



# Hes3 regulates cell number in cultures from glioblastoma multiforme with stem cell characteristics

SUBJECT AREAS:  
CANCER STEM CELLS  
CNS CANCER  
NEURAL STEM CELLS  
STEM-CELL RESEARCH

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Tumors exhibit complex organization and contain a variety of cell populations. The realization that the regenerative properties of a tumor may be largely confined to a cell subpopulation (cancer stem cell) is driving a new era of anti-cancer research. Cancer stem cells from Glioblastoma Multiforme tumors express markers that are also expressed in non-cancerous neural stem cells, including nestin and Sox2. We previously showed that the transcription factor Hes3 is a marker of neural stem cells, and that its expression is inhibited by JAK activity. Here we show that Hes3 is also expressed in cultures from glioblastoma multiforme which express neural stem cell markers, can differentiate into neurons and glia, and can recapitulate the tumor of origin when transplanted into immunocompromised mice. Similar to observations in neural stem cells, JAK inhibits Hes3 expression. Hes3 RNA interference reduces the number of cultured glioblastoma cells suggesting a novel therapeutic strategy.

**G**lioblastoma Multiforme (GBM) is the most common primary brain tumor and is associated with very poor prognosis<sup>1,2</sup>. Tumor initiating cells (Cancer Stem Cells, “CSCs”) have been identified in GBM<sup>3–7</sup> and a race is on to understand how to target them as a potential therapeutic avenue. Although the lineage connection between non-cancerous neural stem cells (NSCs) and CSCs is not established, these cell types share several properties including signaling pathways that contribute to their growth as well as the expression of common markers including nestin and Sox2<sup>8</sup>.

We recently demonstrated that the transcription factor Hes3<sup>9–11</sup> is a marker of established neural stem cell cultures from the fetal and adult rodent central nervous system<sup>12–16</sup>. Factors that promote the expansion of these cells, including Notch ligands, Angiopoietin 2, insulin, as well as inhibitors of the Janus kinases (JAK) and p38MAP kinases, also increase the expression of Hes3. In the adult rodent and primate brains, Hes3 identifies a perivascular cell subpopulation that co-expresses other markers of immature cells, such as Sox2. In this report, we demonstrate that HES3 not only provides a novel identifying marker for the CSC population in GBMs, but is a key mediator of the number of these cells, thereby offering a potential therapeutic target.

## Results

In biopsies from patients with GBM, Hes3 co-localizes with the putative cancer stem cell marker prominin<sup>14,17,18</sup> (Fig. 1a,b). These results agree with the observations that magnetic immunoprecipitation of cells from the rodent brain using an antibody against prominin generate a cell fraction which is enriched in Hes3+/Sox2+ cells<sup>14</sup>. Taken together, these data suggest that Hes3 may be expressed in putative cancer stem cells in GBM and that it may mediate cell number expansion.

