

ORIGINAL ARTICLE

DNMT-dependent suppression of microRNA regulates the induction of GBM tumor-propagating phenotype by Oct4 and Sox2

H Lopez-Bertoni^{1,2}, B Lal^{1,2}, A Li¹, M Caplan¹, H Guerrero-Cázares³, CG Eberhart⁴, A Quiñones-Hinojosa^{3,5,6}, M Glas^{7,8,9}, B Scheffler⁷, J Laterra^{1,2,5,6,10} and Y Li^{1,2,10}

Cancer stem-like cells represent poorly differentiated multipotent tumor-propagating cells that contribute disproportionately to therapeutic resistance and tumor recurrence. Transcriptional mechanisms that control the phenotypic conversion of tumor cells lacking tumor-propagating potential to tumor-propagating stem-like cells remain obscure. Here we show that the reprogramming transcription factors Oct4 and Sox2 induce glioblastoma cells to become stem-like and tumor-propagating via a mechanism involving direct DNA methyl transferase (DNMT) promoter transactivation, resulting in global DNA methylation- and DNMT-dependent downregulation of multiple microRNAs (miRNAs). We show that one such downregulated miRNA, miRNA-148a, inhibits glioblastoma cell stem-like properties and tumor-propagating potential. This study identifies a novel and targetable molecular circuit by which glioma cell stemness and tumor-propagating capacity are regulated.

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INTRODUCTION

Glioblastoma (GBM) contains sub-populations of multipotent stem-like cells (SCs) that grow as spheres (that is, neurospheres) and efficiently propagate tumors in xenograft models, reflecting their self-renewing and tumor-propagating capacity. Substantial evidence indicates that these SCs have a particularly important role in maintaining tumor growth, therapeutic resistance and tumor recurrence.^{1,2} Emerging findings from multiple laboratories reveal that the stem-like tumor-propagating phenotype is dynamically regulated by autocrine/paracrine and environmental signals and that more differentiated cancer progenitor cell subsets have the capacity to dedifferentiate and acquire a stem-like phenotype in response to these contextual cues.³

It is now well recognized that expressing a defined set of ‘Yamanaka transcription factors’ (Sox2, Oct4, Klf4 and c-Myc) can reprogram cells to a stem-like state.⁴ Cell phenotype determination by these transcription factors is context-dependent and regulated by genetic and epigenetic mechanisms that remain poorly defined.^{5,6} In cancer, elevated expression of ‘Yamanaka transcription factors’ correlates with poor prognosis and tumor progression. The expression of one or more of these reprogramming transcription factors has also been shown to switch tumor cells to a more tumor-propagating stem-like state and induce a more aggressive tumor phenotype.⁷ Multiple oncogenic signaling pathways, including receptor tyrosine kinases, have the capacity to serve as upstream drivers of the neoplastic stem-like tumor-propagating state by virtue of their capacity to induce similar

mechanisms involving Oct4, Sox2 and Nanog.^{8,9} Determinants of the tumor-propagating state downstream of these reprogramming transcription factors remain only partially defined.

MicroRNAs (miRNAs) are short noncoding RNAs that inhibit gene expression by targeting messenger RNA (mRNA) for degradation or by blocking translation of target genes.¹⁰ These molecules control a wide range of biologic processes and can function as both tumor suppressors and oncogenes, as well as determinants of tumor cell stem-like states.^{11,12} Reprogramming transcription factors regulate expression of miRNA subsets in embryonic stem cell (ES cells) and expressing a defined set of miRNAs is sufficient to induce dedifferentiation of human and mouse cells, implicating miRNAs in controlling ES cell identity.^{13,14} These and other related findings highlight that miRNAs can act to determine cell fate and cell potency. However, the role and molecular basis for miRNA dysregulation in determining cancer stem-like phenotypes and tumor-propagating capacity remain poorly characterized.

Epigenetic mechanisms such as DNA and histone modifications regulate the expression of coding and noncoding genes, including miRNAs. Conversely, miRNAs modulate the expression of epigenetic modifiers such as DNA methyltransferases (DNMT), histone deacetylases and polycomb group genes involved in cell fate determination.¹⁵ DNA methylation has a particularly prominent role in cell potency and lineage-specific differentiation. Conversions between multipotent stem cells and differentiated cell phenotypes are accompanied by extensive changes in DNA methylation patterns.⁵ Similarly, DNA methylation, mediated by

¹Hugo W Moser Research Institute at Kennedy Krieger, Baltimore, MD, USA; ²Department of Neurology, Johns Hopkins School of Medicine, Baltimore, MD, USA; ³Department of Neurosurgery, Johns Hopkins School of Medicine, Baltimore, MD, USA; ⁴Department of Pathology, Johns Hopkins School of Medicine, Baltimore, MD, USA; ⁵Department of Neuroscience, Johns Hopkins School of Medicine, Baltimore, MD, USA; ⁶Department of Oncology, Johns Hopkins School of Medicine, Baltimore, MD, USA; ⁷Institute of Reconstructive Neurobiology, MediClin Robert Janker Klinik and University of Bonn Medical Center, Bonn, Germany; ⁸Department of Neurology, MediClin Robert Janker Klinik and University of Bonn Medical Center, Bonn, Germany and ⁹Clinical Cooperation Unit Neurooncology, MediClin Robert Janker Klinik and University of Bonn Medical Center, Bonn, Germany. Correspondence: Dr J Laterra or Dr Y Li, Hugo W Moser Research Institute at Kennedy Krieger, 707 North Broadway, Baltimore, MD 21205, USA. E-mail: Laterra@kennedykrieger.org or liyu@kennedykrieger.org

¹⁰These authors contributed equally to this work.

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the combined action of three DNMTs (Dnmt1, Dnmt3a and Dnmt3b), is associated with tumor initiation, progression and specific tumor cell subsets.¹⁶

This study focuses on understanding how reprogramming transcription factors drive the cancer stem-like phenotype through DNA methylation-dependent miRNA regulation. We show that the coordinated actions of Oct4 and Sox2 induce a tumor-propagating stem-like state in GBM cells via a mechanism involving DNMT promoter transactivation, DNA methylation and methylation-dependent repression of multiple miRNAs. We further show that one of the miRNAs repressed by Oct4/Sox2, miRNA-148a, inhibits the GBM stem-like phenotype and that miR-148a repression is required for the induction of GBM tumor-propagating capacity by Oct4/Sox2. These results identify a new methylation-dependent and miRNA-dependent transcriptional axis by which reprogramming transcription factors regulate cancer cell phenotype and tumor-propagating capacity.

