

Review Article

Signaling Pathways in Glioblastoma Cancer Stem Cells: A Role of Stat3 as a Potential Therapeutic Target

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Abstract

Glioblastoma cancer stem cells (GCSCs) play an important role in proliferation, invasion, progression, immune evasion, and resistance to radiation in glioblastoma. Their signaling pathways including receptor tyrosine kinase, Akt, MARK, Wnt, Notch, Hedgehog, and JAK/STAT pathways are complicated, but STAT-3 is a convergence point in several important signaling pathways and contributes to the tumor progression by promoting cell proliferation, cell cycle progression, the inhibition of apoptosis, and tissue invasion. Therefore, STAT-3 is a candidate of therapeutic target of GCSCs. STAT3 is activated through tyrosine phosphorylation by various cytokines and growth factors. STAT-3 is tyrosine phosphorylated by three types of kinases such as receptor tyrosine kinases, JAK family members, and oncogenic kinases including Src and Bcl-Alb. Tyrosine phosphorylated STAT-3 dimerizes and translocates to the nucleus. Active STAT-3 dimers bind to consequences in the promoters of genes such as Bcl-2, Bcl-xL, Mcl-1, and cyclin D1. After induction of target gene expression, multiple STAT-3-endogenous negative regulators such as SOCS3, VHL, and PIAS3 attenuate STAT-3 signaling, and similarly STAT-3 exogenous negative regulators such as pharmacologic JAK inhibitors, dobesilate, and decoy oligonucleotides attenuate STAT-3 activity. In GCSCs, STAT-3 plays a role as a molecular hub in several important signaling pathways that control proliferation, cell cycle progression, anti-apoptosis, invasion, angiogenesis and immune evasion. Therefore, STAT-3 has great potential as a therapeutic target.

Keywords: Signaling pathway; Glioblastoma cancer stem cells; STAT-3; Therapeutic target

Introduction

The cancer stem cells are defined as a cell population residing in tumors, having self-renewal capacity and dividing to give rise to the variety of tumor cells [1,2]. Those cells can reconstitute both the tumor cell hierarchy and the clinical disease state *in vivo* after xeno-transplantation [3]. Although the existence of cancer stem cells in human leukemia is established [1,3], those cells were later discovered to exist in various solid tumors including breast [4], colon [5], lung [6], liver cancers [7], and glioblastoma [8].

Glioblastoma is the most common and lethal brain tumor that shows aggressive natures, with a median overall survival (OS) of less than 15 months after diagnosis. The standard first-line treatment includes resection as much as possible, followed by concurrent radio- and chemotherapy with temozolomide (TMZ), and then 6–12 months of chemotherapy with TMZ. Despite the treatment, recurrence is universal. Glioblastoma is thought to contain a population of self-renewing glioblastoma cancer stem cells (GCSCs) that contributes to treatment resistance [9]. GCSCs are functionally defined with self-renewal measured by serial neurosphere assay *in vitro* and tumor forming capacity through serial transplantation. GCSCs have been shown to differentiate into astrocytes, oligodendrocytes and neurons [10]. Commonly used GCSC markers are as follows: cell surface: CD133, CD15, CD44, CXCR4; integrin alpha-6; protein (cytoplasmic & nuclear): netin, Musashi-1, Bmi-1; transcriptional factor: Sox2, enzyme, ALDH1 [9]. Recently, researches on glioblastoma have

focused on the elucidation of various signaling pathways in GCSCs, particularly aberrant activation. Here, we describe the signaling pathway in GCSCs, particularly focusing on the signal transducer and activator of transcription-3 (STAT-3) as a therapeutic target [10].

Up and downstream signaling pathways of STAT-3 in GCSCs

The activation of several signaling pathways including receptor tyrosine kinase, Akt, MARK, Wnt, Notch, Hedgehog, and JAK/STAT pathways is involved in the progression and proliferation in GCSCs [11]. Among those various signaling pathways in GCSCs, activator of JAK/STAT signal transduction pathway is aberrantly activated in glioblastoma likewise in other solid tumors including breast, lung, ovarian, pancreatic, skin, prostate cancers [12]. STAT-3 contributes to the tumor progression by promoting cell proliferation, cell cycle progression, the inhibition of apoptosis, and tissue invasion [13]. In addition, STAT-3 plays an important role in wound healing, T-cell development, and immune evasion [13,14]. The activation of STAT-3 is implicated in not only controlling critical cellular events involved in tumorigenesis, cell cycle progression, angiogenesis [15], and immune evasion, but also maintaining “stemness” such as self-renewal capacity, ability of differentiation to various tumor cells, and reconstitution of the clinical state *in vivo* after xeno-transplantation [13,16]. Many tumor-derived cell lines require STAT proteins, particularly STAT-3, to maintain a transformed phenotype [17], and a constitutively active STAT-3 mutant, STAT-3C, was sufficient to transform benign tumor

