced when NSCs and INPs are cultured under the colony-forming condition (Hitoshi et al., 2002; Yoon et al., 2004). This is accompanied by precocious neuronal differentiation indicated by the precocious expression of proneural bHLH genes such as Mash1, Math1, and NeuroD (de la Pompa et al., 1997; Lutolf et al., 2002), and the neuron specific marker β-tubulin-III (Yang et al., 2004), and by the decreased expression of the NSC marker, nestin (Lutolf et al., 2002) as well. Mice deficient for other Notch pathway molecules also displayed similar phenotypes (Ishibashi et al., 1995; de la Pompa et al., 1997; Ohtsuka et al., 1999; Handler et al., 2000; Ohtsuka et al., 2001; Hatakeyama et al., 2004). With conditional RBP-J knockout mice, we have reported that RBP-J-mediated signaling might inhibit the differentiation of NSCs into INPs, while support the generation of certain early born neurons at early neurogenic stages, and the differentiation of neurons and glial cells at later neurogenic stages. This study suggested the RBP-J-mediated Notch signaling may regulate neuronal differentiation by the developmental stage-dependent way (Gao et al., 2009).

In addition, the Notch pathway is also crucial for many other binary cell fate decisions during CNS development (Louvi and Artavanis-Tsakonas, 2006). As Notch signaling inhibits NSCs from differentiating into neurons, Notch was also reported to inhibit neuronal while promote glial fate, and to promote the differentiation of glial progenitors into astrocytes versus oligodendrocytes (Tanigaki et al., 2001; Grandbarbe et al., 2003; Taylor et al., 2007). The regulation of binary cell fate decisions by Notch signaling pathway can be related to the pioneer observation on neuroblast and epidermal cell fate decision during *Drosophila* development (Heitzler and Simpson, 1991).

4.2 Notch signaling in GSCs

The many similarities in the growth characteristics and gene expression profiles between NSCs and GSCs suggest that similar signaling pathways should be required for their survival and growth. Notch signaling is known to promote the survival and proliferation of NSCs and to inhibit differentiation (Gaiano and Fishell, 2002). It is reasonable to assume that Notch signaling plays an important role in GSCs.

Aberrantly activated Notch signaling is involved in the generation and progression of many tumors (Radtke and Raj, 2003). Studies from Purow group showed that Notch1, Dll1 and Jagged1 were overexpressed in many glioma cell lines and primary human giloma. When

glioma cell lines were transfected with si-RNA against Notch1 and its ligands, proliferation inhibition and apoptosis induction were observed. This phenomenon was also observed in vivo with mice injected with si-Dll1 and si-Jag1-transfected glioma cells (Purow et al., 2005). However, it is worth to note that the expression pattern of Notch receptors and ligands are variable in different grade glioma, which may indicate that Notch signaling plays divergent roles in different type of glioma (Purow, 2005; Somasundaram, 2005; Phillips, 2006; Kanamori, 2007; Gao, 2007; Zhang, 2008; Hu, 2011).

Recently, further studies about Notch function in GSC maintenance have been published. By measuring HES1 and NICD2, Fan et al. found that Notch activation was elevated in the $CD133^+$ cell fraction in medulloblastoma cell lines. Inhibiting Notch activation by GSI resulted in diminished proliferation, increased neuronal differentiation, reduced $CD133^+$ cell fraction in vitro, and decreased tumorigenicity in vivo. Conversely, $CD133^+$ cell fraction was expanded by activating Notch2 signaling with constitutively activated NICD2 plasmid. In addition, they also found Notch blockade induced more apoptosis in nestin-positive medulloblastoma cells than that in better-differentiated cells lacking nestin. Their data indicate that Notch signaling is important in maintaining GSCs, and Notch signaling blockade can deplete GSCs that is required for in vivo glioma formation by suppressing proliferation and inducing apoptosis or differentiation (Fan et al., 2006).