RESULTS

Oct4 and Sox2 correlate with and induce a GBM stem-like phenotype

Oct4 and Sox2 are core reprogramming transcription factors that physically interact and cooperate to induce ES cell self-renewal and pluripotency.¹⁷ These transcription factors are also over-expressed in human cancers and their expression levels are associated with tumor progression and poor prognosis.¹⁸ We examined Oct4 and Sox2 expression in GBM cell fractions and in regions of human tumors enriched for stem-like tumor-propagating cells. Using two human GBM neurosphere lines previously characterized by us and others,⁸ cell fractions expressing the stem cell markers CD133¹⁹ and SSEA-1²⁰ were separated from marker-negative cells by flow cytometry and Oct4 and Sox2 expression was measured by quantitative reverse transcription-PCR (qRT-PCR). CD133⁺ and SSEA-1⁺ cells expressed 2–4-fold higher levels of Oct4 and Sox2 compared with the CD133⁻ or SSEA⁻ cells (Figure 1a and Supplementary Figure S1). Vescovi and co-workers²¹ and more recently Glas *et al.*²² found that the central cores of clinical GBMs are enriched for cancer SCs relative to the tumor peripheries. We examined the geographic patterns of Oct4 and Sox2 expression in nine GBMs resected at the University of Bonn Medical Center.²² Higher Oct4 and Sox2 expression was found in cells obtained from tumor centers consistent with the preferential localization of SCs (Figure 1b). GBM SCs have the capacity to differentiate along multiple neural lineages in response to experimental conditions of forced differentiation (for example growth factor withdrawal and low serum concentrations), which also inhibit sphere-forming capacity and tumor propagation.² Forced differentiation of GBM spheres resulted in substantial reductions in Oct4 and Sox2 expression (Figure 1c). Using a complementary gain-of-function approach, coexpressing transgenic Oct4 and Sox2 significantly enhanced GBM neurosphere growth (Figure 1d and Supplementary Figure S2A), concurrent with the induction of stem cell markers Bmi-1 and Olig-2 and reduced expression of differentiation markers O4, GFAP and Tuj-1 in the absence of epidermal growth factor/fibroblast growth factor (EGF/FGF) (Figure 1e and Supplementary Figure S1D). Coexpressing Oct4 and Sox2 also generated a cell phenotype resistant to γ -radiation (Figures 1f and h). Taken together, these results show that Oct4 and Sox2 associate with GBM stem-like phenotype and induce heterogeneous populations of GBM neurosphere cells to become more stem-like.

We asked more directly if coexpressing Oct4 and Sox2 induces human GBM cells that lack tumor-propagating potential to transition to a stem-like tumor-propagating state. A172 glioma cells, a non-tumorigenic human glioma line, were transduced with lentiviral vectors coding for vexGFP-Oct4 and mCitrine-Sox2. Cells

expressing both Oct4 and Sox2 were selected by flow cytometry (Supplementary Figure S2B) and cultured under conditions that support somatic cell reprogramming (see Materials and methods for details). Regions containing aggregates of cells with epithelioid morphology appeared within 2 weeks of transduction (Figure 2A, panel b, asterisk). Colonies of these ES-like cells were picked 5 weeks after transduction and maintained in defined neurosphere medium containing EGF and FGF (Figure 2A, panel c). These colonies were found to be positive for alkaline phosphatase, SSEA-1 and SSEA-4 (Figure 2A, panels d–f). Cells comprising these colonies formed large well-defined multicellular spheres (referred to as induced GBM SCs, A172-iGSCs) and maintained the capacity to self-renew through passaging as spheres for at least 6 months (Figure 2B, lower panel). In contrast, control-transfected A172 cells (A172-Con) remained adherent and failed to form self-renewing spheres under identical conditions (Figure 2B, upper panel). A172-iGSCs expressed high levels of stem cell markers and regulators such as CD133, SSEA-1, Bmi-1 and Olig-2 in comparison with A172-control cells (Figure 2C, left panel). Nanog, a critical regulator of stemness, was also substantially upregulated in A172-iGSCs (Figure 2C, right panel). A172-iGSCs also acquired multipotency, a feature of stem-like tumor-propagating cells derived directly from clinical GBM, as evidenced by their capacity to differentiate along GFAP⁺ astroglial, neurofilament M⁺ neuronal and O4⁺ and O1⁺ oligodendroglial lineages (Figure 2D).⁸ A172-iGSCs acquired the capability to efficiently form tumors *in vivo* when injected in the flanks (Figure 2E) or brains (Figure 2F) of immune compromised mice. Histopathologic features of orthotopic xenografts derived from A172-iGSCs included areas of necrosis, pseudopalisading and vascular proliferation similar to clinical GBM specimens (Figure 2G and Supplementary Figure S3). These findings demonstrate that Oct4 and Sox2 activate transcriptional networks that establish a tumor-propagating stem-like phenotype.

Coexpressing Oct4 and Sox2 downregulates a subset of miRNAs via DNA methylation

Specific miRNAs have the capacity to determine cell potency and fate.¹⁴ These and other converging findings led us to ask if specific miRNAs have a role in the GBM cell response to Oct4/Sox2 induction. We used a miRNA PCR array representing 84 mature miRNAs previously associated with nervous system malignancies to examine changes in miRNA expression in response to the induction of the stem-like tumor-propagating phenotype by Oct4 and Sox2. Twenty-three miRNAs were downregulated (≥ 2 -fold) and 10 miRNAs were upregulated (≥ 2 -fold) in GBM neurosphere cells following Oct4 and Sox2 coexpression (Figure 3a). Precursor miRNAs (pre-miRNAs) for a subset of these regulated miRNAs were also quantified by qRT-PCR to validate the array findings. Pre-miR-29a, pre-miR-148a, pre-miR-181, pre-miR-let7b and pre-miR-124a were confirmed to be downregulated (Figure 3b) and pre-miR-23a, pre-miR-10b, pre-miR-138, pre-miR-222 and pre-miR-486-5p were confirmed to be upregulated in GBM cells coexpressing Oct4 and Sox2 (Supplementary Figure S4A).