cells to malignant tumor cells, and these transformed cells could form tumors in nude mice [18]. Furthermore, a dominant-negative mutant of STAT-3 blocked transformation by v-src [19]. STAT-3 is activated through phosphorylation of tyrosine 705 that initiates to form a complex composed of two phosphorylated STAT-3 monomers (pSTAT-3). pSTAT-3 homodimers translocate to the nucleus and bind DNA. STAT3 is activated through tyrosine phosphorylation by various cytokines such as interleukin-6 (IL-6) cytokine families (IL-6, oncostatin M, and leukemia inhibitory factor), IL-4, IL-10, IL-11, and IL-23 and growth factors including platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and epidermal growth factor (EGF) [20]. In addition, STAT-3 is tyrosine phosphorylated by three types of kinases such as receptor tyrosine kinases including EGFR, FGFR, and PDGFR, Janus kinase (JAK) family members, and oncogenic kinases including Src and Bcl-Alb [21]. Active STAT-3 dimers bind to consequences in the promoters of genes such as Bcl-2, Bcl-xL, Mcl-1, p21^{WAF1/CIP1}, and cyclin D1 [22]. As STAT-3 affects transcription of genes involved in cell cycle and antiapoptosis, regulation of STAT-3 activity is required to prevent malignant transformation of cells. After induction of target gene expression, multiple STAT-3-endogenous negative regulators attenuate STAT-3 signaling. Suppressors of cytokine signaling (SOCS)-3 and von Hippel-Lindau (VHL) proteins down-regulate the upstream kinase activity responsible for STAT-3 phosphorylation [23,24], while the protein inhibitors of activated STAT (PIAS)3 inhibits STAT-3 directly [25]. SOCS-3 and VHL proteins belong to BC-box protein families that contain a BC-box motif corresponding to the binding site of elongin BC [26], while PIAS3 belongs to PIAS families that contain a zinc ring finger domain, an NH2-terminal LXXLL motif, a COOH-terminal acidic domain, a serine/threonine-rich domain and PINIT motif involved in the nuclear retention [27]. SOCS-3 and VHL inhibit JAK activation and subsequently attenuate STAT-3 signal transduction in a classic negative feedback loop in the cytoplasm [28], whereas PIAS3 inhibits STAT-3 DNA binding the nucleus and exhibit E3-SUMO (small ubiquitin-like modifier) ligase activity and SUMOylate a variety of transcription factors including p53, c-Jun, and c-Myb [29].

The Notch signaling pathway is involved in cell fate decisions during normal development and in the genesis of glioblastoma [30]. The downstream effects of Notch signaling are highly tissue and time dependent, and Notch has been implicated both in the maintenance of neural progenitors and in the generation of glia during development of the brain [31]. It has been reported previously that in the developing central nervous system, there is cross-talk between the Notch and STAT3 pathways because STAT-3 binds to adjacent site in the Notch1 promoter [32]. Notch pathway genes were up-regulated in GCSCs by constitutive activation of STAT-3 [33], but in contrast the activation and phosphorylation of STAT3 is mediated by the direct binding of several Notch effectors to STAT3 [34].

Inhibiting STAT-3 activity in tumor cells with either dominant-negative or STAT-3 inhibition by STAT-3 siRNA resulted in increased expression of pro-inflammatory mediators including IFN-β, TNF-α, and IL-6 [35]. STAT-3 signaling is dampened by protein tyrosine phosphatases, such as the SH2-domain containing tyrosine phosphatase family (SHP-1, SHP-2), which downregulate STAT-3 activation directly by phosphorylating active STAT-3 complexes [36] (Figure 1).