We found that human glioma cell line SHG-44 stably transfected with NIC plasmid grew significantly faster and had higher colony- and sphere-forming abilities than the parental and control cell lines. These colonies expressed nestin, and could differentiate into three neural lineages, namely neurons, astrocytes or oligodendrocytes. This study indicated Notch signaling play a role in generation and/or maintenance of GSCs in human glioma (Zhang et al., 2008). In a latest study, Hu et al. further demonstrated Notch signaling maintained patient-derived GSCs by promoting their self renewal and inhibiting their differentiation into INP-like cells. Blocking Notch signaling by GSI in cultured tumor sphere can exhaust GSCs from glioma, although some tumor spheres display resistance to GSI treatment (Hu et al., 2011). Taken together, these studies strongly indicate that Notch signaling maintains GSC stemness in glioma as in normal NSCs.

4.3 Notch in stem cell niches

Stem cells reside within specific microenvironment with defined anatomical location termed “niches”. SVZ and SGZ of the hippocampus region are the primary regions in which NSCs reside and support neruogenesis in the brain (Temple et al., 2001; Doetsch et al., 2003). Given the demonstrated similarities between NSCs and GSCs, it is reasonable to presume that GSCs may also, like NSCs, exist within a supportive niche. Through contact-mediated and paracrine signaling interactions between stem cells and the niche microenvironment, the niche maintains and controls critical stem cell properties and functions (Scadden et al., 2006).

The continued tumor growth is often associated with neovascularization (Gimbrone et al., 1972). Soluble factors secreted by tumor cells (such as vascular endothelial growth factor [VEGF]) induce angiogenesis, offering the necessary route for cell dissemination, changing vascular integrity and permeability, and even promoting intravasation and extravasation (Hashizume et al., 2000) . Many studies have shown that proangiogenic growth factors such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), which would ostensibly be in higher concentration near blood

vessels, permit maintenance and expansion of GSCs in culture (Singh et al., 2003, 2004; Yuan et al., 2004; Kondo et al., 2004). Soluble factors (such as VEGF) secreted by vascular endothelial cells have been found to promote self renewal and to inhibit differentiation of NSCs, suggesting that NSCs have a vascular niche (Shen et al., 2004). This finding raises the possibility that GSCs may also rely on interaction with a vascular niche to maintain their stem-like properties, and consequently, their ability to drive tumor growth. Indeed, a very recent study provides compelling evidence that brain tumor stem-like cells are supported by a vascular niche (Calabrese et al, 2007). According to these studies, Folkins et al. combined both antiangiogenic and cytotoxic drugs to treat glioma, and found a significant reduction in sphere forming units (SFU) in tumor. Their work highlights the possibility that selective eradication of GSCs may be achieved by targeting the tumor microenvironment rather than the GSCs directly (Folkins et al, 2007).

Notch signaling pathway has been found to regulate the vascular formation. For example, haplo-insufficiency of Dll4 in mice results in embryonic lethality due to defective vascular development (Gale et al., 2004). Dll4 Expression had been shown to be up-regulated in tumor vessels of several human tumors and its expression correlates with VEGF level in clear cell-renal carcinoma (Malihos et al., 2001; Patel et al., 2005; Li et al., 2007). Pro-angiogenic factors VEGF has been shown to induce Notch1 expression in arterial endothelial cells, which in turn can promote angiogenesis (Liu et al., 2003). Further studies show that mutant mice with disrupted Notch signaling display various defects in blood vessel formation, and deletion of RBP-J can disturb the vascular homeostasis in mouse neural retina by affecting VEGFR1 and VEGFR2 (Dou et al., 2008; Phng & Gerhardt, 2009). In stem cells niches, cell adhesion seems another key factor for the exact location of stem cells. With Notch1, RBP-J and Hes1 deleted mouse models, some studies showed the adherence junctions between retinal progenitor cells (RPCs) in mouse retina are severely disturbed, indicating Notch function on the maintenance of adherence junctions (Jadhav et al., 2006; Zheng et al., 2009). Hypoxic tumor microenvironment plays a key role in the regulation of the GSC phenotype through hypoxia inducible factor 2α (HIF2α) and subsequent induction of specific GSC characteristic genes, including MAML-3, nuclear factor of activated T cells 2 (NFAT2) and aspartate beta-hydroxylase domain-containing protein 2 (ASPHD2) (Seidel et al., 2010). HIF2α has been demonstrated to activate Oct4 that can accelerate cell proliferation as an oncogene (Keith and Simon, 2007). In response to hypoxia, several components of the Notch signaling cascade, such as Notch1, Hes1, Hey1, Dll1 and Dll4, are induced (Poellinger and Lendahl, 2008). Based on those studies, we can speculate that HIF2α may transactivate Oct4 expression through regulating Notch signaling, and then stimulate GSCs proliferation and maintain GSCs niches. However, the interplay mechanism between Notch and hypoxia in glioma still needs to be explored.

