LETTERS

Bone morphogenetic proteins inhibit the tumorigenic potential of human brain tumour-initiating cells

S. G. M. Piccirillo[1,2], B. A. Reynolds[3], N. Zanetti[2], G. Lamorte[2], E. Binda[4], G. Broggi[5], H. Brem[6], A. Olivi[6], F. Dimeco[5,6] & A. L. Vescovi[1,2,4]

Transformed, oncogenic precursors, possessing both defining neural-stem-cell properties and the ability to initiate intracerebral tumours, have been identified in human brain cancers[1]. Here we report that bone morphogenetic proteins (BMPs), amongst which BMP4 elicits the strongest effect, trigger a significant reduction in the stem-like, tumour-initiating precursors of human glioblastomas (GBMs). Transient *in vitro* exposure to BMP4 abolishes the capacity of transplanted GBM cells to establish intracerebral GBMs. Most importantly, *in vivo* delivery of BMP4 effectively blocks the tumour growth and associated mortality that occur in 100% of mice after intracerebral grafting of human GBM cells. We demonstrate that BMPs activate their cognate receptors (BMPRs) and trigger the Smad signalling cascade in cells isolated from human glioblastomas (GBMs). This is followed by a reduction in proliferation, and increased expression of markers of neural differentiation, with no effect on cell viability. The concomitant reduction in clonogenic ability, in the size of the CD133$^+$ population and in the growth kinetics of GBM cells indicates that BMP4 reduces the tumour-initiating cell pool of GBMs. These findings show that the BMP–BMPR signalling system—which controls the activity of normal brain stem cells[2,3]—may also act as a key inhibitory regulator of tumour-initiating, stem-like cells from GBMs and the results also identify BMP4 as a novel, non-cytotoxic therapeutic effector, which may be used to prevent growth and recurrence of GBMs in humans.

Within the past decade accumulating evidence from a number of biological systems, such as the blood[4], breast[5] and brain[6–10], has indicated that transformed, stem-like precursors may represent the cells that drive tumour growth[11]. If tumour-initiating precursors with stem-like properties (TICs) are major culprits in tumour initiation