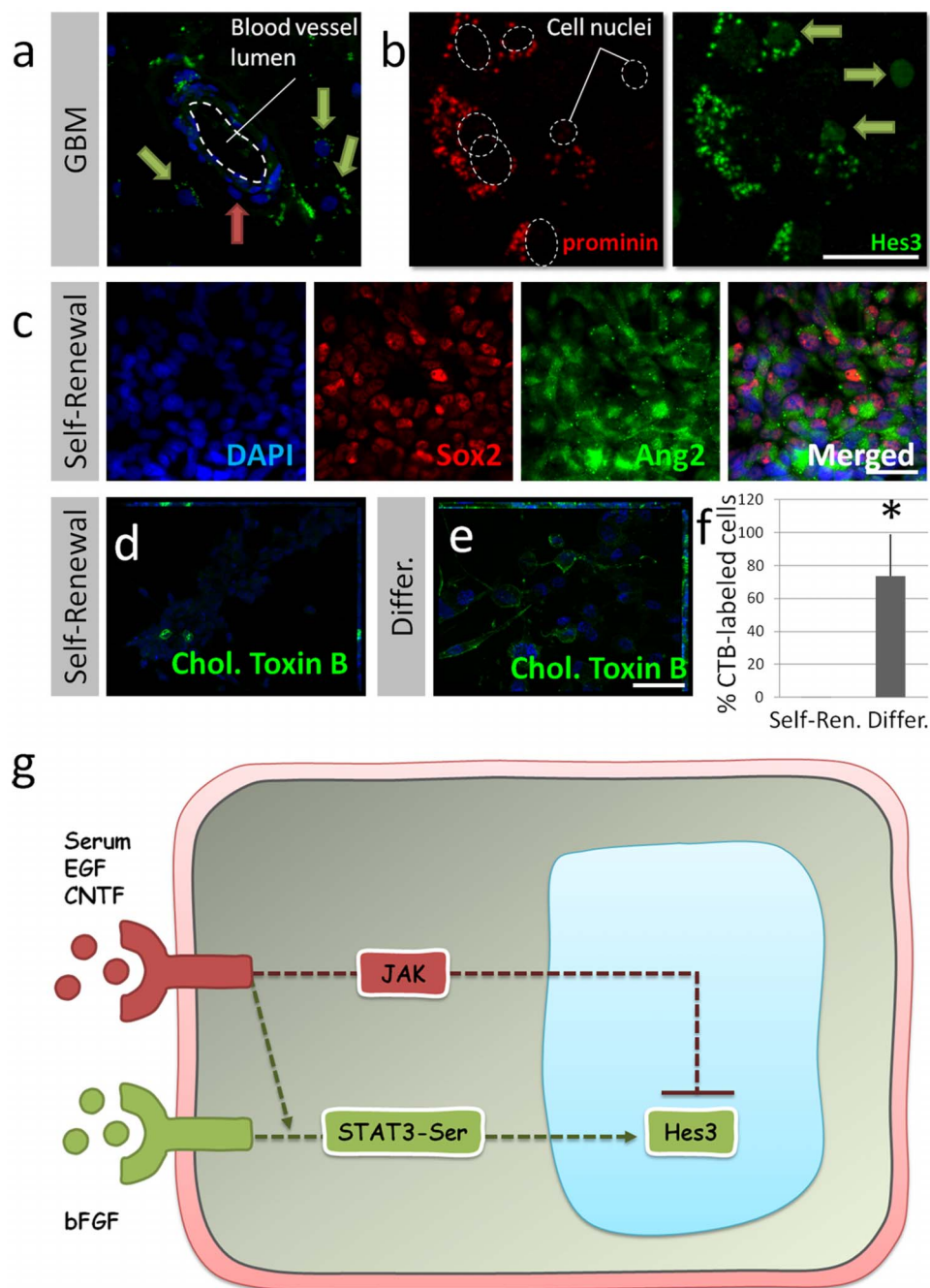
To address the potential value of Hes3 as a target in cancer medicine, we used primary cultured cells that were isolated from GBM biopsies. These cells were maintained in vitro using standard protocols for the establishment of non-cancerous neural stem cells cultures<sup>19–21</sup>. These cells can be expanded with the support of the mitogen Epidermal Growth Factor (EGF) or basic Fibroblast Growth Factor (bFGF)<sup>21</sup>. Our previous work has established that the same cells used in this study can be propagated in culture over long periods of time and many passages, express several markers of NSCs, including, Sox2 and nestin, as well as prominin, can be induced to differentiate



into neurons, astrocytes, and oligodendrocytes, they phenocopy the tumor of origin in xenograph experiments, and they respond to many growth factors that NSCs also respond to<sup>21,22</sup>.

We recently showed that cultured NSCs express Angiopoietin 2 (Ang2) as well as its receptor, Tie2, and that treatment with Ang2 increases cell number both in vitro and in vivo<sup>14,15</sup>. Here we show that GBM cells also express Ang2 together with the more established

marker Sox2 (Fig. 1c). NSCs express a small number of GM1+ gangliosides on their cell surface which can be identified by binding to a fluorophore-conjugated B subunit of cholera toxin<sup>16</sup>; in contrast, their differentiated progeny expresses many binding sites. In our cultured GBM cells (in the presence EGF or bFGF), a very small percentage of cells labeled with conjugated cholera toxin (Fig. 1d-f). In contrast, when cells were treated with serum (which induces the