5. Mechanisms of the regulation of GSCs by Notch signaling

5.1 Downstream effectors of Notch signaling

Hes1, 5, 7 and Hey1, 2, L have been confirmed as direct targets of Notch signaling that are involved in transcriptional repression, and regulate the expression and function of pro-neuronal bHLH proteins, such as Mash-1 and Hash-1 in mammalian and Ac-Sc in *Drosophila* (Kageyama et al, 2005). Moreover, accumulating evidence have defined other Notch targets such as GFAP, cyclin D1, p21, p53, which are regulated by RBP-J dependent Notch signal pathway (Stockhausen et al, 2010).

These downstream molecules of Notch signaling are abnormally regulated in different grades of glioma. Somasundaram et al. showed that Hash1 mRNA was elevated in grade II, III, and secondary GBM, whereas its expression was unchanged in primary GBM compared with normal brain tissue. However, Hes1 expression was elevated in primary GBM (Somasundaram et al., 2005). This study may indicate different activation status of Notch signaling existing in different types of glioma. Recently, a study from Hu et al. showed that Hes1, Hes5 and Glast mRNA level was significantly decreased in cultured patient-derived primary glioma spheres when Notch signaling was blocked by GSI, meanwhile Mash1 expression, a pronerual gene antagonized by Hes genes, was up-regulated. This suggests that Notch-RBP-J-Hes signaling axis may play a critical role in the proliferation and differentiation of GSCs, as its function in NSCs (Hu et al., 2011).

5.2 Crosstalk between Notch signaling and other signaling pathways

In the development of brain, EGFR is expressed in neurogenic regions such as SVZ (Weickert et al., 2000). Given the established Notch/EGFR interplay in several other tumor types (Fitzgerald et al., 2000; Weijzen et al., 2002; Miyamoto et al., 2003; Stockhausen et al., 2005), the high frequency of dysregulated EGFR activity in primary GBM (Tohma et al., 1998; Wong et al., 1987), and the important role of Notch signaling in neurogenesis (Shih & Holland, 2006), it is not surprising that the two pathways have cross-talk in glioma too.

A direct correlation between Notch and EGFR in glioma was provided by Purow et al. who showed that EGFR is under the transcriptional control of Notch signaling, and that EGFR and Notch1 mRNA co-existed and correlated significantly in high-grade astrocytomas (Purow et al., 2008). However, their results did not show the activation status of the Notch signaling, so whether Notch is able to drive the expression of EGFR needs further verify.

EGFR itself has been shown to induce TGFα expression, thus providing the autocrine loop for EGFR activity (Tang et al., 1997). TGFα was detected highly expressed in high grade glioma (Schlegel et al., 1990), and recently one study showed TGFα can up-regulate Hes1 expression and its nuclear translocation in glioma. The nuclear translocation of Hes1 was blocked by MEK1/2 inhibition, indicating that ERK1/2 activity is crucial for this process also (Zheng et al., 2008). However, in another brain tumor neuroblastoma, Hes1 up-regulation by TGFα induction was not dependent ERK1/2 activity (Stockhausen et al, 2005). Therefore, the exact cross-talk mechanism of EGFR-TGFα-Hes1 network seems to be more intriguing and needs further investigation.

