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Squelching Glioblastoma Stem Cells by Targeting REST for Proteasomal Degradation

Peisu Zhang^{1,*}, Justin D. Lathia², William A. Flavahan², Jeremy N. Rich², and Mark P. Mattson^{1,*}

¹Laboratory of Neurosciences, National Institute on Aging Intramural Research Program, Baltimore, MD

²Department of Stem Cell Biology and Regenerative Medicine, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH

Abstract

Glioblastoma brain tumors harbor a small population of cancer stem cells that are resistant to conventional chemotherapeutic and radiation treatments, and are believed responsible for tumor recurrence and mortality. Identifying epigenetic molecular mechanisms controlling self-renewal in glioblastoma stem cells will foster development of targeted therapeutic approaches. The transcriptional repressor REST, best known for its role in controlling cell fate decisions in neural progenitor cells, may also be critical for cancer stem cell self-renewal. Two novel mechanisms for regulating the stability of REST have been revealed recently and involve the telomere-binding protein TRF2 and the ubiquitin E3 ligase SCF β -TrCP. Reduced TRF2 binding to REST and increased SCF β -TrCP activity target REST for proteasomal degradation thereby inhibiting cancer stem cell proliferation. Neurological side effects of treatments that target REST and TRF2 may be less severe than conventional brain tumor treatments because postmitotic neurons do not express REST and have relatively stable telomeres.

Keywords

Glioblastoma; REST; TRF2; neurogenesis; neuroblastoma; telomere erosion

Introduction

Glioblastoma multiforme is the most common primary brain tumor in adults with an incidence of three per 100,000 [1]. The lethality of these tumors is underscored by a recent study which reported the 1 year survival rate to be 18% and the 2 year survival rate to be 3% for newly diagnosed glioblastomas [2]. Current standard-of-care therapies for glioblastoma include tumor resection, radiation and temozolomide (an alkylating agent) [3]. However, these conventional cytotoxic therapies have for the most part done little to improve overall patient survival, and with many failed attempts at therapies, there is a clear need for a better understating of brain tumor biology which in turn is likely to result in more effective therapies.

The failure to cure many cancers has been attributed to the fact that a minor subpopulation of cells can survive and initiate cancer recurrence after conventional chemotherapy or radiation treatments, even when the bulk of the tumor mass had been eliminated [4,5]. Stem-like cells can be isolated from wide variety of cancers, including leukemia, multiple myeloma, colon

*Correspondence: mattsonm@grc.nia.nih.gov; zhangpe@grc.nia.nih.gov.

cancer and brain tumors, which has led to the new concept of cancer stem cells or tumor initiation cells [6–12]. Like most cancers, glioblastomas are composed of a heterogeneous population of cells, and recent findings suggest that glioblastomas harbor cancer stem cells that are resistant to conventional therapies. In 2003, Singh et al. [13] reported the identification and purification of a cancer stem cell from human brain tumors that possesses a marked capacity for proliferation, self-renewal and differentiation. Other groups then reported that stem cells can be isolated from human adult glioblastomas that express the neural stem cell marker CD133, a surface antigen [14–16] (Figure 1). Like normal neural stem cells, glioblastoma stem cells exhibit a high capacity for self-renewal and, interestingly, are capable of differentiating into neuron- and glia-like cells when exposed to differentiation signals in culture [16–18]. When grafted into mice CD133+ cells, but not CD133– cells, are able to generate brain tumors that phenotypically recapitulate the patient's original tumor (19,20). Cancer stem cells derived from human glioblastoma surgical specimens and xenografts display resistance to radiation due to enhanced activity of the DNA damage check point [21]. Considerable evidence therefore suggests a key role for cancer stem cells in tumor growth and recurrence of human glioblastomas.