**Figure 1 | Hes3 is expressed in putative cancer stem cells in glioblastoma.** (a) Hes3+ cells are located adjacent to blood vessels in biopsies from GBM patients. (A blood vessel lumen is marked in white; green arrows point to Hes3+ cells; the red arrow points to nuclei of vascular cells). (b) Another example of Hes3+ cells in a GBM tumor show co-localization of Hes3 with prominin (red and green split channels are shown side-by-side). White circles show the location of the nuclei, some of which appear positive for Hes3 signal (Marked by green arrows). (c) Cultured GBM cells co-express Sox2 and Ang2. (d-f) GBM cells in the presence of EGF and the absence of serum (“Self-Renewal”) express low amounts of GM1+ gangliosides, identified by labeling with a fluorophore-conjugated cholera toxin B subunit; addition of serum for 4 days increases labeling. (g) Diagram showing positive regulation on Hes3 transcription by bFGF via phosphorylation of STAT3-serine, and opposition by JAK activity. Hes3 is positively regulated by signals that promote STAT3-serine phosphorylation in the absence of STAT3-tyrosine phosphorylation. In NSCs, such signals include bFGF, insulin, the Notch ligands Delta4 and Jagged1, and Angiopoietin 2. Signals that induce the JAK/STAT pathway (which leads to STAT3-tyrosine phosphorylation) do not induce Hes3 transcription. Such signals include CNTF, EGF, and components of serum. [Nuclei are stained with DAPI and appear blue. Size bar: 100 micrometers].



differentiation of NSCs), the number of cholera toxin binding sites greatly increased. These results further support that the cultured GBM cells used express markers commonly associated with neural stem cells and that they possess the potential to differentiate into cells representing the main cell types of the nervous system.

Our previous work with NSCs showed that Hes3 expression is opposed by the actions of JAK. Its activity can be assessed by measuring STAT3 phosphorylation on tyrosine residue 705 (STAT3-Tyr), which is downstream of JAK<sup>23</sup>. In contrast, Hes3 expression is supported in conditions where STAT3 is phosphorylated on serine residue 727 (STAT3-Ser)<sup>12</sup>. NSCs cultured in bFGF exhibit low Hes3 mRNA levels, but these can be increased by concomitant exposure to Ciliary Neurotrophic Factor (CNTF) and JAK inhibitor (JAK I)<sup>12</sup>, a treatment which elevates STAT3-Ser and inhibits STAT3-Tyr (Suppl. Fig. 1a,b). Similar results can be obtained by low concentrations of CNTF<sup>24</sup> that also elevate STAT3-Ser but not STAT3-Tyr phosphorylation (Suppl. Fig. 1c). The potential of JAK I to promote the undifferentiated state in NSC cultures is also demonstrated by the fact that it inhibits the expression of the glial marker GFAP in the presence of bFGF (Suppl. Fig. 1d). Low CNTF concentrations or concomitant high concentrations of CNTF and JAK I increase the number of cultured NSCs in the presence of bFGF (Suppl. Fig. 1e).

We hypothesized that GBM cells cultured in the absence of serum and the presence of mitogen would induce the expression of Hes3 and, therefore, they would resemble, in that respect, the cells of their origin in the patient. We tested the following four conditions: (a) EGF, (b) EGF + JAK I, (c) bFGF, and (d) bFGF + JAK I. We found that the highest STAT3-Ser and lowest STAT3-Tyr phosphorylation state was in the FGF + JAK I condition (Fig. 2a). In these conditions, cells were able to proliferate; in fact, their number was greater than in the other conditions, after one week of treatment (Fig. 2b). We note that inclusion of the JAK inhibitor allowed cell cultures to reach a higher terminal cell density, as assessed by cell number counting following a 2 week treatment (Fig. 2c,d). Hes3 expression varied among the four conditions; the highest incidence of Hes3+ cells was in the bFGF + JAK I condition (Fig. 2e,f). Since the culture system presented here lacks serum to support cell growth, the presence of a mitogen (bFGF or EGF) is required. As a consequence, we studied the effects of treatments (e.g. JAK I) in the presence of these defined factors.