In addition, the cross-talk between Notch signaling and EGFR signaling has been revealed in tumor angiogenesis (Zeng et al., 2005). VEGF promotes angiogenesis by stimulating Notch signaling. In glioblastoma cells EGFR transcriptionally up-regulates VEGF expression by PI3K signaling. However, inhibition of PI3K or EGFR did not completely abolish induction of VEGF mRNA by hypoxia, indicating that transcriptional regulation of the VEGF promoter by EGFR appears to involve other signals and to be distinct from signals induced by hypoxia (Maity et al., 2000). In fact, in solid tumor such as glioblastoma, although hypoxia elicits an angiogenic response, tumor vessels are often malformed and occlusions are frequent, as such intratumoral hypoxic areas remain. In this hypoxic environment, HIF1α and HIF2α are stabilized, and as a consequence, VEGF is up-regulated and participates in new blood vessels forming, and TGFα is subsequently up-regulated (Birlik et al., 2006). There is growing evidence that the cellular response to hypoxia and Notch signaling are intimately connected both in normal cells and cancer cells (Poellinger &

Lendahl, 2008). For example, Notch ligand Dll4 has been shown to be induced by hypoxia, and more specifically by HIF1α, VEGF and bFGF (Mailhos et al., 2002; Patel et al., 2005; Liu et al., 2003). Consistently, Dll4 expression in a glioma xenograft model significantly enhanced tumor growth accompanying with Hes1 mRNA elevation in host endothelial cells. Here the tumor displayed decreased vessel density and number, although the vessels were larger and better perfused. Recently, our lab also reported that general blockade of Notch signaling in tumor-bearing mice could lead to defective angiogenesis in tumors through inhibition of HIF activation (Hu et al., 2009). These observations indicate a potential cooperation network in glioma angiogenesis among EGFR, hypoxia, VEGF, and Dll4-Notch signaling pathway (Stockhausen et al., 2010).

EMT represents a molecular change on decreasing cell adhesion and acquiring tumor invasion. Together with transforming growth factor (TGFβ), Jagged1-Notch pathway activates Hey1 to trigger EMT of epithelial cells of human, murine and canine origins (Zavadil et al., 2004). The Jagged1-Notch-Snail2 cascade has also been shown to induce EMT in human breast tumor cells (Leong et al., 2007). One extracellular matrix glycoprotein tenascin-c (TNC), that induces proliferation and migration of neuronal precursors in neurogenic zones of embryonic and postnatal mouse brain, has been shown to be highly expressed in invasive GBM (Garcion et al., 2001; Nishio et al., 2005). The molecular mechanism through which Notch signaling induces TNC-dependent glioma cell motility is based on the trans-activation of the TNC promoter by RBP-J (Leins et al., 2003). Recently, a parallel study in childhood ependymomas has shown the association between tumor recurrence and frequent amplification of 9qter, precisely at the location of both Notch1 and TNC genes (Puget et al., 2009). However, the cross-talk between TGFβ signaling and Notch signaling during glioma EMT still needs to be explored.

6. Notch signaling as a therapeutic target

6.1 Inhibitors of Notch signaling

The observations described above suggest that blocking Notch pathway may be a promising strategy for glioma therapy. In fact, GSI MK0752 (Merck) as an inhibitor of Notch activation has recently been used in a phase I clinical trial of relapsed or refractory T-acute leukemia and lymphoma (T-ALL) (NCT00100152), and phase I breast cancer or others solid tumors (NCT00106145). A new clinical trial has just started for treating patients with recurrence or progressive GBM with GSI RO4929097 (NCT01122901).

However, as GSI affects all Notch receptors, it is difficult to distinguish the specific outcome on a particular cell type. In addition, GSI are not specific for Notch receptors and effects on other targets are to be expected. Moreover, unwanted side effects using GSI have been observed, for example gastrointestinal toxicity (Van Es et al., 2005). The shortcoming of GSI administration in patients may suggest that different targeting strategies or drug combinations are needed. When considering the cross-talk between Notch signaling and the RAS/MEK/ERK and PI3K/AKT pathways downstream of EGFR and their roles in experimental glioma, it is tempting to speculate that simultaneous inhibition of several pathways could lead to improved treatment of glioma patients. In addition, based on the specific role of Dll4-Notch1 in neovascularization, anti-Dll4 has been proposed as sharper therapeutic agents that can reduce tumor burden and prolong survival of the Dll4 expressing tumors, especially devoid of side effects (Ridgway et al., 2006).