Although it is as yet unknown whether cancer stem cells originate from transformed normal stem cells or a different cell type, Hemmati et al. demonstrated that both normal and tumor-derived neurospheres display increased self-renewal capacity resulting from enhanced activity of the oncogene *bmi-1*, which encodes a member of polycomb transcriptional factor involved in the epigenetic repression of differentiation and apoptosis [9]. In addition, cells isolated from cortical glial tumors contain neural stem-like cells expressing glial and neuronal markers and tumor-related genes such as Notch [8]. In contrast, exposure of human brain cancer stem cells to astroglial fate-inducing factors such as BMP4, enforces terminal differentiation and renders the cells vulnerable to cytotoxic therapies [22]. It is therefore reasonable to consider that molecular mechanisms that regulate the proliferation and differentiation of normal neural stem cells also determine the fate of glioblastoma stem cells. In this regard the transcriptional repressor REST, which negatively regulates genes critical for neuronal differentiation [23], is of particular interest. REST is expressed in high amounts in many different tumor cells including human neuroblastoma and tetracarcinoma [24] cells and glioblastoma stem cells (Figure 1). Because of the importance of REST for maintaining neural stem cells and cancer cells in a self-renewing state, and recent evidence that REST can be targeted for proteasomal degradation [24,25], there is a rationale for therapeutic interventions that target REST in the treatment of glioblastoma.

Cancer Stem Cells and Glioblastomas

Cancers are complex cellular systems that are aberrantly regulated. They are formed from neoplastic cells with associated vasculature, inflammatory cells, and recruited non-neoplastic cells [4]. Within these neoplastic compartments (areas associated with hypoxia or perivascular regions in brain tumors), there exist cells with heterogeneous phenotypes including differences in morphology and proteomes, suggesting a potential hierarchy in differentiation status [17]. The outgrowth of a fraction of tumor cells has been hypothesized to occur by two general mechanisms, a stochastic model in which all tumor cells are equally prone to acquire further genetic changes creating a growth advantage, and a hierarchical model in which a fraction of cells are more neoplastic and capable of rapidly generating the bulk of the cells in a tumor. These two mechanisms are likely not mutually exclusive. The hierarchical model of tumor growth supports the presence of tumor cells that share some characteristics with normal adult stem cells. They are long-lived and are able to self-renew and differentiate into the relevant cellular identities of a given tissue; in the case of the central nervous system (CNS) the cell types include neurons, astrocytes and oligodendrocytes. Cancer stem cells (also termed tumor stem cells, tumor initiating cells, or tumor propagating cells) are populations that possess the

ability to self-renew and propagate tumors. The existence of cancer stem cells was demonstrated in seminal studies of leukemias which showed that a rare cell fraction was capable of propagating tumors [9,14]. Evidence for the existence of cancer stem cells has been extended to solid cancers, including several primary brain tumors, suggesting that cellular heterogeneity exists in many (but not necessarily all) tumors [10–16]. However, the tumor stem cell hypothesis is poorly understood and highly controversial. Not all tumors must be propagated by cancer stem cells, nor are cancer stem cells necessarily rare [25].

In the adult mammalian CNS small numbers of neural stem cells are retained in the subependymal zone adjacent to the lateral ventricle and in the subgranular zone of the hippocampus (Figure 2), and are believed responsible for the replacement of neurons in the olfactory bulb and hippocampal dentate gyrus, respectively. Upon stress or brain injury, these neural stem cells can undergo amplification via asymmetric divisions that propagate the multi-lineage progenitors which then transit to mature neurons or glia. Though not yet established, emerging evidence suggests that neural stem or progenitor cells may be a source of the cancer stem cells believed to generate glioblastomas [26] (Figure 2). For example, loss of p53 in the adult brain provides a proliferative advantage to the slow- and fast-proliferating neural progenitor cells; when combined with a mutagenic stimulus these cells transform and may give rise to glial tumors [27]. In addition, glial progenitor cells can form high-grade glioma cells when transformed with c-myc and H-ras [28]. Many cancer stem cells express cell-surface proteins such as CD133 that are also found in normal stem cells, but may also harbor mutations in tumor suppressor genes such as PTEN and p53 [19,29,30]. However, neural progenitor cells may not be the only source of cancer stem cells. As evidence, leukaemia-associated proteins can induce adjacent somatic cells to acquire cancer stem cell-like properties and support tumor formation [31]. During the tumorigenesis process, dysfunctional oncoproteins (gain-of-function effect) or tumor suppressor proteins (loss-of-function effect) play intrinsic roles in impairing innate tumor-suppressive mechanisms that trigger terminal differentiation and apoptosis or senescence. Therefore, tumor stem cells are most likely to either directly or indirectly hijack self-renewal and survival mechanisms used by normal stem cells [32]. Finally, normal glial cells are also a potential source of glioblastoma stem cells. Indeed, we now know that somatic cells can be reprogrammed to a pluripotent state by expression of a small number of genes, so-called induced pluripotent stem cells [33].

