Cell Stem Cell
Article

An RNAi Screen Identifies TRRAP as a Regulator of Brain Tumor-Initiating Cell Differentiation

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DOI 10.1016/j.stem.2009.11.002

SUMMARY

Glioblastoma multiforme (GBM) is a highly aggressive form of brain cancer associated with a very poor prognosis. Recently, the initiation and growth of GBM has been linked to brain tumor-initiating cells (BTICs), which are poorly differentiated and share features with neural stem cells (NSCs). Here we describe a kinome-wide RNA interference screen to identify factors that control the tumorigenicity of BTICs. We identified several genes whose silencing induces differentiation of BTICs derived from multiple GBM patients. In particular, knockdown of the adaptor protein TRRAP significantly increased differentiation of cultured BTICs, sensitized the cells to apoptotic stimuli, and negatively affected cell cycle progression. TRRAP knockdown also significantly suppressed tumor formation upon intracranial BTIC implantation into mice. Together, these findings support a critical role for TRRAP in maintaining a tumorigenic, stem cell-like state.

INTRODUCTION

Glioblastoma multiforme (GBM; WHO grade IV astrocytoma/glioma) is the most aggressive and most common brain tumor in adults. Most deaths occur within 2 years of diagnosis despite advances in surgery, radiation, and chemotherapy (Grossman and Batara, 2004). In addition to its highly brain-infiltrative character, GBM shows a strong phenotypic and genotypic heterogeneity, further complicating therapeutic intervention. Recent evidence has indicated that primary brain tumors possess a hierarchical organization of heterogeneous cell populations, which differ in their tumor-forming potential. Tumor-driving capacity has been attributed to poorly differentiated, brain tumor-initiating cells (BTICs, also termed cancer stem cells or tumor stem cells) (Lee et al., 2006; Nakano and Kornblum, 2006; Singh et al., 2003). BTICs share several features with neural stem cells (NSCs) including the expression of neural markers such as Nestin and Sox2 (Gangemi et al., 2008; Mangiola et al., 2007), the ability to migrate within the brain (Sanai et al., 2005), the capacity to self-renew and to undergo multilineage differentiation (Fael Al-Mayhani et al., 2009; Galli et al., 2004; Lee et al., 2006; Nakano and Kornblum, 2006; Piccirillo et al., 2006; Pollard et al., 2009; Singh et al., 2003), and responsiveness to similar signaling cues (for example Hedgehog, Notch, and Pten/Akt signaling) (Clement et al., 2007; Purow et al., 2005; Zheng et al., 2008). Furthermore, it has been recently reported that bone-morphogenetic protein (BMP), as well as distinct microRNAs, can suppress the tumorigenicity of BTICs by promoting their differentiation (Lee et al., 2008; Piccirillo et al., 2006; Silber et al., 2008). Thus, it may be possible to target BTICs by developing differentiation-inducing therapies for GBM tumors. This approach requires an understanding of the genes and pathways that are involved in the maintenance of the poorly differentiated, tumor-initiating cell state. In this study, we established and characterized BTICs from several primary GBM tumors and identified genes regulating their differentiation through an unbiased, kinome-wide RNA interference (RNAi) screen (~500 kinase targets). To further validate the screen results, we show that one candidate gene known as transformation/transcription domain-associated protein (*TRRAP*) is required for BTIC tumorigenicity in vitro and in vivo.

RESULTS

In Vitro Expansion and Characterization of Patient-Derived BTICs

To establish BTIC expansion and culture conditions suitable for the automated imaging required for this large-scale RNAi screen, we optimized adherent cell culture conditions that

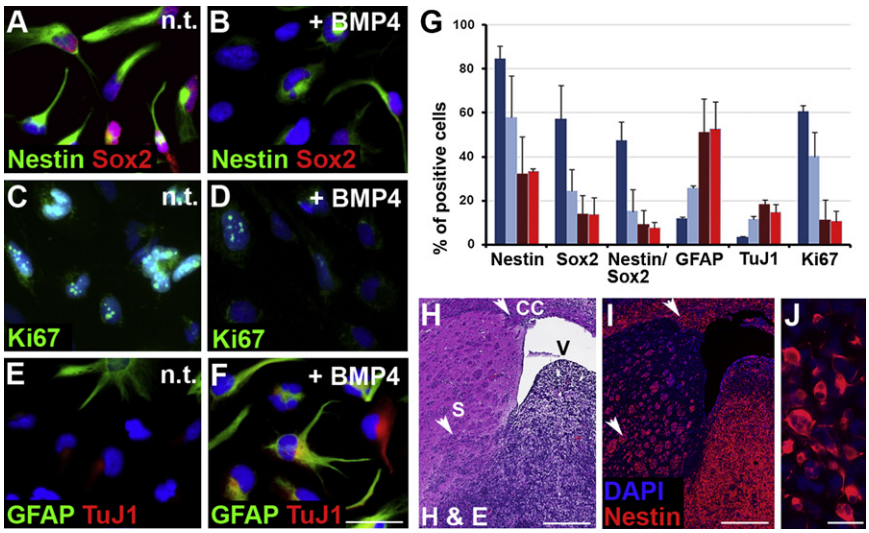

Figure 1. Phenotypic Characterization of BTICs

(A–F) Expression of Nestin, Sox2, and Ki67 (A and C) decreases in cultured BTICs in the presence of BMP4 (50 ng/mL; B and D) whereas expression of GFAP and TuJ1 increases compared to nontreated cells (n.t., E and F); DAPI-stained nuclei (blue). Scale bar represents 25 μm.