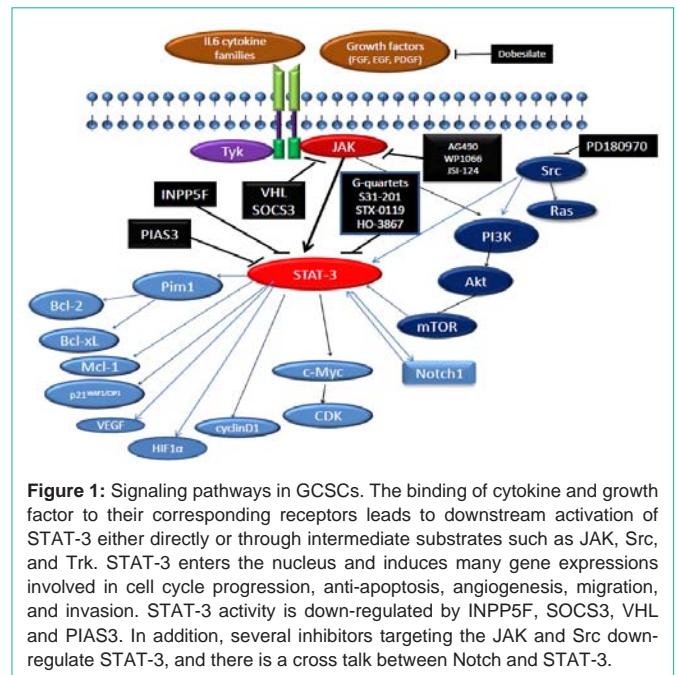


Figure 1: Signaling pathways in GCSCs. The binding of cytokine and growth factor to their corresponding receptors leads to downstream activation of STAT-3 either directly or through intermediate substrates such as JAK, Src, and Trk. STAT-3 enters the nucleus and induces many gene expressions involved in cell cycle progression, anti-apoptosis, angiogenesis, migration, and invasion. STAT-3 activity is down-regulated by INPP5F, SOCS3, VHL and PIAS3. In addition, several inhibitors targeting the JAK and Src down-regulate STAT-3, and there is a cross talk between Notch and STAT-3.

Inhibitory regulators of STAT-3

STAT-3 is a promising target for GCSCs, not only because it is a convergence point in several important signaling pathways that promote proliferation [15], invasion [37], immune evasion [14,38,39], anti-apoptosis [40], anti-autophagy [41], formation of peritumoral edema [42] and maintenance of “stemness” [15] but also because aberrant STAT-3 activation results from upstream dysregulation [43] (Table 1). There are two approaches to inhibit STAT-3: (1) through exogenous regulators such as RNA interference and chemical inhibitors, and (2) through endogenous regulators such as PIAS3, SOCS-3, and VHL [13] (Table 2). Direct STAT-3 inhibition has been achieved with dominant negative constructs, oligonucleotides, or, phosphopeptidic agents that mimic the native tyrosine 705 containing binding sequence or non-native STAT3-binding sequences [10]. Some platinum compounds interfere with STAT-3 activation and abrogate signaling mediated by constitutively active STAT-3 [44]. Direct inhibition of STAT-3 activity using RNA interference induced apoptosis and inhibited survival [17]. Among decoy oligonucleotides of STAT-3 binding site, G-quartets, having competitive inhibitory

Table 1: Roles of STAT-3 associated with glioblastoma / glioblastoma cancer stem cells (GCSCs).

Authors & Year [Reference]	Role of STAT-3
Hirano T, et al. 2000 [39]	B cell growth and differentiation
Rahaman SO, et al. 2002 [40]	Apoptosis inhibition
Sherry MM, et al. 2009 [16]	Proliferation and maintenance of multipotency in GCSCs
See AP, et al. 2012 [14]	Modulation of immune microenvironment
Yokogami K, et al. 2013 [15]	VEGF upregulation
Zheng Q, et al. 2014 [37]	Tumor invasion
Assi H, et al. 2014 [38]	Dendritic cell differentiation
Wang XF, et al. 2014 [42]	Peritumoral edema formation
Yaun G, et al. 2014 [41]	Autophagy suppression

Table 2: STAT-3 inhibitors.

Authors & Year [Reference]	STAT-3 inhibitors
Bromberg JF, et al. 1998 [19]	RNA interference
Dorsey JF, et al. 2000 [52]	PD180970
Leong PL, et al. 2003 [45]	Decoy oligonucleotide
Blaskovich MA, et al. 2003 [50]	JSI-124
Cuevas P, et al. 2005 [54]	Dobesilate
Shuai K. 2006 [25]	PIAS3
Iwamaru A, et al. 2007 [49]	WP1066
Yoshimura A, et al. 2007 [28]	SOCS-3
Iwamaru A, et al. 2007 [49]	AG490
McFarland BC, et al. 2011 [51]	AZD1480
Mir SA, et al. 2012 [47]	S31-201
Kanno H, et al. 2013 [24]	VHL
Ashizawa T, et al. 2013 [46]	STX-0119
Rath KS, et al. 2014 [60]	HO-3867
Kim HS, et al. 2014 [61]	INFP5F