Based on the information described above, several basic therapeutic principles for combating cancer stem cells have been proposed: 1) treatments that promote terminal differentiation of the cancer stem cells; 2) therapy to arrest malignant proliferation; 3) suppression of self renewal; 4) immune modulation; and 5) niche targeting [5,34]. At the molecular level the goal would then be to induce a switch from the self-renewal program to terminal cell differentiation or death (Figure 2). We believe that the transcriptional repressor REST is one such molecular switch that is an attractive therapeutic target for at least some neural tumors.

REST: A Key Regulator of the Fates of Neural Stem Cells and Cancer Cells

REST (repressor element 1-silencing transcription factor), also known as NRSF (neuron-restricted silencing factor), is a master transcriptional repressor recognized as a negative regulator of genes devoted to many aspects of neuronal functions [35–38]. A zinc finger domain in REST recognizes conserved 21–23 base pair repressor elements (RE-1 elements) within regulatory regions of genes, including a large number of genes that encode neuron-specific proteins such as type II sodium channels, the neurotrophic factor BDNF (brain-derived neurotrophic factor) and the cell adhesion molecule L1 [29,36,39]. Genome- and transcriptome-wide analyses focused on the REST binding RE-1 sequence have revealed a REST-regulated network with over 700 genes and 22 microRNAs in humans [29,39]. In addition, REST also recruits multiple co-repressors via two repressor domains located at the

N- and C-termini of the protein. REST is expressed ubiquitously in non-neuronal tissues and stem cells, wherein it suppresses neuronal differentiation. Thus, the transition from stem cell and progenitor cells to terminally differentiated neurons requires removal of REST from RE-1 binding sites resulting in the de-repression of neuron-specific target genes.

While REST represses neuronal genes, it appears to have paradoxical roles in cancer cell biology; it can exert tumor suppressor activity in some settings and oncogenic activity in others. For example, in certain non-neuronal tumors, such as breast, colon and small cell lung cancers, reduced transcription of REST occurs resulting in the upregulation of neuroendocrine genes [28,40,41]. Inactivation of REST or RNAi-mediated REST depletion can promote transformation, proliferation, and migration of human mammary epithelial cells [42]. However, oncogenic roles for REST have been observed in several types of brain tumor cells including neuroblastoma and medulloblastoma, childhood malignancies believed to arise from neural progenitors [24,43]. Analysis of 161 nervous system tumors including astrocytomas, glioblastomas, oligodendrogliomas, oligoastrocytomas, medulloblastomas, meningiomas and schwannomas, suggest that REST is expressed and is neither activated nor inactivated via mutations in these neural cell-derived tumors [44]. High levels of REST activity coupled with Myc upregulation in may be play a central role in the malignant transformation of at least some of these brain tumors [43]. Consistent with this possibility, we found that REST is expressed at high levels in human neuroblastoma [24] and glioblastoma (Figure 1) cells.

By maintaining neural progenitor cells in a self-renewing state REST plays critical roles in embryonic development and adult neurogenesis [35]. Perturbation of REST expression or function results in early embryonic lethality and ectopic expression of neuronal genes in non-neuronal tissues [45,46]. Forced expression of a dominant negative form of REST efficiently suppresses endogenous REST activity and converts non-neuronal cells to neuron-like cells that express voltage-dependent sodium channels [47]. Additional evidence suggests that the REST regulation network is intimately related to the regulatory repertoire of core pluripotency factors (Oct4, Sox2 and Nanog) in human and mouse where REST is recruited to the genes for *Nanog* and the *microRNA mir-21* [37,48]. Data suggest that mir-21 acts in a negative feedback loop to suppress the expression of *Sox2* and *Nanog* [37]. Thus, REST may have both direct and indirect regulatory roles in maintaining pluripotency by integrating its regulatory mechanisms with those of the Oct4-Sox2-Nanog system.