To address whether Hes3 is a mediator of the expansion of GBM cells and, therefore, a putative anti-cancer target, we performed transfections with Hes3 siRNA. We tested two different control ("scrambled") siRNA products and three different Hes3 siRNA products for corroboration. We performed the experiments in both EGF and bFGF + JAK I conditions to show the sturdiness of the result and to demonstrate that the effect of Hes3 interference is not an artifact of a particular cell culture system. In all cases, Hes3 interference caused the reduction of Hes3 expression, as expected, and a reduction in cell number (Fig. 2g). We confirmed the results using two additional GBM cell cultures established from different patients (Fig. 2h–j). A cell survival assay showed a reduction in viability in the Hes3 siRNA – transfected cells, suggesting that, partly, the reduction in cell number is due to a reduction in viability (Fig. 2k).

## Discussion

Brain tumors contain multiple cell types, some of which may carry the lion's share of the regenerative properties of the tumor, manifested as metastasis and recurrence. Different subpopulations of cells express different sets of markers (with plenty of overlap) allowing for the ability to distinguish among these cell types to a certain degree. These cells may also differ in terms of the signal transduction pathways they utilize in order to grow. Given the variations in extracellular signals within the tumor microenvironment, this is a logical postulate. It is, therefore, critical to study and define different

signaling systems that contribute to the growth of tumors and design manipulations to counteract tumor growth.

In this manuscript we applied findings that we generated during our previous work with non-cancerous NSCs<sup>12–16</sup>. This work showed that NSCs utilize non-canonical signal transduction pathways to regulate their survival and numbers in vitro and in vivo. Key to these non-canonical pathways is the phosphorylation state of STAT3. When STAT3 is phosphorylated on serine 727 in the absence of detectable phosphorylation on tyrosine 705, cells survive and proliferate in a self-renewing manner. In contrast, when the tyrosine residue is also phosphorylated, NSCs differentiate with a preference towards glial fates<sup>19,25,26</sup>. STAT3-Ser phosphorylation promotes the expression of the transcription factor Hes3, which we showed to be a novel biomarker of established NSCs. In addition, Hes3 identifies new pools of cells in various areas of the adult brain which can be placed in culture where they exhibit self-renewal and multi-potentiality, generating neurons, astrocytes, and oligodendrocytes.

We also showed that Hes3 is expressed in tumor tissue from glioblastoma patients, and that it co-localizes with the putative CSC marker prominin. Here, we extended our studies to show that (1) primary GBM cell cultures also express Hes3 under conditions that support STAT3-Ser phosphorylation and suppress STAT3-Tyr phosphorylation and that (2) Hes3 interference reduces the numbers of cells in culture.