structures comprised of guanine-rich oligonucleotides, inhibited STAT-3 binding to endogenous gene promoters and subsequently attenuated STAT-3-induced gene expression by competitively binding activated STAT-3 [45]. STX-0119 (a N-[2-(1,3,4-oxadiazolyl)]-4-quinolinecarboxamide derivative) inhibited STAT3 dimerization and suppressed the growth of transplanted tumors of GCSCs. STX-0119 inhibited proliferation and sphere formation in GCSCs by down-regulating the gene expression of STAT3-target genes including cyclin D1, survivin, Bcl-xL, c-myc, MMP2, VEGF and HIF-1 α [46]. Similarly, S31-201 inhibited STAT-3 homodimer formation by inhibiting STAT-3 DNA binding, and showed the antitumor activity by inhibiting STAT-3 induced gene expression [47]. In addition, small molecule, nonphosphorylated STAT-3 inhibitor, 31 (SH-4-54) that strongly binds to STAT3 protein. SG-4-54 effectively suppresses STAT3 phosphorylation and its downstream transcriptional target genes and potently kills GCSCs. Moreover, in vivo, SH-4-54 inhibited pSTAT-3 in vivo and potently controlled glioblastoma tumor growth with exhibition of blood-brain barrier permeability [10].

Inhibition of upstream kinases of STAT-3 leads to abrogation of STAT-3 activity. EGFR inhibitor gefitinib, an upstream kinase of STAT-3, led to inhibition of STAT-3 tyrosine phosphorylation [48]. Since the activation of STAT-3 depends on its direct phosphorylation by tyrosine kinases, several pharmacologic JAK inhibitors [AG490, WP1066, JSI-124 (cucurbitacin I), and AZD1480], FGF signaling pathway inhibitor [Dobesilate], and phosphorylation inhibitor [HO-3867] led to STAT-3 inhibition [49-56]. The JAK inhibitor AG490 decreased cell survival and induced apoptosis by blocking constitutively active STAT-3 in glioblastoma [49]. AG490 inhibited expression of STAT-3 target- pro-survival genes including Bcl-xL, Bcl-2, and Mcl-1 and led to apoptosis [49]. Afterwards the structure of AG490 was modified to produce the more potent and active compound WP1066 that successfully crossed the blood brain barrier and that inhibited the growth of xenografts compared with untreated controls [53]. WP1066 blocked tyrosine phosphorylation of STAT-3 by JAK, which resulted in a failure of STAT-3 to

translocate to the nucleus and to mediate its transcriptional effects. WP1066 treatment of glioblastoma cells decreased STAT-3-mediated expression of Bcl-xL, Mcl-1, and c-Myc, induced apoptosis, and reduced viabilities [53]. WP1066 also reversed immune tolerance by blocking STAT-3-mediated immune suppression. WP1066-stimulated proliferation of T cells obtained from glioblastoma patients and enhanced immunogenic responses in glioma-infiltrating microglia, macrophages, and peripheral blood monocytes [53]. Another Jak inhibitor JSI-124 significantly enhanced apoptosis and inhibited proliferation of glioblastoma [49]. Furthermore, it was demonstrated that STAT-3 inhibition by JSI-124 sensitized GBM cells to temozolomide, bis-chloroethylnitrosourea, and cisplatin, enhancing the anti-proliferative effects of these clinically utilized chemotherapeutic agents. Similar additive effects of anti-Stat3 inhibitors with temozolomide or Taxol [50]. In addition, another JAK inhibitor, AZD1480, has been observed to significantly reduce glioblastoma cell growth in both cell culture and animal xenograft studies by blocking STAT-3 activity [51]. Furthermore, Src inhibitor PD180970, a novel pyrido[2,3-d]pyrimidine derivative, reduced STAT3 activity thorough Src inhibition, cell cycle arrest in G2, and reduced viability of cells accompanied by induction of apoptosis [52]. Dobesilate (Dihydroxy-2,5-benzenesulfonate) inhibits activation of STAT-3 by attenuating the upstream of FGF signaling pathway and constitutive expression of tyrosine phosphorylated STAT-3, and blocks neovascularization [54-56]. In addition, dobessilate inhibits activation of the MAPK extracellular signal-regulated protein kinases [57] and expression of the pro-survival proteins Bcl-xL and cyclin D1 [58]. These facts suggest that dobessilate increased apoptosis and decreased cell growth and survival partially by blocking STAT-3 activation. The kinase inhibitors at upstream of STAT-3 have significant potential as chemotherapeutic agents for glioblastoma. Furthermore, inhibition of various upstream kinases in combination with direct STAT-3 inhibitors may also down-regulate GCSCs and be effective in glioblastoma therapy. Recently it has been shown that inhibition of a single tyrosine kinase pathway provides little benefit in reducing glioma cell survival and growth. However, a combinatorial strategy to inhibit multiple upstream tyrosine kinases proved to significantly reduce intracellular signaling, survival, and anchorage-independent growth of glioma cells [58]. Then, STAT-3 inhibition by direct, indirect, or a combinatorial approach using existing pharmacologic inhibitors has promise as a clinical target in glioblastoma.