Posttranslational Regulation of REST by SCF β -TrCP

A novel and fascinating aspect of posttranslational regulation of REST was discovered recently by Westbrook et al [49] and Guardavaccaro et al [50]. Both groups established that the half-life of the REST protein is regulated by the highly versatile ubiquitin ligase (E3) SCF β -TrCP that ubiquitinates REST, thereby targeting REST for proteasomal degradation. SCF β -TRCP (F-box protein β -TRCP) is a multiprotein E3 ubiquitin ligase responsible for the degradation of REST. Recent findings suggest that two adjacent SCF β -TRCP-binding motifs in REST are involved in targeting REST for ubiquitin-mediated proteasomal degradation, but the two motifs differentially mediate effects of REST on mitotic checkpoint regulation and neuronal differentiation. Overexpression of a REST mutant lacking the SCF β -TRCP target site stabilizes REST and attenuates M phase entry, resulting in genomic instability characterized by aberrant sister chromatid separation and anaphase progression [50]. In contrast, wild-type REST is readily degraded during the G2 cell-cycle phase even when overexpressed. SCF β -TRCP is required for neural differentiation only when REST is present, and failure to degrade REST inhibits differentiation; however, overexpression of SCF β -TRCP can cause REST degradation and the oncogenic transformation of some types of epithelial cells [49].

The identification of SCF β -TRCP as a regulator of REST stability provides a new perspective on the molecular mechanisms that regulate the fate of neural progenitor cells and cancer cells. However, when considering SCF β -TRCP as a potential therapeutic target it is important to recognize that REST is not the only target of the SCF β -TRCP pathway. Indeed, recent findings have shown that SCF β -TRCP functions in diverse pathways by targeting various proteins for proteasomal degradation; examples include proteins involved in the regulation of NF- κ B, Wnt signaling, calcium signaling, cell contact and polarity, cell-cycle regulation, DNA damage and replication [51]. However, recent findings described in the next section provide evidence that REST can be more selectively targeted for proteasomal degradation.

TRF2 Regulates REST DNA Binding and Protein Stability

Telomeres at the end of chromosomes are specialized structures responsible for maintaining chromosome stability; in mammalian cells telomeres consist of repetitive DNA (TTAGGG)_n and an array of associated proteins. Stem cells and cancer cells maintain telomere length mostly through the activity of telomerase, a reverse transcriptase [52]. As with most cancer cells, high telomerase activity is observed in cells isolated from glioblastoma multiforme [18]. In the absence of telomerase, telomeres shorten with every cell division in proliferating somatic cells and normal stem cells. After about 50–70 cell doublings, telomere shortening eventually reaches a critical point and triggers telomere erosion and a DNA damage response involving ATM kinase and a p53-mediated cell cycle arrest signaling pathway [53,54]. Thus, telomere attrition usually leads to cell senescence or apoptosis or senescence.

Whereas telomerase has become an attractive target for cancer therapy, other telomere-associated proteins, including TRF2 (telomere repeat-binding factor 2), have also emerged as targets. TRF2 is a 66 kDa protein with a C-terminal Myb DNA-binding domain and a N-terminal dimerization domain. TRF2 binds to telomeric TTAGGG repeat sequences where it facilitates telomere t-loop formation [53]. TRF2 functions as a key component of the so-called telomere ‘shelterin’ complex, also referred to as the telomere cap, to prevent exposure of the free end of the chromosome and consequent abnormal chromosome rearrangements, or engagement of cell cycle arrest or apoptosis (Figure 3). In contrast to the gradual effects of decreased telomerase activity, TRF2 removal in proliferating human and mouse cells rapidly triggers a telomeric DNA damage response and cell cycle arrest to promote either senescence or apoptosis, depending on cell type and state [55,56]. Conversely, increased levels of TRF2 may promote cancer cell proliferation and survival. TRF2 expression is elevated in several solid cancers, including skin, gastric, breast and hepatic tumors [57–59]. Forced overexpression of TRF2 in skin stem cells in a mouse model promotes spontaneous tumors associated with a dramatic increase of chromosomal aberrations in keratinocytes [57].