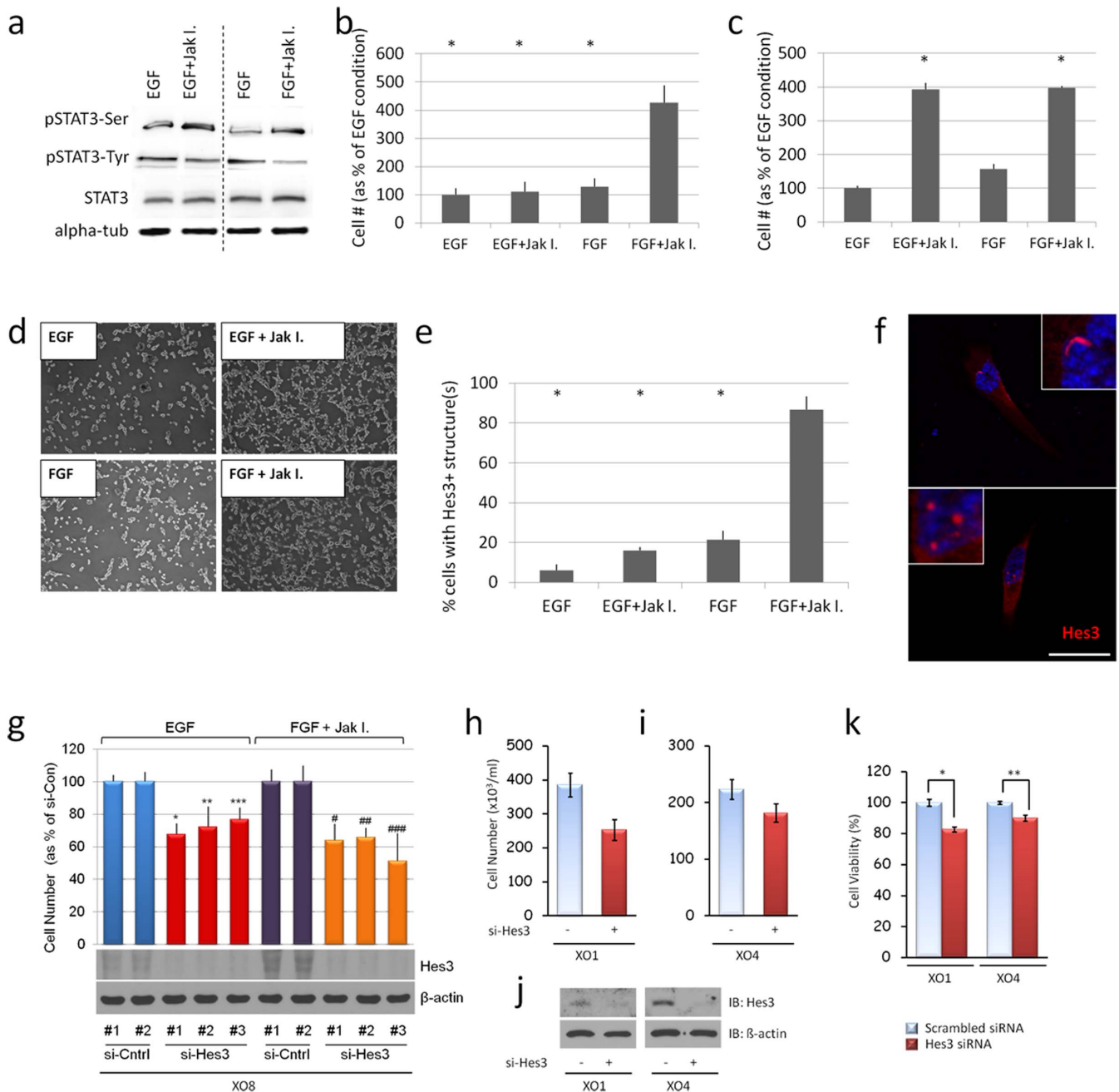
These early results suggest that Hes3 is a new marker of GBM cells and a mediator of cell expansion. Future studies will address the relevance of Hes3 in cultures from different GBM subtypes (e.g., as defined by The Cancer Genome Atlas). It will also be important to address the mechanisms that regulate the cell number changes reported here. For example, our results help distinguish between cell expansion rates and maximal cell density. Specifically, whereas inclusion of the JAK I in the presence of EGF did not alter cell number during the early culture phase, it did allow cells to reach higher densities. Given that JAK is implicated in the canonical Notch signaling pathway and subsequent gliogenic differentiation<sup>27</sup>, it is intriguing to speculate that non-canonical Notch signaling branches which are opposed by JAK<sup>12</sup> may be competing with canonical pathways and that Hes3 may be a critical regulator of the balance between these two branches.

Our previous work with non-cancerous NSCs showed that activation of the STAT3-Ser/Hes3 signaling pathway promotes cell survival without altering the cell cycle duration<sup>12</sup>. Whether this applies to GBM cells, will have to be determined in future studies.

It is difficult to define a cancer stem cell population. The GBM-derived cells used in this work, however, do exhibit CSC properties, as suggested by their (1) expression of undifferentiated markers, (2) ability to differentiate into neuron and glia-like cells, and (3) ability to phenocopy the tumor of origin in immunocompromised mice. These results suggest that Hes3 may be relevant as a target for the elusive CSC population of GBM and, possibly other solid tumors such as breast, colon and pancreatic cancers<sup>28–32</sup>. Preliminary results show that in spontaneously arising brain tumors in adult mouse null for the p53 tumor suppressor gene, Hes3+ cells are clearly seen both within the tumor mass (identified by morphology and staining for KI67), as well as outside the borders of the tumor (data not shown). Additional work will require validation in multiple in vitro and in vivo paradigms.