DNA methylation contributes to cell-fate determination programs in stem cells^{5,23} and epigenetic silencing of tumor suppressor microRNAs by CpG island hypermethylation is a common hallmark of cancer.²⁴ We found that the pan-DNMT inhibitor 5-azacytidine (5-Aza), under conditions that inhibited global DNA methylation in GBM neurosphere cells (Supplementary Figure S4B), abrogated the downregulation of miRNAs by Oct4 and Sox2 coexpression. Specifically, miR-148a, miR-124, miR-200, miR-17*, miR-217, miR-296 and miR30c were both inhibited ≥ 2 -fold by Oct4/Sox2 compared with untreated controls and upregulated ≥ 2 -fold by Oct4/Sox2+5-Aza compared with Oct4/Sox2 alone (Figure 3c). These array findings were further examined by quantifying the effects of 5-Aza and an additional DNMT inhibitor, zebularine, on the expression of the precursor

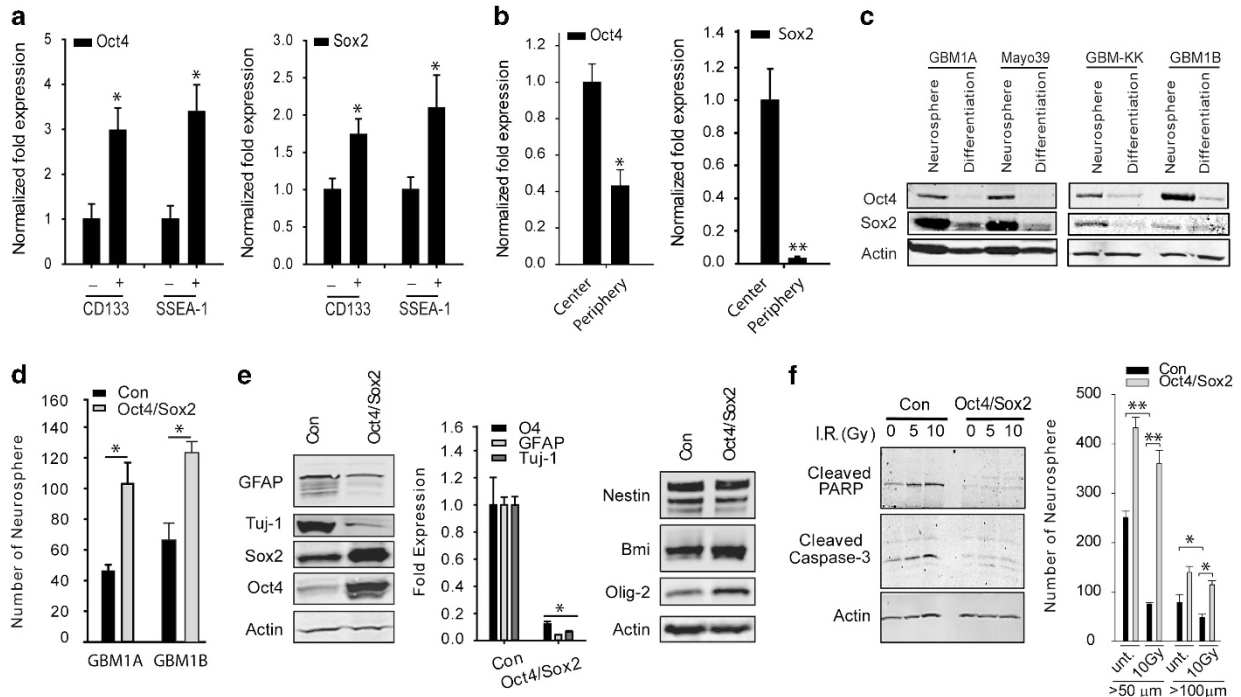


Figure 1. Oct4/Sox2 expression and function in GBM neurosphere cells. **(a)** GBM neurosphere cells expressing GBM SC markers CD133 or SSEA were isolated by flow cytometry, respectively. CD133⁺ cells express higher levels of Oct4 and Sox2 compared with CD133⁻ cells as determined by normalized qRT-PCR. Similar results were observed in the SSEA⁺ cells. **(b)** Oct4 and Sox2 expression levels in cells obtained from the centers and peripheries of nine clinical GBMs were determined by qRT-PCR. Oct4 and Sox2 expression levels are higher in tumor centers relative to peripheries. **(c)** Immunoblot shows decreased expression of Oct4 and Sox2 following forced differentiation of GBM1A, Mayo39, GBM-KK and GBM1B neurospheres. **(d)** Equal numbers of GBM1A and GBM1B neurosphere cells transfected with Oct4/Sox2-expressing or control vectors were cultured in neurosphere medium containing EGF/FGF for 7 days and neurosphere numbers (>100 μ m diameter) were quantified by computer-assisted image analysis. Oct4/Sox2 coexpression significantly enhanced GBM neurosphere self-renewal. **(e)** GBM1A neurospheres were grown in media without EGF/FGF for 2 days. Immunoblot (left panel) and qRT-PCR (middle panel) show decreased expression of GFAP and Tuj-1, markers of astroglial and neuronal lineage differentiation and increased expression of stemness markers Bmi-1, Olig-2 (right panel) in GBM neurospheres following Oct4/Sox2 coexpression. **(f)** GBM1A neurospheres coexpressing Oct4/Sox2 or control vector were treated with γ -radiation for 48 h. PARP and caspase-3 cleavage were evaluated by immunoblot (left panel). Equal numbers of GBM1A neurosphere cells expressing Oct4/Sox2 or control vectors were treated with γ -radiation (10 Gy) and the number of neurospheres was quantified 7 days after treatment using computer-assisted image analysis (right panel). Oct4 and Sox2 coexpression significantly enhanced radiation-resistance compared with the control neurospheres. * $P < 0.05$ and ** $P < 0.01$.

forms of these seven miRNAs by qRT-PCR. Both 5-Aza and zebularine rescued the precursor forms of miR-148a, miR-124, miR-200, miR-296-5p (Figure 3d and Supplementary Figure S4C) and miR-217 (data not shown) from the inhibitory effects of Oct4 and Sox2 coexpression. Bisulfite sequencing revealed that Oct4 and Sox2 coexpression in GBM neurosphere cells increased promoter CpG island methylation (<http://services.abc.uni-stuttgart.de/BDPC/BISMA/>) from 45 to 91% for miR-148a, from 58 to 92% for miR-124-2, from 67 to 100% for miR-296 (Figure 3e) and from 76 to 82% for miR-200 (data not shown).

DNMT expression is induced by Oct4/Sox2 and associated with the GBM stem-like phenotype

DNA methylation is mediated by the combined actions of three DNMTs, Dnmt1, Dnmt3a and Dnmt3b. Immunoblot analysis showed that coexpressing Oct4 and Sox2 increased DNMT expression in multiple cell lines (Figure 4a). Moreover, quantification of whole-cell 5-methylcytosine levels showed that coexpressing Oct4 and Sox2 increased global DNA methylation, demonstrating that DNMT upregulation by Oct4 and Sox2 translates to global downstream methylation events in GBM neurosphere cells (Figure 4b). *In silico* analysis of the *Dnmt1*, *Dnmt3a* and *Dnmt3b* promoters 5' from the translation start sites (<http://algggen.lsi.upc.es/>) identified potential binding sites for both Oct4 and Sox2 in all three promoters (Figures 4c and d and