TRF2 increases the resistance of cancer cell lines and human gastric cancer cells to several chemotherapeutic agents [60]. Exposure of cancer cells to radiation and DNA-damaging drugs such as etoposide results in TRF2 up-regulation at the transcriptional and translational levels. A tight correlation between drug resistance and TRF2 elevation was demonstrated in studies of multidrug resistant gastric carcinoma-derived cell lines in which the multidrug resistant variants exhibit higher levels of TRF2 than their parental cell lines upon exposure to several chemotherapeutic drugs [61]. Moreover, overexpression of TRF2 prevented the activation of DNA repair proteins (ATM and γ H2AX phosphorylation) and cell cycle arrest pathways by chemotherapeutic treatments, whereas depletion of TRF2 by siRNA exacerbated DNA damage responses [61]. Thus, TRF2 elevation promotes uncontrolled cell cycle progression by impairing DNA damage-repair and cell cycle checkpoint pathways resulting in drug resistance, at least in certain types of cancers.

Displacement of TRF2 from telomeres by overexpression of a dominant-negative TRF2 mutant induces cell cycle arrest and differentiation or apoptosis in many types of tumor cells [61] including those of neural origin [24]. Preclinical studies have demonstrated efficacy of dominant negative TRF2 overexpression in suppressing the tumorigenicity of melanoma cells [62]. Dominant negative TRF2 overexpression appears to have no adverse effects on fully differentiated postmitotic neurons which normally express low levels of TRF2 [63]. However, it should be noted that neural progenitor cells and newly generated neurons may be vulnerable to TRF2 inhibition [64], suggesting a potential adverse effect of treatments that target TRF2 on plasticity in brain regions that contain neural progenitor cells (i.e., hippocampus and subventricular zone).

While one major function of TRF2 is to secure the telomere capping structure, recent findings suggest that TRF2 may serve as a platform for recruiting a spectrum of nuclear proteins. TRF2 can suppress or otherwise modulate the function of protein binding partners in nuclear processes such as DNA replication (WRN and BLM) [65], DNA recombination (XPF/ERCC1) [66], and responses to DNA damage (ATM and the MRE11 complex [67,68]. It has been shown that TRF2 binds the autophosphorylation site of ATM and thereby inhibits the ability of ATM to activate downstream signaling transducers (e.g. NBS1) in pathways for arresting cell proliferation [67]. TRF2 has been detected at non-telomeric DNA damage sites prior to ATM activation after ionizing radiation treatment [69]. The latter finding provides a potential explanation at the molecular level for why an increased amount of TRF2 is a critical factor for drug resistance in some cancer cells.

Telomeres and telomere-associated proteins have been implicated in the regulation of gene expression. Evidence from yeast studies suggests the existence of a telomere loop effect, in which telomeres are able to juxtapose with distant gene loci facilitated by transient interaction between telomere binding proteins and transcription factors, thereby providing a mechanism for gene repression [70]. However, recent findings suggest that TRF2 influences the processes of cell proliferation, differentiation and survival through specific interactions with nuclear proteins in locations other than telomeres [69]. In support of an extra-telomeric function of TRF2 in mammalian cells, we recently found that TRF2 and REST interact in nuclear foci that co-localize with PML bodies in human neuroblastoma and tetracarcinoma cells [24], as well as in glioblastoma stem cells (Figure 1 and the authors' unpublished data). Downregulation of TRF2 expression and selective disruption of TRF2 binding to REST (by overexpressing a dominant-negative TRF2 mutant) results in ubiquitination and proteasomal degradation of REST (Figure 3). As a consequence, genes regulated by REST (including neuronal genes encoding β 3-tubulin, synaptophysin, BDNF, L1CAM and SCN3A) are de-repressed and the cells stop dividing and acquire several phenotypic properties of differentiated neurons [24]. Interestingly, we also found that TRF2 removal is not harmful to postmitotic neurons and can even enhance their differentiation [63]. Although it remains to be established if the TRF2-REST complex is localized close to telomeres, our data suggest that terminal differentiation and senescence, two potential tumor suppression mechanisms, can be simultaneously evoked by selectively reducing the level of TRF2 or its interaction with REST in proliferating normal and cancerous brain cells. We therefore propose that, in addition to its well-known function in maintaining telomere integrity at chromosome ends, TRF2 binds and stabilizes REST thereby facilitating the physiological self-renewal of neural progenitor cells and the pathological uncontrolled proliferation of cancer cells.