(G) Quantitation of cell marker expression of GBM surgical specimens (mean, error bars represent the standard error of the mean) grown under BTIC conditions (blue columns), upon withdrawal of FGF/EGF (light blue columns), upon further addition of 10% serum (dark red columns), and under BTIC conditions plus BMP4 (red columns).

(H and I) H&E and Nestin staining on adjacent sections detecting GBM-1-derived tumor mass below the ventricle (V), 15 weeks postimplantation. Arrowheads illustrate tumor cells infiltrating the corpus callosum (CC) and striatum (S) of the mouse brain. Scale bars represent 500 μm.

(J) Higher-power image of Nestin-expressing tumor cells within the CC. Scale bar represents 20 μm. Additional immunohistochemistry of xenograft tumors is shown in Figure S1.

have been previously reported for the undifferentiated growth of NSCs and BTICs (Gage et al., 1995; Lee et al., 2006; McKay, 1997). Under these BTIC conditions (poly-D-lysine/laminin-coated culture dishes and Neurobasal medium supplemented with the mitogens FGF/EGF), monolayer-cultured cells were reproducibly derived from five different primary human GBM specimens (GBM-1 to GBM-5; Figure S1 available online). These maintained both distinct global gene expression patterns and differential expression of several glioma candidate genes, reflecting GBM tumor heterogeneity (Figure S1). We next evaluated the neural stem cell-like characteristics and differentiation properties of early culture passage GBM-derived monolayer cells by using established in vitro and in vivo assays (Gage et al., 1995; Lee et al., 2006, 2008; McKay, 1997; Nakano and Kornblum, 2006). Under BTIC conditions, the majority of these cells maintained an undifferentiated phenotype as indicated by a high percentage of cells positive for the neural precursor/glioma markers Nestin and Sox2 (~80% and 60%, respectively; Figures 1A and 1G) and the proliferation marker Ki67 (~60%; Figures 1C and 1G), as well as a low percentage of differentiated cells expressing the astroglial marker GFAP (<10%) or the neuronal marker TuJ1 (<5%; GFAP-TuJ1 double-positive cells were <2%; Figures 1E and 1G). Upon withdrawal of the mitogens FGF/EGF, the percentage of differentiated cells increased by ~2-fold, while a substantial cell population remained proliferative (~40% Ki67-positive cells), consistent with an autocrine mitogenic activity reported previously for mitogen-depleted cultures of glioma stem cells (Li et al., 2009). Expectedly, addition of serum induced a prodifferentiation effect of all specimen-derived cells as reflected by a ~4-fold increase of GFAP- or TuJ1-positive cells and a concurrent reduction of Nestin, Sox2, and Ki67 immunoreactivity (Figure 1G). Addition of the cytokine BMP4 (50 nM) to the FGF/EGF-containing BTIC medium elicited a differentiation phenotype in all specimens at a level comparable to mitogen-depleted, serum-treated cultures.

Patient-Derived BTIC Xenograft Tumors Show Characteristics of High-Grade Gliomas

To evaluate the in vivo tumorigenicity of the patient-derived monolayer cultured cells, they were genetically tagged with firefly luciferase (*F-luc*) and their growth was monitored by noninvasive bioluminescence imaging after implantation into the forebrain of CB17/SCID mice (Figure S1). Injection of 10,000 or 30,000 early passage cells led to efficient tumor growth over 15 weeks in 100% of the cases. Subsequent histological analysis and staining for GFAP, Ki67, and human-specific Nestin confirmed a large tumor burden in animals 15 weeks after implantation (Figures 1H–1J; Figure S1). Importantly, the xenograft tumors showed characteristics of high-grade gliomas including high cellularity, tumor heterogeneity, proliferation, and massive infiltration into areas adjacent to the implantation site and even into relatively distant brain areas (e.g., the olfactory bulbs). In line with recent reports (Fael Al-Mayhani et al., 2009; Lee et al., 2006; Pollard et al., 2009), our findings indicate a stem cell-like and tumor-initiating character of monolayer-cultured specimen-derived BTICs, which also reflect hallmark features of human brain tumors in the xenograft model.

A Kinome-wide RNAi Screen for Induced BTIC Differentiation

We next established a quantifiable assay of BTIC differentiation suitable for a high-throughput RNAi screen. BTICs were grown in precoated 384-well microtiter plates (~1000 cells/well) and transduced with lentiviral particles produced from a nontargeting shRNA control plasmid encoding GFP (shGL3). Viral transduction was efficient as indicated by 60%–90% GFP-expressing cells in distinct experiments. To establish a positive control for a BTIC differentiation phenotype, shGL3-transduced BTICs were treated with BMP4 and analyzed after 6 days by automated image analysis after fixation and nuclear staining (Figures 2A–2C; Figure S2). To minimize potential screen artifacts, several parameters were combined to quantitatively

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