Supplementary Figure S5, top left panel). Therefore, we hypothesized that Oct4 and Sox2 directly transactivate DNMT gene expression and thereby alter the methylation landscape and inhibit miRNA expression in GBM neurospheres. Quantitative chromatin immunoprecipitation showed that Oct4 and Sox2 complex with the predicted promoter sites (Figures 4c and d and Supplementary Figure S5, right and middle panels). Promoter-reporter assays were used to determine if Oct4 and Sox2 transactivate these DNMT promoters. Regions of the human *Dnmt1* promoter containing an Oct4 binding site (*Dnmt1*-Oct4) and Sox2 binding site (*Dnmt1*-Sox2), and the *Dnmt3b* promoter containing both Oct4 and Sox2 binding sites (*Dnmt3b*-3) were separately cloned into a luciferase reporter cassette. Compared with control, coexpressing Oct4 and Sox2 induced *Dnmt1*-Oct4/luciferase activity ~17-fold, *Dnmt1*-Sox2/luciferase activity ~11-fold and *Dnmt3b*-3/luciferase activity ~20-fold (Figures 4c and d, right panel). We asked if the expression and/or function of DNMTs associate with clinical GBM specimens and primary isolates of stem-like sphere-forming cells. *Dnmt1* and *Dnmt3b* expression levels, as measured by qRT-PCR analysis, were found to be elevated in clinical GBM specimens relative to normal brain (Supplementary Figure S6). More specifically, simple regression analyses showed that *Dnmt1* and *Dnmt3b*, but not *Dnmt3a* expression, significantly correlated with Sox2 expression ($R^2 = 0.719$ and 0.676 , respectively, $P < 0.01$) and with Oct4 expression ($R^2 = 0.473$, $P < 0.01$ and $R^2 = 0.384$, $P = 0.05$,

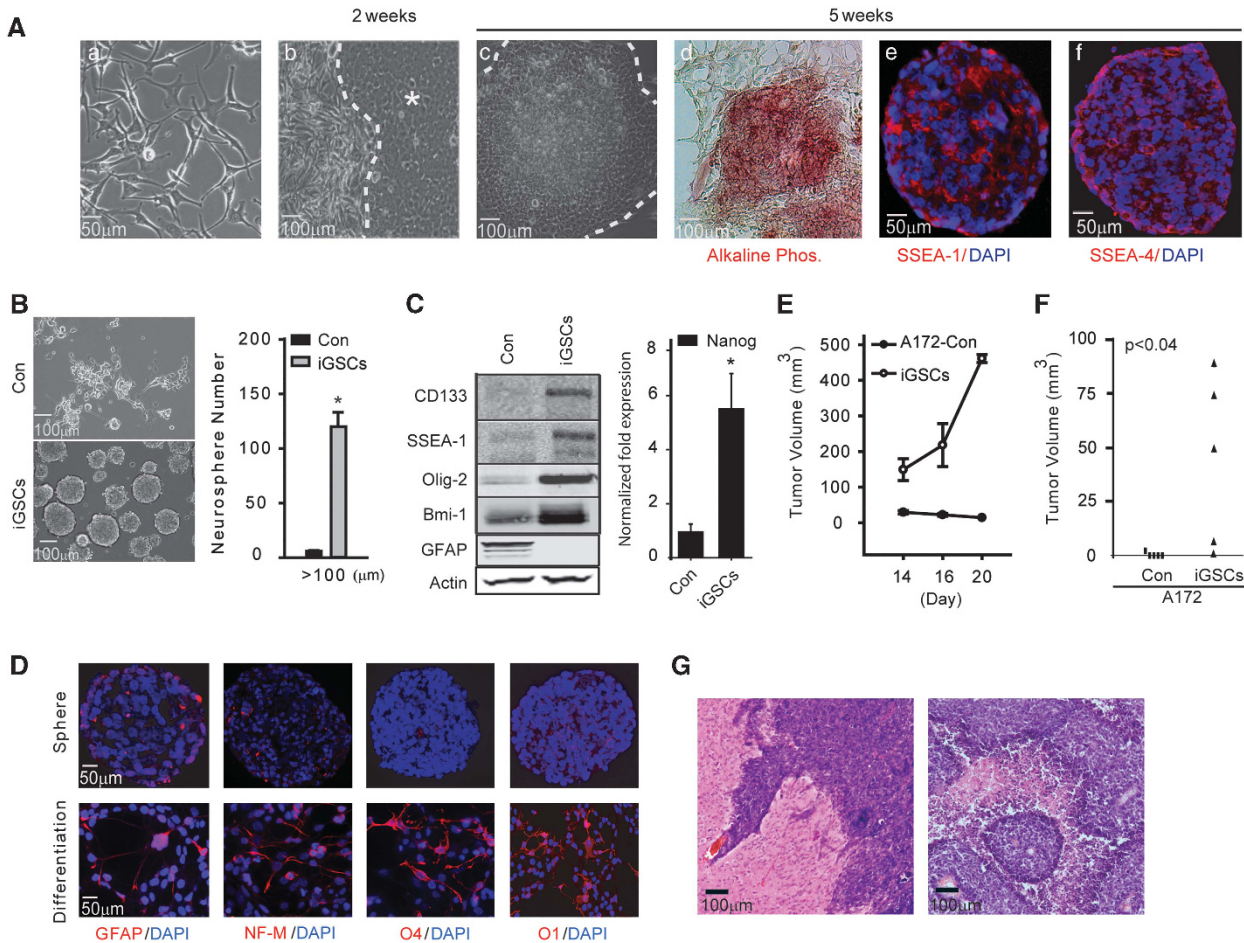


Figure 2. Oct4/Sox2 expression induces stem-like phenotype in GBM cells. **(A)** **(a)** Morphology of A172 cells grown in Dulbecco's modified Eagle's medium (DMEM) medium containing 10% FBS; **(b)** and **(c)** Morphologic transition and colony formation of A172 cells 2 weeks after transgenic Oct4/Sox2 coexpression; **(d)** A172-derived colonies stained for alkaline phosphatase activity, **(e)** red) SSEA-1 and **(f)** red) SSEA-4. Nuclei are stained blue with DAPI (4',6-diamidino-2-phenylindole). **(B)** Oct4/Sox2-transfected A172 cells, but not A172-con cells, form neurospheres (A172-induced glioma SCs, A172-iGSCs) when grown in GBM neurosphere medium (left). Equal numbers of A172-iGSCs and A172-con cells were cultured in neurosphere medium for 1 week and spheres > 100 μ m in diameter were quantified by computer-assisted image analysis (right). **(C)** Immunoblot shows increased expression of stemness markers (CD133, SSEA-1, Olig-2 and Bmi-1) and decreased expression of astroglial lineage marker GFAP in the A172-iGSCs compared with A172-con cells grown in GBM neurosphere medium for 24 h (left panel). Total RNA was isolated from cells and subjected to quantitative RT-PCR for Nanog (right panel), $*P < 0.01$. **(D)** Forced differentiation induced A172-iGSCs to express differentiation markers GFAP (astroglial), neurofilament M⁺ (neuronal) and O4⁺/O1⁺ (oligodendroglial). **(E)** Equal numbers of A172-iGSCs and A172-con cells were injected into mouse flanks and tumor growth quantified by caliper measurements. **(F)** Equal numbers of A172-iGSCs and A172-con cells were injected into the right striatum of mice. Animals were killed by perfusion fixation 12 weeks after implantation. Volumes of measurable tumors were quantified from H&E-stained serial brain sections. **(G)** H&E-stained sections of A172-iGSCs xenografts showed infiltration, necrosis and pseudopalisading, hallmarks of the GBM histopathology. $*P < 0.05$.