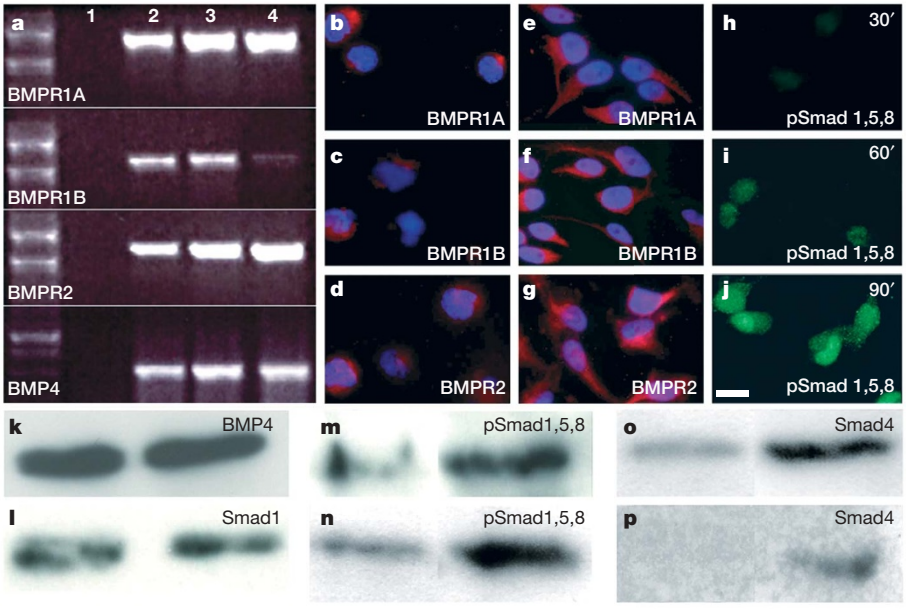

Figure 1 | Expression and activation of BMP receptors in human GBM cells. **a**, Messenger RNA transcripts for BMP receptors and BMP4. Lane 1, negative control; lane 2, acutely dissociated GBM CD133$^+$ cells; lane 3, briefly cultured CD133$^+$ cells; lane 4, MCF7 cells (positive control). **b**–**g**, BMPR immunoreactivity in acutely isolated (**b**–**d**) or briefly cultured (**e**–**g**) CD133$^+$ cells. **h**–**j**, Phosphorylation of Smad proteins (using anti-phospho(p)Smad1,5,8) after exposure of briefly cultured cells to BMP4 for 30 min (**h**), 60 min (**i**) or 90 min (**j**) (Supplementary Fig. 2b; Supplementary Table a). **b**–**j**, Scale bar, 15 μm, as in **j**. **k**–**p**, Western blotting. **k**, BMP4 in acutely dissociated (left) or briefly cultured CD133$^+$ cells (right). **l**, Unchanged Smad1 levels in BMP4-treated (right), briefly cultured cells (left, untreated control). **m**, **n**, Increased Smad1,5,8 phosphorylation by BMP4 (left, untreated control; right, BMP4-treated) in acutely dissociated (**m**) or briefly cultured (**n**) cells. **o**, **p**, Increased Smad4 expression in briefly cultured (**o**) or acutely dissociated (**p**) cells by BMP4 (untreated control, left; BMP4-treated, right).

[1]Department of Biotechnology and Biosciences, University of Milan–Bicocca, 20126 Milan, Italy. [2]Unit of Cancer Stem Cell Biology, StemGen Spa, University of Milan–Bicocca, 20126 Milan, Italy. [3]Laboratory for Neural Stem Cell Biology, Queensland Brain Institute, University of Queensland, 4072 Brisbane, Queensland, Australia. [4]Stem Cell Research Institute, H. S. Raffaele, 20132 Milan, Italy. [5]National Neurological Institute 'C. Besta', 20133 Milan, Italy. [6]Department of Neurosurgery, John Hopkins University, Baltimore, 21287 Maryland, USA.

and development, then conventional approaches targeting the overall population of neoplastic cells may well spare TICs owing to their idiosyncratic properties[12].

Similar to normal neural stem cells, brain TICs (BTICs) represent a small fraction of GBM cells[8], belong to a $CD133^+$ pool[9], display self-renewal *in vitro*, generate a large number of progeny and are multipotent[8,13,14]. BTICs can establish GBMs at the clonal level and perpetuate across serial transplantation[8,9].

Bone morphogenetic proteins (BMPs) elicit a plethora of actions[15–19] and have an instructive role in the adult brain stem cell niche, favouring the acquisition of an astroglial fate[2,3]. Given this and the involvement of BTICs in brain tumours, we examined the putative effects of BMPs on human GBMs.