6.2 Exhausting GSCs by inhibiting Notch signaling

Based on the observations that Notch signaling participates in regulating multiple aspects of GSCs, especially on their "stemness" maintenance, to exhaust GSCs by inhibiting Notch signaling seems a more exciting option. In 2006, Fan et al. reported to block Notch pathway by GSIs to reduce neurosphere growth and clonogenicity in vitro. The putative GSCs markers CD133, Nestin, Bmi, and Olig2 were reduced following Notch blockade. Using in vitro and in vivo assays, these authors demonstrated that Notch pathway blockade depletes stem-like cells in GBMs, suggesting that GSIs may be useful as chemotherapeutic reagents to target GSCs in malignant gliomas. Notch pathway inhibition appears to deplete stem-like cancer cells through reduced proliferation and increased apoptosis associated with decreased AKT and STAT3 phosphorylation (Fan et al., 2006, 2010). Further, Hu et al reported Notch signaling contributes to the maintenance of both normal NSCs and patient-derived GSCs. Inhibition of Notch signaling by GSI can dramatically decrease the number of secondary tumor spheres, and GSCs can be induced to differentiate into mature neural cell types in differentiation medium. However, GSI might not effect in the same way on different gliomas, especially at the early stage of therapy. Thus, this study suggested drug combination may be more useful for targeting GSCs and consequently exhausting GSCs (Hu et al., 2011).

Hoey et al. reported that the application of anti-human and anti-mouse Dll4 can impede tumor proliferation and neovasculogenesis, and that anti-human Dll4 reduces cancer stem cell frequency (Hoey et al., 2009). Zhen et al. investigated the ability of As(2)O(3) to inhibit the formation of tumor in three different glioma cell lines. Their results revealed the negative regulation of GSCs by As(2)O(3). In addition, Western blot analysis revealed decreased levels of Notch1 and Hes1 proteins due to As(2)O(3) treatment. The authors concluded that As(2)O(3) has a remarkable inhibitory effect on GSCs in glioma cell lines in vivo and in vitro; in addition, they determined that the mechanism of GSC inhibition involves the down-regulation of Notch activation (Zhen et al., 2010).

Radiotherapy represents the most effective non-surgical treatments for gliomas. However, gliomas are highly radioresistant and recurrence is nearly universal. Inhibition of Notch pathway with GSI rendered GSCs more sensitive to radiation at clinically relevant doses. In a study by Wang et al., GSI enhanced radiation-induced cell death and impaired clonogenic survival of GSCs, but not non-stem glioma cells. Moreover, knockdown of Notch1 or Notch2 sensitized GSCs to radiation and impaired xenograft tumor formation (Wang et al., 2010). This indicated that the Notch signaling plays a critical role in the regulation of radioresistance of GSCs, and demonstrated that inhibition of Notch signaling holds promise to improve the efficiency of current radiotherapy in glioma treatment.

It has been commonly recognized that CSCs contributes to the tumor angiogenesis. It is found that GSCs produce much higher levels of VEGF in both normoxic and hypoxic conditions than the non-GSC population, and this GSC-mediated VEGF production leads to enhanced endothelial cell migration and tube formation in vitro (Bao et al., 2006). A VEGF-overexpression glioma model has recently provided supportive evidence for this as well by showing that glioblastoma GSCs overexpressing VEGF produce larger, more vascular, and highly hemorrhagic tumors (Oka et al., 2007). When applying the humanized monoclonal anti-VEGF antibody bevacizumab (Avastin) to cultured GSCs, the in vitro endothelial migration and vascular formation were blocked. Moreover, in vivo administration of bevacizumab potently inhibited the growth, vascularity, and hemorrhage of xenografts derived from GSCs, whereas no effects were seen on xenografts from non-GSCs (Bao et al.,

2006). However, drug resistance and recurrence of tumors has been observed, even treatment was initially effective (Bergers & Hnanhan, 2008). This is most likely due to the activation of pro-angiogenic pathways other than VEGF, especially Dll4-Notch signaling involved in tumor angiogenesis (Li et al., 2007). This suggested that targeting Notch by soluble Dll4 plus anti-VEGF antibody in GSCs would result in improved glioma treatment outcome. Moreover, the latest report from Ying et al showed that glioblastoma stem-like cells were depleted with all-trans retinoic-acid (RA) treatment, accompanied with down-regulation Notch signaling pathway targets, such as Hes5, Hey1 and Hey2. These data indicate that Notch pathway downregulation mediates RA effects on stem cell pool loss. However, this paper does not observe the combinatory inhibition effect from GSI blockade of Notch signaling and RA downregulation of Notch signaling, and the detailed mechanism of targeting GBM-stem cells by drug combination still need to be further elucidated (Ying et al., 2011).