Concluding Remarks

Glioblastomas are non-capsulated brain tumors that are typically refractive to conventional therapies, apparently because they harbor cancer stem cells that are relatively resistant to radiation and DNA-damaging agents. Therefore, specifically targeting these resilient cancer

stem cells, without harming nearby postmitotic neurons and proliferating glial cells, is a major challenge. A solution to this problem would include the identification of a molecular target critical for the self-renewal of cancer stem cells, and the development of a therapeutic intervention that is selective for that target. It may be possible to target REST for proteasomal degradation by inhibiting the interaction of REST with TRF2, or by enhancing activity of the ubiquitin E3 ligase SCF β -TrCP. Recent studies using cultured cells suggest that REST can indeed be selectively targeted for proteasomal degradation by viral vector-based depletion or inhibition of TRF2, or overexpression of SCF β -TrCP, resulting in suppression of cell proliferation. Because neurons and glial cells express little or no REST, they may be not be adversely affected by such a therapeutic approach, although adult neural stem cells would likely be inhibited. Combining a REST/TRF2-based treatment with low doses of existing chemotherapeutic agents might further improve the outcome in patients with glioblastomas.

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Figure 1. Protocol for the isolation of glioblastoma stem cells from surgical specimens

(a) Glioblastoma surgical specimens are disassociated into a single-cell suspension and separated by fluorescence activated cell sorting (FACS) using antibodies against markers for cancer stem cells. The conventional marker used in the example here is the cell surface antigen CD133, but no definitive marker exists and additional markers have been suggested including L1CAM, A2B5, and CD15 [71–73]. CD133⁺ cells are self-renewing stem cells as indicated by their ability to form neurospheres in culture and tumors when grafted into the brains of immunodeficient mice; in contrast CD133⁻ cells form neither neurospheres in culture nor tumors in vivo. (b) CD133⁺ cancer stem cells isolated from a glioblastoma that had been surgically removed from a patient are shown. The cells were immunostained using antibodies against REST (red) and TRF2 (green). The cells were also labeled with the DNA-binding dye DAPI (blue) and the lower image is a merged image of all three labels and shows that REST and TRF2 are often co-localized in nuclear foci (arrows).

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Figure 2. Characteristics and fates of normal neural progenitor cells and cancer stem cells

Cancer stem cells within a brain tumor may arise from normal stem cells or neural progenitor cells that harbor mutations. Cancer stem cells within glioblastomas may be particularly resistant to cytotoxic therapies, and therefore are a source of cells for cancer recurrence. To meet the challenge of a cure for glioblastomas, new therapies that selectively suppress proliferation and/or kill cancer stem cells must be found.

Figure 3. Mechanisms by which TRF2 and SCF β -TrCP regulate cancer stem cell fate via interactions with REST

REST is a transcriptional silencer that represses a large network of neural genes; this gene network is also intimately related to the regulatory repertoire of core pluripotency factors that control the self-renewal capacity of stem cells. (i) REST represses genes by binding to specific RE1 elements, and several proteins interact with REST to modify its activity including CoREST, histone deacetylase 2 (HDAC2) and telomere repeat-binding factor 2 (TRF2). The proteasomal degradation of REST is essential for de-repressing neural genes and neuronal differentiation. (ii) TRF2 binds to and stabilizes REST, thereby preventing its degradation, whereas activity of the ubiquitin E3 ligase SCF β -TrCP accelerates proteasomal degradation of REST. (iii) TRF2 is also a critical component of the shelterin protein complex that protects and stabilizes telomeres. Gene therapies that deplete or neutralize TRF2 will therefore have a dual adverse effect on cancer stem cells by exposing telomere ends to induce a DNA damage response involving the proteins ATM and γ H2AX which triggers cell cycle arrest and apoptosis, and by depleting REST to engage the neuronal cell differentiation program. Increased expression of SCF β -TrCP would also deplete REST and inhibit cancer stem cell growth.