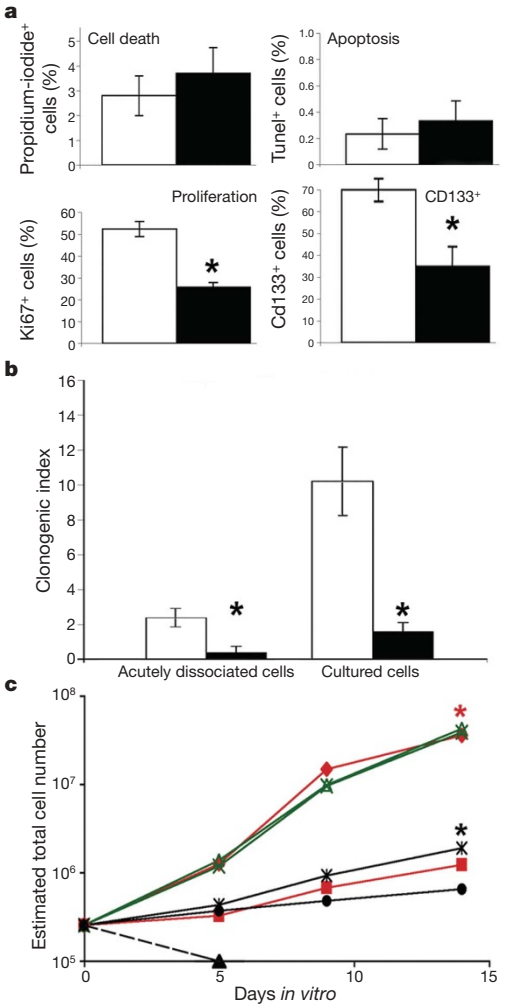

Figure 2 | BMP4 depletes the GBM BTIC population *in vitro*. **a**, BMP4 doesn't alter cell death or apoptosis (Tunel, terminal-deoxynucleotidyl-transferase-mediated dUTP nick end-labelling), but reduces proliferation (Ki67 immunoreactivity) and the $CD133^+$ BTIC population[9] in GBM cultures. Untreated control, empty column; BMP4-treated, solid column; mean ± s.e.m., $n = 3$; $^*P < 0.005$. **b**, BMP4 reduces the clonogenic index in acutely dissociated and briefly cultured GBM cells. Untreated control, empty column; BMP4-treated, solid column; mean ± s.e.m., $n = 3$; $^*P < 0.005$. **c**, Acutely dissociated GBM cells could not be expanded in the presence of BMP4 (black dotted line, triangles). After brief expansion with mitogens alone, (red rhombuses), acutely dissociated cells received BMP4, resulting in the reduction of the growth kinetics (red squares), similar to human fetal neural stem cells (black stars, untreated control; black circles, BMP4-treated) but unlike U87 human glioma lines (green triangles, untreated control; green stars, BMP4). Mean ± s.e.m., $n = 3$; $^*P < 0.005$ for red asterisk and $^*P < 0.01$ for black asterisk).

The *in vitro* findings below describe data from GBM cells[8,9] sorted for $CD133^+$ using fluorescence activated cell sorting, and either cultured for 48 h (ref. 9), and thus referred to as 'briefly cultured cells', or used soon after sorting from GBM tissue (acutely dissociated). Equivalent results were obtained with additional samples, including unsorted, briefly cultured or acutely dissociated cells from human GBMs[8,9] (for detailed sample specifics and complete data, see Supplementary Tables a–i and respective legends). First, we demonstrated that BMP4 and its receptor (BMPR) transcripts and proteins are expressed in GBM cells, particularly in the $CD133^+$ putative BTIC population[9]. Transcripts for BMPR subtypes 1A, 1B and 2 and BMPs were found in acutely dissociated and briefly cultured GBM cells (Fig. 1a; Supplementary Figs 1 and 10a). The cognate receptor (Fig. 1b–g) and BMP4 proteins (Fig. 1k) were found in both $CD133^+$ and $CD133^-$ fractions (not shown). BMPRs were known to be functional because Smad 1-5-8 phosphorylation was observed (Fig. 1h–j and Supplementary Table a) following addition of BMP4 ($100\ ng\ ml^{-1}$; Supplementary Fig. 2a); the p38 mitogen-activated protein kinase (MAPK) pathway was not engaged (Supplementary Fig. 2b).

We next investigated the effect of BMP4 on GBM cells. Unlike other cell systems[20], BMP4 didn't induce cell death or apoptosis, but reduced proliferation in response to mitogens (Fig. 2a; Supplementary Tables b–d). BMP2, 5, 6, 7 and 8b, but not BMP1, 3 or 3b, yielded similar effects (Supplementary Fig. 3). This was confirmed by the concomitant increase in the number of GBM cells in G0/G1 phase and a decrease of those in S phase in response to BMP4 (Supplementary Fig. 4b and Supplementary Table e). Transforming growth factor (TGF)βs, found in GBMs[21] and known to engage overlapping signalling pathways with BMP4[22,23], elicited no effects (Supplementary Fig. 3), underlining the specificity of the BMP4 action on GBM cells.

Two assays—one determining the percentage of clone-forming neural precursors (the clonogenic index)[24] and a second assessing the expansion of the neural-stem-cell pool by growth kinetics analysis[25]—confirmed that the cytostatic effect of BMP4 impinged on the BTIC population in GBM cells. BMP4 produced a greater than 70% reduction in the clonogenic index in GBM cells *in vitro* (Fig. 2b). Furthermore, exposure of GBM cells to BMP4 soon after isolation from the primary tumour specimen prevented their expansion in culture (Fig. 2c). Addition of BMP4 to GBM cells that were already expanding *in vitro* greatly decreased their growth rate (Fig. 2c; Supplementary Fig. 4a).

Because BMP4 reduces the growth of BTICs (Fig. 2), we investigated its effects on the $CD133^+$ subpopulation, known to be enriched in BTICs[9]. BMP4 decreased the size of the $CD133^+$ pool by approximately 50%, both in acutely dissociated and briefly cultured GBM cells (Fig. 2a; Supplementary Fig. 5 and Supplementary Table g). These data indicate that BMP4 targets the BTIC population.