respectively) in primary GBM neurosphere isolates (Figure 5a and Supplementary Figure S7). Additionally, multivariate linear regression analyses revealed a strong correlation between Oct4 and Sox2 coexpression levels with Dnmt1 ($R^2 = 0.882$, $P < 0.01$) and Dnmt3b ($R^2 = 0.9391$, $P < 0.01$) (Figure 5b). We also found that DNMT expression associated with SC subsets consistent with the correlative and functional associations between Oct4 and Sox2 and the GBM cell stem-like phenotype. CD133⁺ cells isolated from GBM-derived neurospheres were found to express higher DNMT levels compared with CD133⁻ cells (Figure 5c). Treating GBM-derived neurospheres with the DNMT inhibitors 5-Aza or zebularine significantly decreased sphere-forming capacity concurrent with decreased expression of prominin (CD133) and nestin (Figures 5d and e). Global cell methylation was reduced under conditions of forced differentiation, further strengthening the association between DNA methylation pathways and the SC phenotype (Figure 5f).

DNMT-dependent repression of miR-148a mediates induction of a GBM stem-like tumor-propagating phenotype by Oct4/Sox2

MiR-148a was found to be one of the most significantly decreased miRNAs in GBM neurospheres following Oct4 and Sox2 coexpression (Figure 3a). Coexpressing Oct4 and Sox2 downregulated both pre- and mature miR-148a in GBM1A and A172-iGSC neurosphere cells (Figure 6a). Furthermore, its expression inhibition coincided with promoter CpG island methylation and was abrogated by DNMT inhibitors, indicating a DNMT-dependent mechanism activated by Oct4/Sox2 expression (Figure 3e). We next asked if miR-148a associates with GBM cell stemness. CD133⁺ cell subpopulations isolated from patient-derived GBM neurosphere lines expressed lower levels of miR-148a compared with CD133⁻ cells (Figure 6b). Forced differentiation of GBM neurospheres, shown above to inhibit Oct4 and Sox2 expression and global DNA methylation (see Figures 1c and 5f), increased miR-148a expression (Figure 6c). These associations led us to hypothesize that