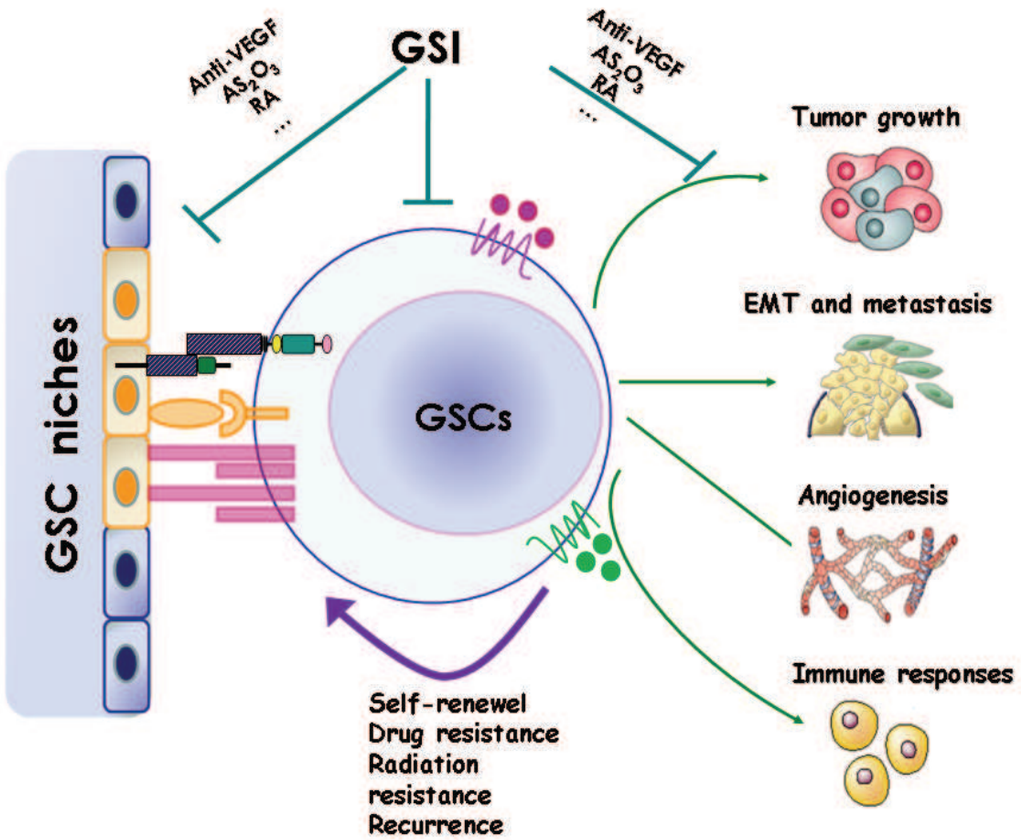

Fig. 2. Roles of Notch signaling in GSCs, and the potential therapeutic strategies targeting Notch signal with GSI in combination with the other drugs, such as anti-VEGF, AS_2O_3, RA, and so on

7. Conclusion

Glioma is the most prevalent and the most aggressive brain tumor resistant to conventional therapies, and as a result, the frequency of recurrence after treatment is definitely very high.

Notch signaling has recently been shown to be responsible for GSCs proliferation, apoptosis inhibition and invasion, specifically for GSCs maintenance, metastasis and chemo-and radio-therapy resistance. To target Notch signaling in GSCs holds a promising treatment strategy against glioma. Although GSI has been applied in clinical trials, considering glioma heterogenicity and tumor microenvironment, combination of Notch signal inhibitor and other therapies will hopefully provide promising ways to improve patient outcome in future (Fig. 2).

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9. References

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Cancer Stem Cells - The Cutting Edge

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