BMP4 induced overt morphological changes in GBM cells *in vitro*. Relative to cells grown with mitogens alone, BMP4-treated cells adopted a more differentiated morphology (flat, phase-dark with elaborated processes; Fig. 3a, b; Supplementary Fig. 4c). We also observed an increase in the expression of astroglial markers (Glial fibrillary acidic protein (GFAP) immunoreactivity), together with a similar but reduced increase in neuronal (ßIII-tubulin and MAP5) and oligodendroglial (Galactocerobroside C (GalC) immunoreactivity) antigens (Fig. 3c–h; Supplementary Fig. 4c).

The aberrant co-expression of neuronal and glial antigens in undifferentiated BTICs makes it impractical to quantify the pro-differentiation effects of BMP4 by direct counting of immunoreactive cells (Fig. 3c, f; Supplementary Fig. 6). Therefore, we used cytofluorimetry to measure fluorescence signal intensity. Relative to control cells, BMP4-treated cultures exhibited a greater than twofold increase in GFAP immunoreactivity (Fig. 3i; Supplementary Table h) and a consistent, yet not marked, increase in βIII-tubulin (Fig. 3j) and GalC immunoreactivity (Fig. 3k; Supplementary Table h).

Altogether, the results above suggested that BMP4 produces a pro-differentiation action on GBM cells (which, predominantly, acquire

an astroglial-like fate) and depletes the pool of tumorigenic BTICs. From this we inferred that the *in vitro* reduction in the stem-cell-like BTIC would correspond to a similar decline in the ability of BMP4-treated cells to form tumours *in vivo*. To test this, both acutely dissociated and briefly cultured $CD133^+$ GBM cells were exposed to BMP4 for 48 h, before unilateral, intrastriatal injection (3×10^5 viable cells) into immunodeficient mice. Tumour formation and expansion were compared with those of control animals receiving GBM cells maintained under identical conditions without BMP4. All animals receiving untreated GBM cells developed large tumour masses (Fig. 4a; Supplementary Fig. 7) that showed characteristic glioblastoma features, including nuclear atypia, expression of aberrant glia, extensive neovascularization and high mitotic activity[8] (Supplementary Fig. 7), and invaded the lateral, third and fourth ventricles (Supplementary Figs 7, 8). Conversely, BMP4-treated cells did not form invasive tumours, but small, delimited lesions, which were confined to the injection site, had a low mitotic index and showed no ventricular invasion (Fig. 4b; Supplementary Fig. 7). Three to four months post-injection all control animals died, whereas nearly all mice receiving BMP4 pre-treated cells survived (Fig. 4i). Identical results were observed when the residual $CD133^+$ fraction from BMP4-treated cells was re-purified by FACS and its tumorigenicity compared with an equal number (3×10^5 $CD133^+$ cells per animal) of $CD133^+$ cells from matched control animals (Supplementary Fig. 7m, n). Also, we could always re-culture $CD133^+$ BTICs (average clonogenic frequency: $9.0 \pm 1.3\%$ ($n = 2$), 90 days post-transplant) from the brain of mice receiving acutely dissociated control GBM cells. When re-transplanted these cells gave rise to large secondary brain tumours (see Supplementary Fig. 7). Conversely, this was never possible when animals received BMP4-pretreated GBM cells in the primary transplant, nor was it possible by direct injection of 3×10^5 cells, which were acutely dissociated from the primary tumours from these same mice. Thus, transient exposure to BMP4 depletes the BTIC population and produces a significant decrease in the *in vivo* tumour-initiating ability of GBM cells.

Next, we sought to establish if delivery of BMP4 *in vivo* might prevent intracerebral tumour establishment and growth. Transplantation of acutely dissociated or briefly cultured GBM cells was accompanied by injection of vehicle(control)- or BMP4-saturated polyacrylic beads (releasing BMP4 for 1 week; unpublished data), at the site of cells' engraftment, either at the same time as cell implantation (co-treatment) or ten days later (post-treatment). In all experiments, animals receiving control beads developed large, malignant tumours (Fig. 4c, e, g) and died (Fig. 4i), whereas mice implanted with BMP4-releasing beads displayed small, confined lesions (Fig. 4d, f, h), surviving significantly longer (Fig. 4i). Control tumours contained pleiomorphic, neoplastic elements, with reactive chromatin and malignant, infiltrating cells (Fig. 4g). Conversely, lesions in BMP4-treated animals included few neoplastic cells, but had mature elements and macrophages (Fig. 4h). The mitotic index was significantly lower in BMP4-treated animals (co-treatment: control 3.78 ± 0.17 versus BMP4 0.20 ± 0.11; $P < 0.01$, mean $\pm$ s.e.m., $n = 4$, two-tailed Student's *t*-test; post-treatment: control 2.88 ± 0.46 versus BMP4 0.63 ± 0.29; $P < 0.05$, mean $\pm$ s.e.m., $n = 4$, two-tailed Student's *t*-test). *In vivo,* immunofluorescence revealed the presence of astrocytes and nestin-positive cells, but not oligodendroglial or neuronal cells, in control and BMP4-treated tumours (Supplementary Fig. 9).