Beyond the possible value of Hes3 as a CSC target, these results show that the same cell (or cell line) is capable of utilizing distinct signaling pathways to ensure its growth. Glioma cells are often cultured in the presence of serum, or in the absence of serum with EGF. Here we show that the same GBM cell cultures can be cultured in EGF or FGF + JAK I in the absence of serum. EGF and serum components activate the JAK-STAT pathway which suppresses Hes3 expression, whereas bFGF activates the STAT3-Ser pathway which induces Hes3 expression. Therefore, the same cell can grow





**Figure 2 | Hes3 regulates the number of GBM cells in culture.** (a) Culture conditions affect the phosphorylation states of STAT3 of GBM cells. (b) GBM cells grow efficiently in the four conditions described in the text (7-d). (c) JAK inhibition increases the terminal cell density that GBM cells reach (14-d). (d) JAK inhibition increases the terminal cell density that GBM cells reach (14-d; representative phase contrast images). (e) Culture conditions regulate the probability that GBM cells will express Hes3 (7-d). (f) Examples of Hes3 staining in human GBM cells, in the FGF+JAK I conditions (7-d; size bar: 25 micrometers. Nuclei are stained with DAPI and appear blue). (g) Hes3 knockdown in GBM cells (line “XO8”) reduces the amount of Hes3 protein and cell number (5-d). Each control bar represents a different control (“scrambled”) siRNA product and each of the three “Hes3” labeled bars represents a different Hes3 siRNA product for corroboration. (h–j) Similar results are obtained using two additional GBM cell cultures (“XO1” and “XO4”) in the presence of EGF. (k) A cell viability assay (Promega MTS) shows reduced viability following Hes3 interference (5-days).

under conditions that not only are starkly different, but even opposing. This complexity at the level of signal transduction of a single cell confers growth options to the cell and may be a major contributor to the difficulties in combating cancer.

## Methods

**Neural stem cell culture.** Neural Stem Cell cultures from the mid-gestation (E13.5) mouse embryo cortex were grown as previously described<sup>14</sup>. Tissue was dissected under a dissection microscope, triturated in 1 ml N2 medium containing bFGF

with a 1 ml pipette until no tissue clumps were seen; the triturate was allowed to settle for 1 min and the top 0.9 ml was collected, diluted in N2 containing bFGF and plated. Cells were expanded in serum-free DMEM/F12 medium with N2 supplement and bFGF (20 ng/ml) for 5 days under 5% oxygen conditions and were re-plated fresh or from frozen stocks at 1,000–10,000 cells per cm<sup>2</sup>. bFGF was included throughout our experiments, unless otherwise stated. To induce differentiation, the culture medium was replaced with fresh culture medium which does not contain bFGF and the cells were incubated for 5 days. Treatments were as described in the main text. In the experiments involving the treatment of cells with CNTF and the JAK inhibitor, the inhibitor was added to the culture medium 30-min prior to the CNTF.



**Glioblastoma cell culture.** Three GBM cell lines (“X01”, “X04”, and “X08” lines) were established from acutely resected human tumor tissues (Glioblastoma multiforme). Resected tissue was treated using the protocol described above for mouse fetal neural stem cells. Cells were cultured under the same conditions that were used for rodent neural stem cells. Variations in their mitogenic support are noted in the text. Treatments were as described in the main text.

**Pharmacological Treatments.** bFGF and EGF were purchased from RnD Systems and were used at a concentration of 20 ng/ml. JAK inhibitor was purchased from Calbiochem and was used at a concentration of 200 nM.

**Human biopsies of solid tissue.** All human tissues in this study were obtained during surgical resections from patients with newly diagnosed or recurrent tumors. Materials in excess of pathological evaluation were used for research purposes in accordance with protocols approved by the Institutional Review Board of the National Institutes of Health. Written consent was obtained and all research tumor tissues were de-identified.