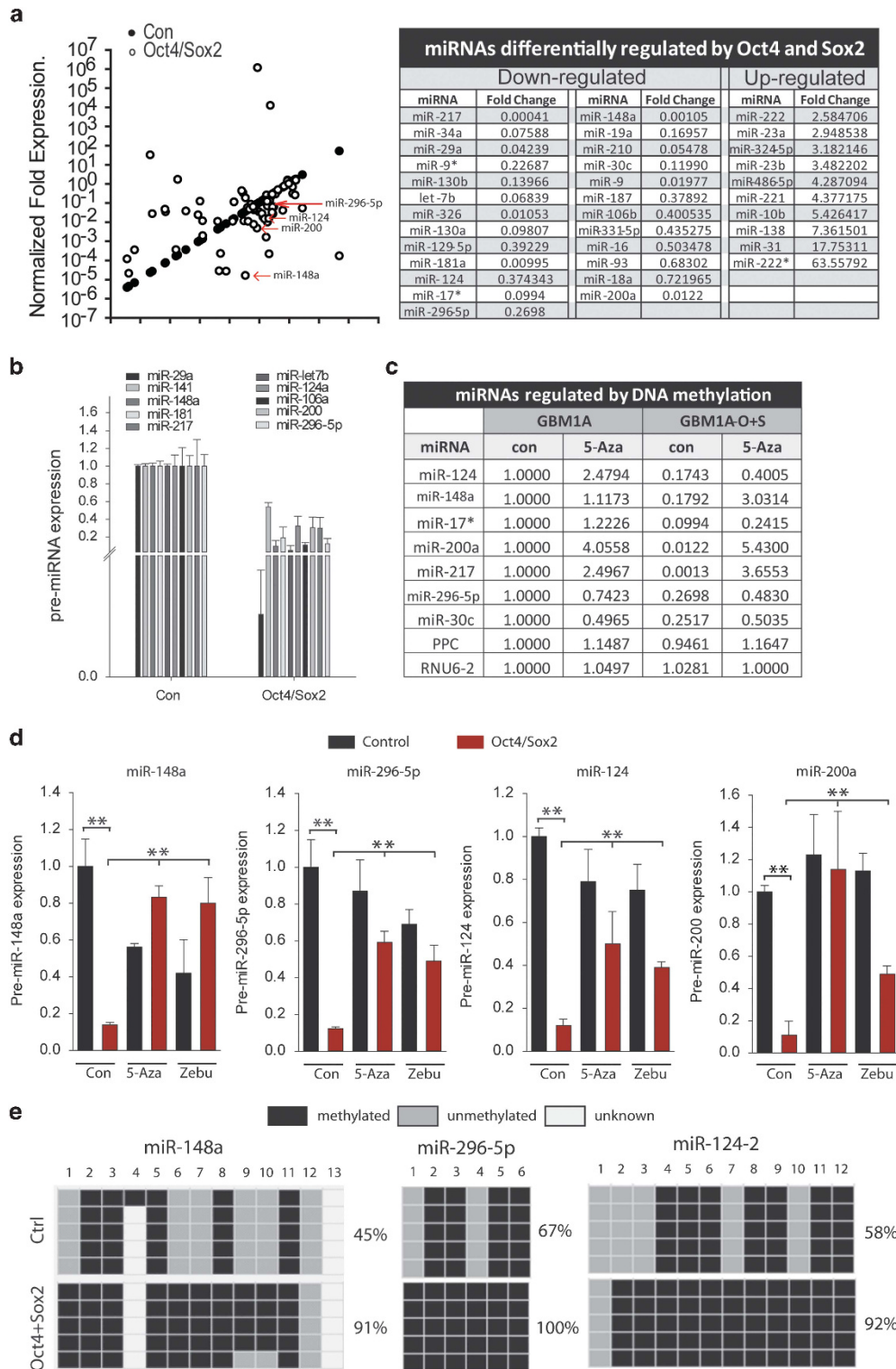


Figure 3. Oct4/Sox2 downregulates microRNA expression in a methylation-dependent manner. **(a)** Differential miRNA expression was determined in GBM1A neurospheres coexpressing Oct4/Sox2 relative to control GBM1A spheres using a Human Brain Cancer miRNA PCR array (left). Table shows miRNAs that changed ± 2 -fold in response to Oct4/Sox2 coexpression. **(b)** Effects of Oct4/Sox2 coexpression on pre-miRNA quantified by qRT-PCR. Shown are miRNAs detected in **(a)** to be downregulated. **(c)** GBM1A neurospheres coexpressing transgenic Oct4/Sox2 or controls were treated \pm DNMT inhibitor 5-Aza ($1 \mu\text{M}$) for 72 h. miRNA expression was determined using a Human Brain Cancer miRNA PCR array. Table shows miRNAs found to be both ≥ 2 -fold downregulated by Oct4/Sox2 (compared with untreated controls) and ≥ 2 -fold upregulated in cells treated with Oct4/Sox2+5-Aza (compared with Oct4/Sox2 alone). **(d)** GBM1A neurospheres expressing transgenic Oct4/Sox2 or control spheres were treated \pm DNA methylation inhibitors 5-Aza ($1 \mu\text{M}$) or zebularine ($10 \mu\text{M}$) for 72 h. The indicated pre-miRNA were quantified by qRT-PCR. **(e)** Methylation status of promoters for miR-148a, miR-296-5p and miR-124-2 was determined by bisulfite sequencing. Five clones were sequenced for each condition, each row represents one clone. ****** $P < 0.01$.

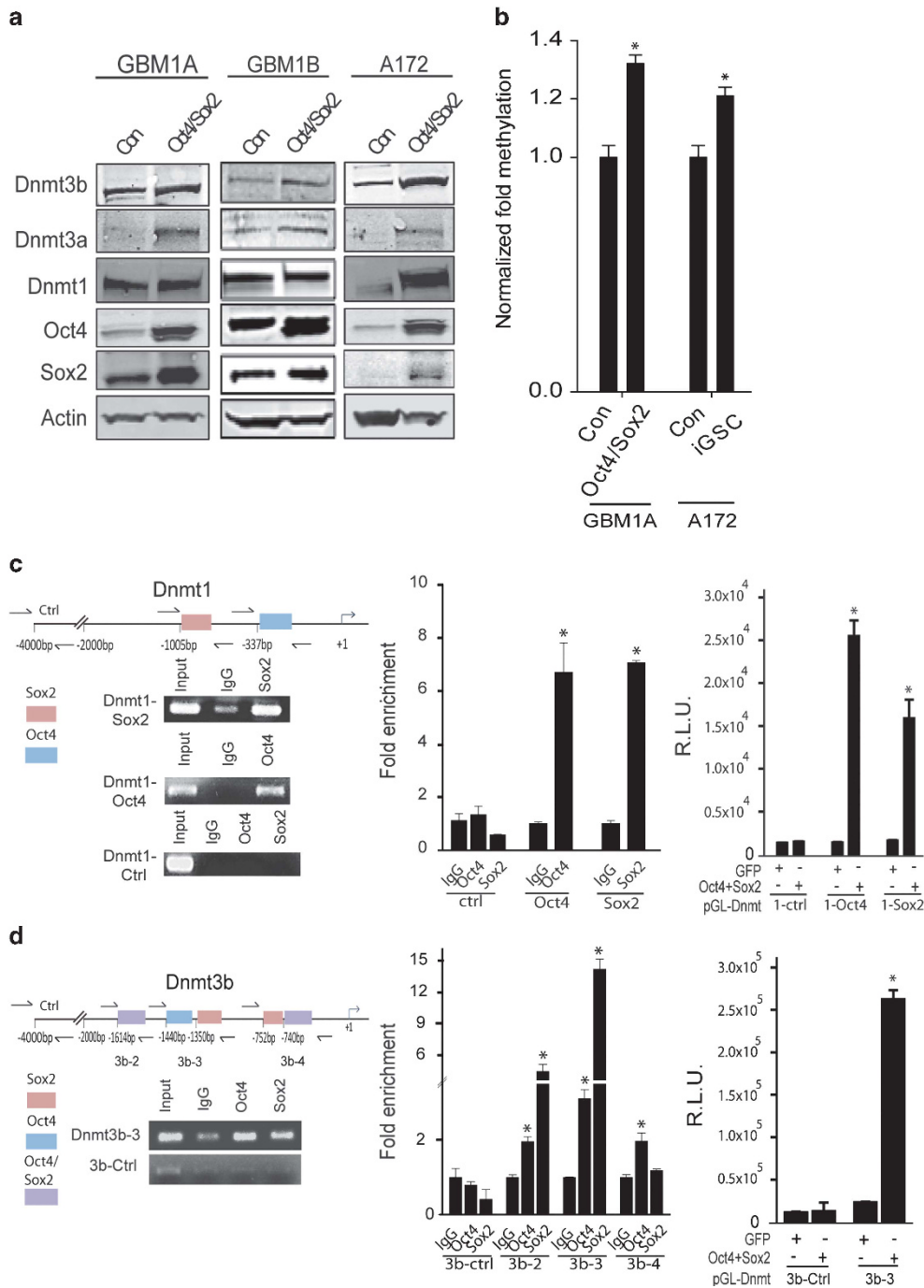


Figure 4. Oct4 and Sox2 bind *Dnmt* promoters and induce DNMT expression. **(a)** Immunoblot shows increased expression of DNMTs in response to transgenic Oct4/Sox2 coexpression in GBM cells compared with their respective parental controls. **(b)** Enzyme-linked immunosorbent assay (ELISA)-based quantification of whole-cell 5-methylcytosine shows increased global DNA methylation in GBM neurospheres coexpressing Oct4/Sox2 compared with control neurospheres. **(c and d** left panels) Oct4 and Sox2 binding sites on human *Dnmt1* and *Dnmt3b* promoters were predicted by PROMO search tools. Arrows indicate primer sites used for PCR analyses. Agarose gel electrophoresis shows relative efficiencies of Oct4 and Sox2 binding to *Dnmt1* and *Dnmt3b* promoters. **(c and d** middle panels) DNA purified from chromatin immunoprecipitation was analyzed by qRT-PCR using primer pairs designed to amplify fragments containing Dnmt1-Oct4, Dnmt1-Sox2, Dnmt1-control, Dnmt3b-2, Dnmt3b-3, Dnmt3b-4, and Dnmt3b-control. **(c and d** right panels) Co-expressing Oct4/Sox2 induces luciferase reporter activity driven by regions of the *Dnmt1* and *Dnmt3b* promoters containing the indicated Oct4 and/or Sox2 binding sites. * $P < 0.05$.