BTICs are implicated in the initiation and development of GBMs[1], the most common adult malignant brain tumour[26]. The fact that BTICs possess some properties of normal stem cells[12,13,27] may explain GBMs' therapy-resistance and common recurrence[1,12,28]. Hence, discovering shared regulatory mechanisms between normal neural stem cells and BTICs may identify new targets for GBM therapy. Our data show that BMP4, a neural-stem-cell regulator, dramatically hinders the tumorigenicity of human GBM cells. The available methods used

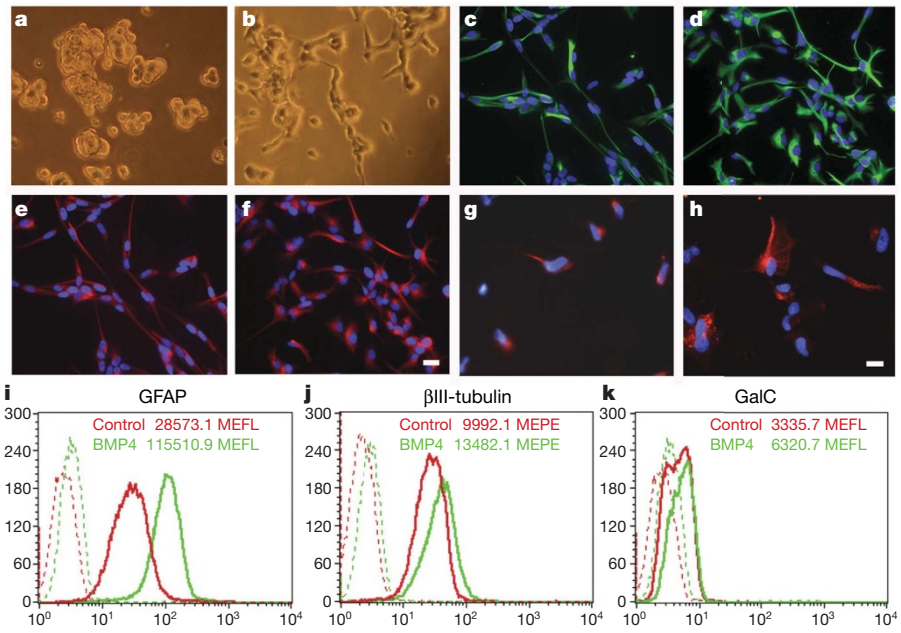

Figure 3 | BMP4 elicits a pro-differentiation effect on GBM cells. **a**, **b**, Relative to mitogens alone (control; **a**) more differentiated (that is, adherent and phase-dark) cells (**b**) are seen on addition of BMP4 to briefly cultured cells. **c**–**h**, BMP4 increases GFAP immunoreactivity (**c**, untreated control; **d**, BMP4-treated), ßIII-tubulin immunoreactivity (**e**, untreated control; **f**, BMP4-treated) and GalC immunoreactivity (**g**, untreated control; **h**, BMP4-treated) relative to controls. Representative micrographs of three experiments are shown. **a**–**f**, Scale bar, 20 μm, as in **f**. **g**, **h**, Scale bar, 15 μm, as in **h**. **i**–**k**, Cytofluorimetric quantification in untreated control (red) versus BMP4-treated (green), briefly cultured GBM cells, revealing an increase in the molecules of equivalent fluorescein (MEFL) for GFAP immunoreactivity (**i**). A slight increase in equivalent phycoerythrin molecules (MEPE) for ßIII-tubulin (**j**) or MEFL for GalC immunoreactivity (**k**), was observed (Supplementary Table h).