**Animal handling.** Animals were handled and housed according to the German Federal guidelines for the use and care of laboratory animals, and the study was approved by the Landesdirektion Dresden. For research performed at the National Institutes of Health (USA), all research involving animals was conducted in accordance with NINDS ACUC (National Institute of Neurological Disorders and Stroke/Animal Care and Use Committee) guidelines and after their approval.

**RNA interference.** We used the Amaxa nucleofector equipment to electroporate siRNA into GBM cell cultures. Two control siRNA products were used (Santa Cruz Biotechnology, sc37007 and OriGene Tech, SR3004) and three anti-Hes3 siRNA products (Santa Cruz Biotechnology, sc88003 and OriGene Tech, SR318208A and SR318208C). The day before transfection, 5 million cells were dissociated from a culture flask and transferred to T25 Falcon culture flask with suspension medium containing EGF or bFGF. Cells were then transfected with siRNA using a nucleofecting electroporator according to the manufacturer’s protocol (Amaxa Inc., Gaithersburg, MD, USA). After 24 h, the medium was replaced, and cells were harvested for additional experiments. Typical transfection efficiency was at approximately 80%<sup>21,22</sup>.

**Reverse transcriptase PCR.** RNA was extracted from cell cultures with Trizol (Invitrogen, 15596-026), and PCR reactions were performed with the ProSTAR First-Strand RT-PCR Kit (Stratagene, 200420).

**Immunohistochemistry.** Under deep anesthesia, animals were perfused transcardially with a rinse of saline, followed by 4% formaldehyde fixative (pH 7.4). Brains were removed immediately, stored in the fixative solution overnight, and then in 30% sucrose for 3 days. Brains were frozen-sectioned at 16 or 30 micrometers.

**Immunocytochemistry.** Cells were fixed onto the culture plate by replacing the culture medium with ice cold 4% formaldehyde fixative (pH 7.4), for 30 min. PFA was washed twice with PBS and the cells were immunostained using standard techniques.

**Western Blot analysis.** Western Blot analyses were performed using standard protocols. Briefly, Laemmli Sample Buffer from Bio-Rad was used as the harvesting buffer. Samples were boiled for 15 min and approximately 40 microgram of protein was loaded per well. Primary antibodies were used at a dilution of 1 : 500 and secondary antibodies at 1 : 4,000. Antibodies were diluted in 5% BSA in PBST. Primary antibodies were incubated overnight, with mild shaking, at 4 degrees Celsius.

**Cell viability assays.** Cell viability was measured by the MTS assay (Promega).

**Reagents.** We used the following reagents and antibodies: bFGF (233-FB), fibronectin (1030-FN), from R&D; JAK Inhibitor I (420099), from Calbiochem; Polyoornithine (P-3655), insulin (I9278) from Sigma; Alexa-Fluor-conjugated secondary antibodies from Invitrogen; DAPI (D-8417) from Sigma, ECL reagents (34080) from Pierce, polyacrylamide gradient gels from Invitrogen, HRP-conjugated secondary antibodies from Jackson Immunoresearch, and general chemicals from Sigma. For immunohistochemical staining and Western Blotting, we used antibodies against the following markers: Hes3 (25393), STAT3 (482), pSer727-STAT3 (8001-R), pTyr705-STAT3 (7993) from Santa Cruz;  $\alpha$ -tubulin from Sigma. (T-6074). Hes3 siRNA was from Santa Cruz Biotechnology and OriGene Tech (please see Electroporation section).

**Statistical analysis.** Results shown are the mean  $\pm$  S.D. Asterisks identify experimental groups that were significantly different (p-value<0.05) from control groups by the Student’s t-test (Microsoft Excel), where applicable.

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## Author contributions

A.A.T. conceived the project, A.A.T., D.M.P. and S. Poser designed experiments, A.A.T., D.M.P., J.J., J.M., S.M., D.E., S.S., R.R., N.P., S. Pacioni and S. Poser performed experiments, A.A.T., D.M.P., J.J., R.D.M. and S. Poser analyzed data. A.A.T., D.M.P. and S. Poser wrote the main manuscript text. All authors reviewed the manuscript.

## Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

**Competing financial interests:** The authors have submitted two patents relating to this work.

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