miR-148a functions to inhibit the stem-like phenotype in GBM, a function that would be consistent with its repression by Oct4/Sox2 expression. Therefore, we asked if forced miR-148a expression abrogates the capacity of Oct4 and Sox2 to induce a tumor-propagating stem-like state. Retroviral-based miR-148a expression

decreased the neurosphere growth capacity of patient-derived GBM neurosphere cells and A172-iGSCs and induced morphologic differentiation characterized by cell process formation and transition to an adherent growth pattern (Figures 6d and e and Supplementary Figure S8). These effects were accompanied by

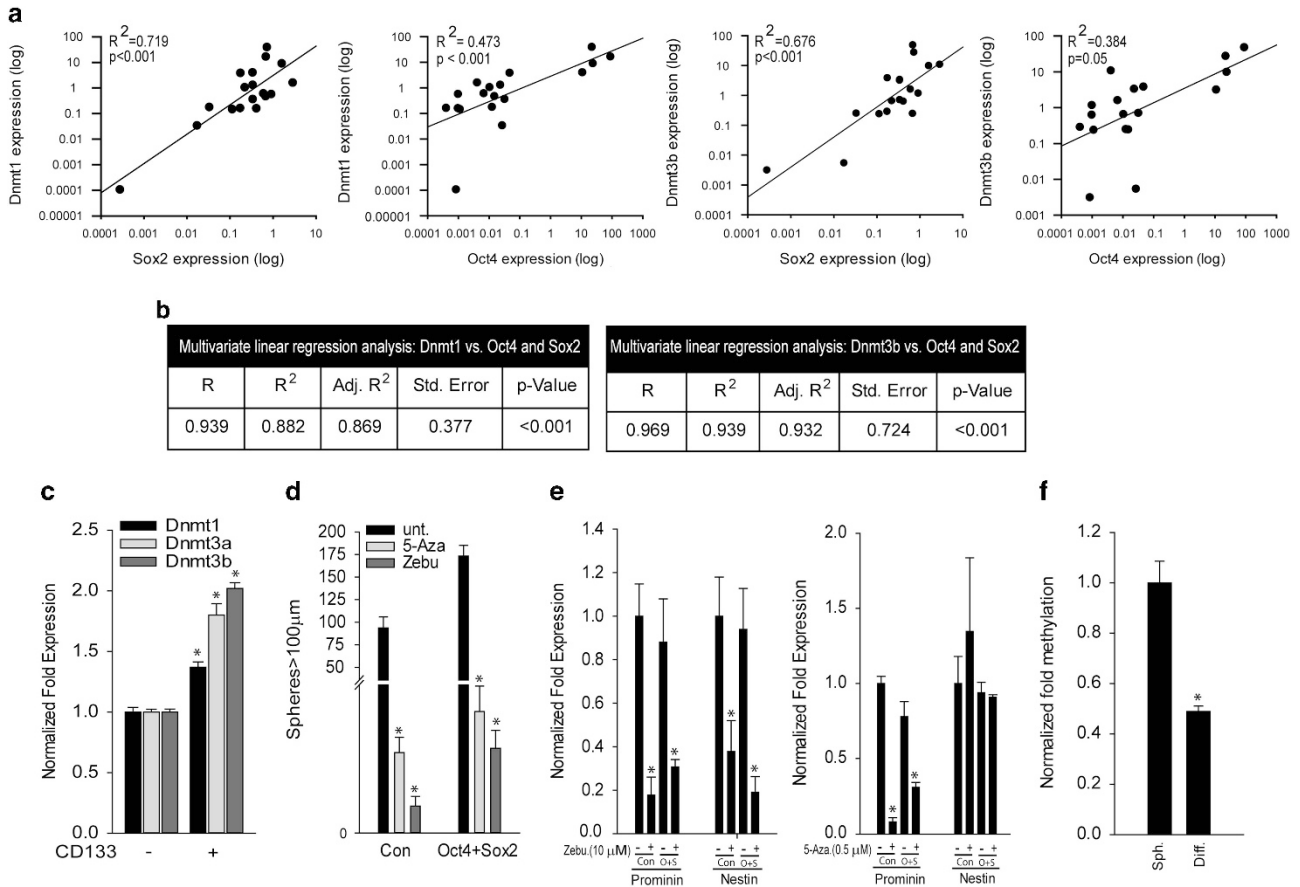


Figure 5. DNMT expression is associated with GBM stem-like phenotype. **(a)** Simple linear regression showing significant correlation between Dnmt1 with Sox2 and Oct4 expression (left two panels, $R^2=0.719$ and $R^2=0.473$, respectively, $P < 0.001$), Dnmt3b with Sox2 and Oct4 expression (right two panels, $R^2=0.676$, $P < 0.001$ and $R^2=0.384$, $P=0.05$, respectively) in 18 primary GBM neurosphere isolates established independently from clinical GBM tissues, as determined by qRT-PCR analysis. **(b)** Table showing a strong correlation between Oct4 and Sox2 coexpression levels with Dnmt1 (left panel, $R^2=0.882$, $P < 0.001$) and Dnmt3b (right panel, $R^2=0.9391$, $P < 0.001$) using multivariate linear regression analysis in primary GBM neurosphere isolates, as determined by qRT-PCR analysis. **(c)** CD133⁺ and CD133⁻ cells were isolated from GBM neurospheres by flow cytometry and the expression of DNMTs was quantified by qRT-PCR. **(d)** GBM1A neurospheres coexpressing transgenic Oct4/Sox2 or controls were treated with DNMT inhibitors 5-Aza (1 μM) or zebularine (10 μM) and sphere formation capacity was measured 7 days after treatment. **(e)** DNMT inhibition results in reduced expression of prominin and nestin as quantified by qRT-PCR. **(f)** Enzyme-linked immunosorbent assay (ELISA)-based quantification of methylated DNA in GBM1A neurosphere cells 5 days after serum-induced differentiation.

increased expression of the differentiation markers GFAP and O4 (Figure 6f) and decreased expression of prominin (CD133) and nestin (Figure 6g). To evaluate the effects of miR-148a on tumor propagation, patient-derived GBM neurosphere cells and A172-iGSCs were retrovirally transduced to express transgenic miR-148a (or miR-control) and implanted orthotopically to mice. Seven of eight mice (88%) implanted with GBM1A-miR-control and four of four mice (100%) implanted with A172-iGSCs-miR-control cells generated tumors, whereas only one of seven mice (14%) implanted with GBM1A-miR-148a-transduced cells and zero of five mice (0%) implanted with A172-iGSCs-miR-148a-transduced cells developed detectable tumors (Figure 6h). Taken together, these results show that miR-148a expression inhibits GBM stem-like phenotype and suppresses the tumor-propagating capacity of GBM neurospheres.