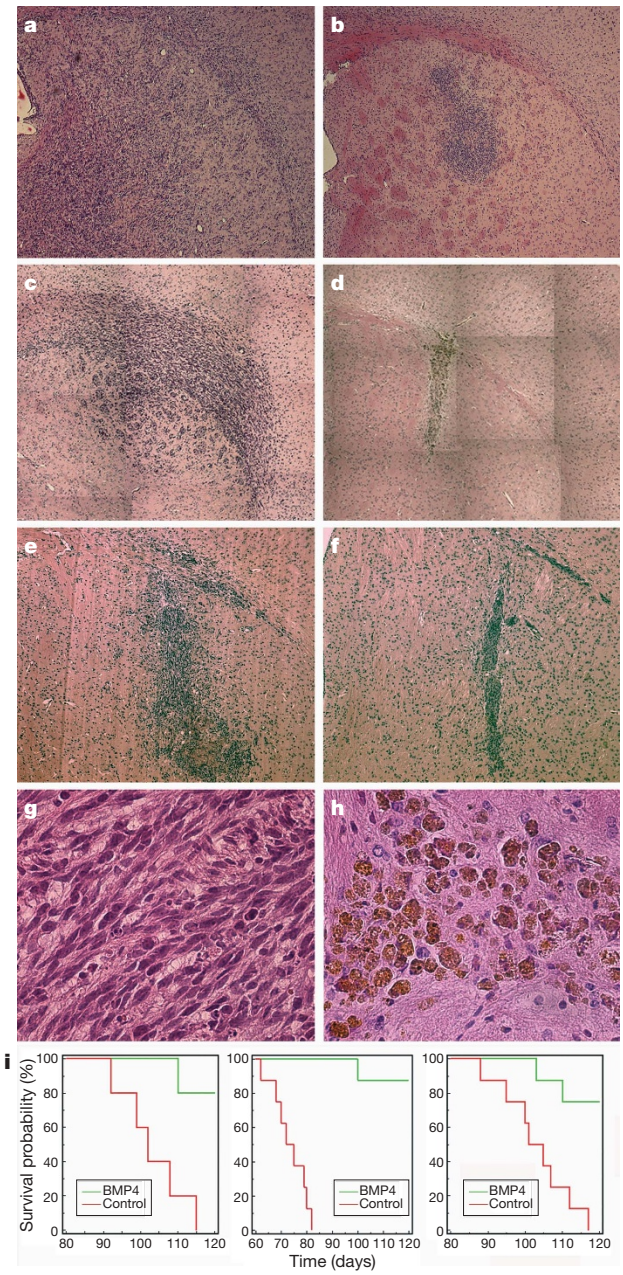

Figure 4 | BMP4 inhibits tumorigenicity of GBM cells. a, **b**, Forty-eight hour exposure of acutely dissociated GBM cells to BMP4 *in vitro*, before transplantation into the striatum of *scid/bg* mice, dramatically reduces tumour-initiating ability (**a**, untreated control; **b**, five-weeks post-injection; see Supplementary Fig. 7 for data on briefly cultured cells). **c**–**f**, Polyacrylic beads, pre-adsorbed with BMP4 and implanted at the cell injection site inhibit tumour growth when co-injected at the same time as cells (co-treatment; **c**, untreated control; **d**, BMP4-treated; **c**, **d**, briefly cultured GBM cells, four-weeks post-injection, $n = 14$) or 10 days thereafter (post-treatment; **e**, untreated control; **f**, BMP4-treated; **e**, **f**, acutely dissociated GBM cells, 4 weeks from cell injection, $n = 5$). The mitotic index was always significantly higher in untreated controls compared with BMP4-treated animals, in co-transplantation (3.78 ± 0.17 versus 0.20 ± 0.11, respectively, $n = 4$; $P < 0.01$, two-tailed Student's *t*-test) and post-transplantation experiments (2.88 ± 0.46 versus 0.63 ± 0.29, respectively, $n = 4$; $P < 0.05$, two-tailed Student's *t*-test). Tumours in untreated control mice contain pleiomorphic, neoplastic and malignant, infiltrating cells (**g**). Lesions in BMP4-treated animals contained differentiated cells and macrophages (**h**). Magnification was ×5 in **a**–**f**, and ×40 in **g**, **h**. **i**, Survival of BMP4-treated animals was enhanced in pre- (left) co- (centre) and post-transplantation treatment (right) paradigms (Logrank test, $P < 0.001$, $P < 0.001$ and $P < 0.005$, respectively).

here do enable significant enrichment of GBM BTICs[8,9], but still yield preparations containing additional cell types. Thus, although BMP4 targets the GBM stem-like BTIC pool, it is also likely to affect these heterogeneous GBM cell populations as a whole.