HO-3867, a novel curcumin analog and a member of the diarylidenyl piperidone class decreased expression of tyrosine-phosphorylated STAT3 (pTyr705) and STAT3's downstream targets cyclin D1 and Bcl-2, and significantly inhibited cancer stem cell growth in a dose- and time-dependent manner [59,60]. INPP5F (inositol polyphosphate-5-phosphatase F), one of the polyphosphoinositide differentially expressed in GCSCs, inhibited STAT-3 activity via inhibition of STAT-3 phosphorylation and suppressed glioma tumorigenesis [60]. Constitutively expressed INPP5F showed to suppress self-renewal and proliferation potentials of glioblastoma cells and reduced tumorigenicity of glioblastoma. In addition, loss of INPP5F gene in gliomas is significantly correlated with lower overall patient survivals. INPP5F is a potential tumor suppressor in gliomas via endogenous inhibition of STAT3 pathway [61].

SOCS-3 and VHL are not only endogenous inhibitors in STAT-3 signaling [24,62] and they work in a negative-feedback loop to suppress STAT-3 signaling [63,64]. In contrast, elevated SOCS-3 or VHL expression was reported in some cancer tissues [65-70]. It was shown that glioblastoma tissues expressed higher levels of SOCS-3 than did control brain tissues and that SOCS-3 promoter activity and mRNA expression is inhibited by ectopic PIAS3 expression [71]. Moreover, constitutive expression of SOCS-3 in glioblastoma is related to cell proliferation and resistance to radiation [72]. U87-MG glioma cells constitutively express SOCS-3 [64], while both those cells and GCSCs derived from U87-MG glioma do not express VHL [24]. A unique characteristic of GCSCs is the expression of the cell surface marker CD133 that is a hallmark of neural precursor cells [73]. Since the expression of SOCS-3 in glioblastoma cells was shown to correlate with resistance to ionizing radiation [71,72], CD133+ cells might also express elevated levels of SOCS-3 as well as activated STAT-3.

Similarly, VHL inhibited JAK2 and STAT3. In addition, VHL up-regulated PTEN expression. This fact suggested that VHL affected the JAK/STAT pathway as well as the PTEN/PI3K/Akt pathway; they also suggest that up-regulation of PTEN by VHL gene transfer may affect the PI3K/Akt pathway, since PTEN is a PI3K/Akt pathway inhibitor [73]. VHL inhibited the implantation ability as well as soft agar colony and neurosphere formation, all of which are characteristics of cancer stem cells. Our results suggest that these characteristics may be related to the JAK/STAT or PTEN/PI3K/Akt pathways in GCSCs. Overexpression of VHL up-regulated PTEN and down-regulated the JAK/STAT pathway. In addition, overexpression of VHL significantly down-regulated the proliferation of U87-GCSCs. However, the overexpression of VHL inhibited cell proliferation of U87-GCSCs to a lesser extent than it did soft agar colony and neurosphere formation, or implantation capacity into SCID mice. These results suggest that VHL inhibited the tumorigenicity and self-renewal ability via the JAK/STAT pathway and also affected the PTEN/PI3K/Akt pathway, both of which are critical in GCSCs [24] (Figure 1).

Conclusion

In GCSCs, STAT-3 plays a role of a molecular hub in several important signaling pathways that control proliferation, cell cycle progression, anti-apoptosis, invasion, angiogenesis and immune evasion. There are two approaches to inhibit STAT-3: (1) through endogenous inhibitors (PIAS3, SOCS-3, VHL) and (2) through exogenous inhibitors (JAK inhibitors, Src inhibitor, EGFR inhibitor, decoy oligonucleotides, RNA interference). STAT-3 has great potential as a therapeutic target.

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