Blocking endogenous BMP4 (Fig. 1 and Supplementary Fig. 10a) reduces Smad signalling and increases GBM cell growth (Supplementary Fig. 10b, c). Thus, endogenous BMP4 may operate in GBM cells, regulating the balance between proliferation and differentiation, and favouring the production of the differentiated astroglial-like cells normally found within GBMs.

Regarding the mechanisms underlying the growth inhibitory effects of BMP4 on GBM BTICs: BMP4 may reduce the frequency of BTICs by decreasing symmetric cell cycles that generate two BTICs on division; trigger differentiation of a subpopulation of BTICs; or block proliferation of BTICs and their progeny. Although not mutually exclusive, the above scenarios would all reduce the BTIC population.

We show that a critical system that regulates normal neural-stem-cell fate[2,29] operates in the BTICs of human GBMs. It also unveils an undocumented role for BMPs in human GBM biology: their ability to promote differentiation and to deplete the pool of BTICs. The finding that *in vivo* delivered BMP4 blocks tumour development raises the potential of use in patients following surgery. These results support a new approach to GBM treatment—inducing differentiation of the tumour-initiating cells, rather than killing them. The delivery of BMP4 or BMP-mimetic drugs, combined with classical therapy, may help reduce patient lethality due to GBM.

METHODS

Samples. Data are from cells dissociated from adult human GBM specimens, obtained and classified according to the guidelines of the World Health Organization.

Primary cell culturing and propagation. Cells were isolated from GBMs as described[8] and sorted for $CD133^+$ immunoreactivity and used as such (acutely dissociated cells) or following plating in NeuroCult NS-A medium (Stem Cell Technologies) and culturing with mitogens for 48 h (briefly cultured) (see Supplementary Methods). Culture propagation, clonogenic assay and population analysis were performed as previously described[8,9]. BMP4 pre-treatment was performed by adding 100 ng ml^{-1} of BMP4 to growth medium, 48 h before transplantation.

Immunocytochemistry. GBM cells were plated onto Matrigel-coated glass coverslips in the presence of FGF2/ EGF and treated with BMP4 (100 ng ml^{-1}) for 48 h. Cells were washed, fixed in 4% paraformaldehyde and processed for immunofluorescence of neural antigens (GFAP, Dako Corporation; neuronal βIII-tubulin, Babco; GalC, Chemicon) as described[8]. Ki67 staining (1:1,000, NovoCastra) detected proliferating cells. For the remaining immunocytochemical analysis see Supplementary Methods.

Evaluation of tumorigenicity by orthotopic injection and immunohistochemistry. Cells were orthotopically transplanted following washing and resuspension in PBS (10^8 cells per ml). Three microlitres were injected stereotactically into the right striatum of immunodeficient mice as previously described[8]. Polymer-based delivery of BMP4 used BMP4-loaded heparin acrylic beads (100 beads per animal; Sigma). Beads were incubated for 1 h at 37 °C in PBS alone or containing BMP4 (0.65 μg μl^{-1}), rinsed three times with PBS and implanted. Haematoxylin- and eosin-staining and immunohistochemistry were performed on paraffin-embedded, 4-μm thick sections and processed as previously described[8,30].

Kaplan Meier survival analysis. A downward sloping plot of the cumulative chance (*y*-axis) of survival time was performed by MedCalc software (Mariakerke). Differences were analysed by Logrank test.

Received 6 April; accepted 12 October 2006.

1. Vescovi, A. L., Galli, R. & Reynolds, B. A. Brain tumour stem cells. *Nature Rev. Cancer* **6**, 425–436 (2006).
2. Lim, D. A. *et al.* Noggin antagonizes BMP signaling to create a niche for adult neurogenesis. *Neuron* **28**, 713–726 (2000).
3. Panchision, D. M. & McKay, R. D. The control of neural stem cells by morphogenic signals. *Curr. Opin. Genet. Dev.* **12**, 478–487 (2002).
4. Lapidot, T. *et al.* A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* **367**, 645–648 (1994).
5. Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J. & Clarke, M. F. Prospective identification of tumorigenic breast cancer cells. *Proc. Natl Acad. Sci. USA* **100**, 3983–3988 (2003).

6. Ignatova, T. N. *et al.* Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers *in vitro*. *Glia* **39,** 193–206 (2002).
7. Hemmati, H. D. *et al.* Cancerous stem cells can arise from pediatric brain tumors. *Proc. Natl Acad. Sci. USA* **100,** 15178–15183 (2003).
8. Galli, R. *et al.* Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Res.* **64,** 7011–7021 (2004).
9. Singh, S. K. *et al.* Identification of human brain tumour initiating cells. *Nature* **432,** 396–401 (2004).
10. Taylor, M. D. *et al.* Radial glia cells are candidate stem cells of ependymoma. *Cancer Cell* **8,** 323–335 (2005).
11. Sell, S. Stem cell origin of cancer and differentiation therapy. *Crit. Rev. Oncol. Hematol.* **51,** 1–28 (2004).
12. Reya, T., Morrison, S. J., Clarke, M. F. & Weissman, I. L. Stem cells, cancer, and cancer stem cells. *Nature* **414,** 105–111 (2001).
13. Potten, C. S. & Loeffler, M. Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development* **110,** 1001–1020 (1990).
14. Weiss, S. *et al.* Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. *J. Neurosci.* **16,** 7599–7609 (1996).
15. Shah, N. M., Groves, A. K. & Anderson, D. J. Alternative neural crest cell fates are instructively promoted by TGFβ superfamily members. *Cell* **85,** 331–343 (1996).
16. Furuta, Y., Piston, D. W. & Hogan, B. L. Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. *Development* **124,** 2203–2212 (1997).
17. Li, W., Cogswell, C. A. & LoTurco, J. J. Neuronal differentiation of precursors in the neocortical ventricular zone is triggered by BMP. *J. Neurosci.* **18,** 8853–8862 (1998).
18. Iantosca, M., McPherson, C., Ho, S. & Maxwell, G. Bone morphogenetic proteins-2 and -4 attenuate apoptosis in a cerebellar primitive neuroectodermal tumor cell line. *J. Neurosci. Res.* **56,** 248–258 (1999).
19. Chen, D., Zhao, M. & Mundy, G. R. Bone morphogenetic proteins. *Growth Factors* **22,** 233–241 (2004).
20. Hallahan, A. R. *et al.* BMP-2 mediates retinoid-induced apoptosis in medulloblastoma cells through a paracrine effect. *Nature Med.* **9,** 1033–1038 (2003).
21. Kjellman, C. *et al.* Expression of TGF-β isoforms, TGF-β receptors, and SMAD molecules at different stages of human glioma. *Int. J. Cancer* **89,** 251–258 (2000).
22. Canalis, E., Economides, A. & Gazzerro, E. Bone morphogenetic proteins, their antagonists, and the skeleton. *Endocr. Rev.* **2,** 218–235 (2003).
23. Golestaneh, N. & Mishra, B. TGF-β, neuronal stem cells and glioblastoma. *Oncogene* **24,** 5722–5730 (2005).
24. Reynolds, B. A. & Weiss, S. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev. Biol.* **175,** 1–13 (1996).
25. Reynolds, B. A. & Rietze, R. L. Neural stem cells and neurospheres–re-evaluating the relationship. *Nature Methods* **2,** 333–336 (2005).
26. Barker, F. G. II. *et al.* Survival and functional status after resection of recurrent glioblastoma multiforme. *Neurosurgery* **42**, 709–720; discussion 720–3 (1998).
27. Dean, M., Fojo, T. & Bates, S. Tumour stem cells and drug resistance. *Nature Rev. Cancer* **5,** 275–284 (2005).
28. Pardal, R., Clarke, M. F. & Morrison, S. J. Applying the principles of stem-cell biology to cancer. *Nature Rev. Cancer* **3,** 895–902 (2003).
29. Rajan, P., Panchision, D. M., Newell, L. F. & McKay, R. D. BMPs signal alternately through a SMAD or FRAP–STAT pathway to regulate fate choice in CNS stem cells. *J. Cell Biol.* **161,** 911–921 (2003).
30. Vescovi, A. L. *et al.* Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. *Exp. Neurol.* **156,** 71–83 (1999).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements This work was supported by the Associazione Italiana per la Ricerca sul Cancro, the Plurigenes EEC grant, funding from the Brain Tumours Funders' Collaborative, from Neurothon Onlus, and by a donation from BMW Italia. We are grateful to R. Galli, J. Laterra, C. L. Solero and F. McBlane for helpful comments and suggestions.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to A.L.V. (vescovi@tin.it).