DISCUSSION

GBM SCs display self-renewing, multipotent, tumor-propagating properties and contribute disproportionately to therapeutic resistance and tumor recurrence. Although the origins of GBM SCs remain unclear, emerging evidence highlights the plasticity of

GBM SCs and the roles for autocrine/paracrine signaling in the generation of SC subsets from more differentiated transit-amplifying tumor cells. Specific transcriptional networks involving the 'Yamanaka transcription factors' have essential roles in somatic cell reprogramming^{25,26} and maintaining the stemness and proliferation potential of pluripotent cells.¹⁷ These transcription factors are commonly overexpressed in neoplastic SCs, including GBM SCs, suggesting that they function to generate and/or maintain the cancer stem-like phenotype. How reprogramming transcription factors function cooperatively and which transcription factor networks are essential for maintaining the neoplastic stem-like state remain poorly understood. Earlier findings from our laboratory established that 'Yamanaka transcription factors' and Nanog are downstream constituents of the c-Met signaling pathway that induces glioma stem-like phenotypes *in vitro* and *in vivo*.^{8,9} We now extend those earlier findings by demonstrating that coexpressing Oct4 and Sox2 induces DNMT expression and methylation events that repress miRNAs, including miR-148a, which we show functions to inhibit GBM tumor propagation (Figure 7). These findings identify a novel molecular axis consisting of Oct4 and Sox2, DNMT-dependent DNA methylation and repression of multiple microRNAs that drives cancer cell

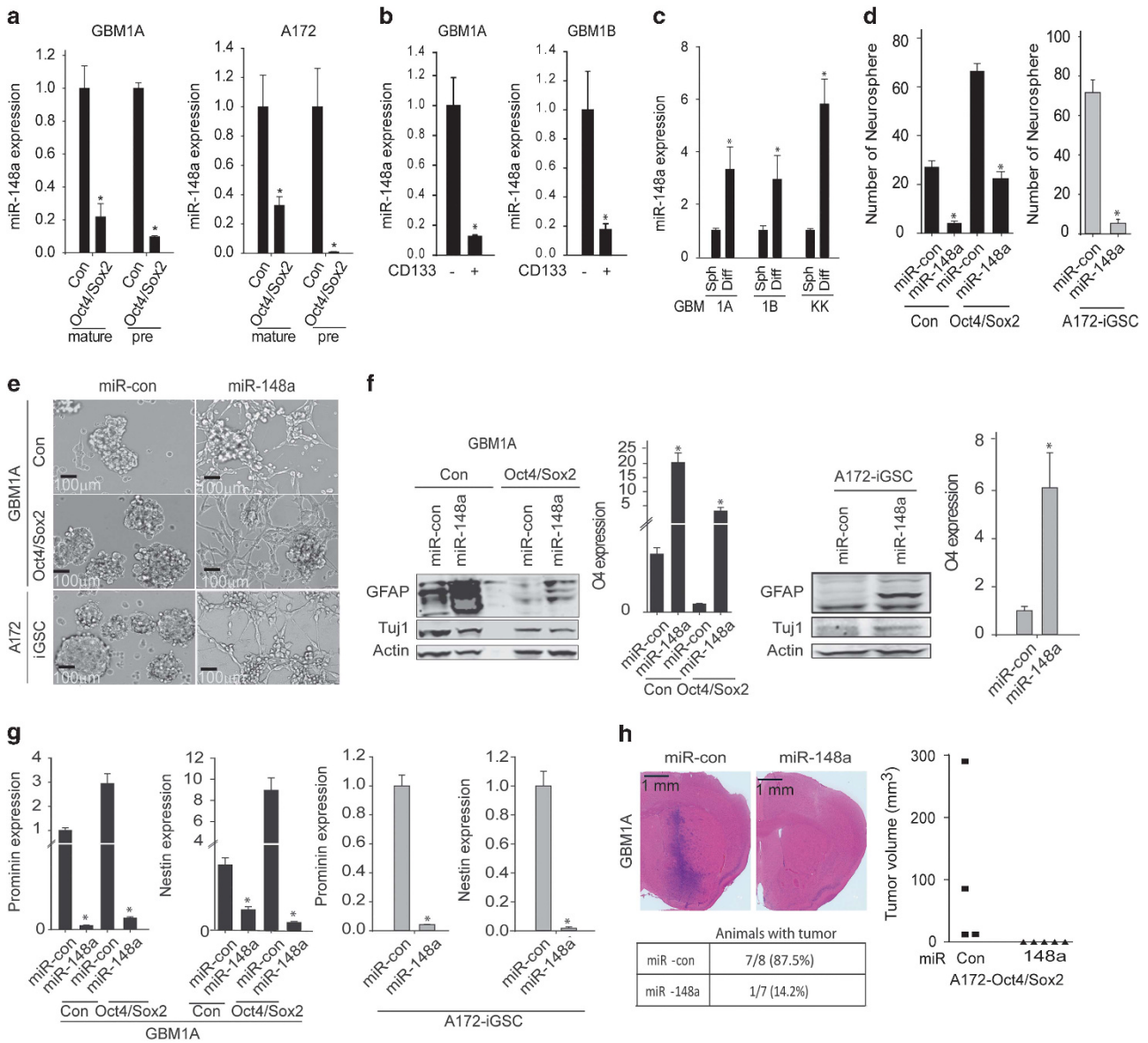


Figure 6. miR-148a expression inhibits Oct4/Sox2-induced GBM stem-like tumor-propagating phenotype. **(a)** Effects of transgenic Oct4/Sox2 on pre- and mature miR-148a expression quantified by qRT-PCR in patient-derived GBM1A neurospheres and A172-iGSC neurospheres. **(b)** CD133⁺ and CD133⁻ cells were separated from GBM neurospheres by flow cytometry. CD133⁺ cells express lower levels of miR-148a compared with CD133⁻ cells as determined by normalized qRT-PCR. **(c)** Pre-miR-148a expression is induced in GBM neurospheres by forced differentiation as determined by qRT-PCR. **(d)** GBM1A neurospheres coexpressing Oct4/Sox2 or control neurospheres, and A172-iGSCs were transduced with retrovirus expressing miR-148a or miR-control (miR-con). Neurosphere-forming capacity (> 100 μ m diameter) was quantified by computer-assisted image analysis. **(e)** Transgenic miR-148a expression induces neurospheres to transition to an adherent growth pattern. **(f and h)** GBM1A and A172-iGSCs spheres under normal neurosphere growth conditions containing EGF/FGF were transfected with miR-148a and miR-con; the effects of transgenic miR-148a on lineage-specific marker expression were determined by immunoblot (GFAP, Tuj1) and qRT-PCR (O4). GBM stemness markers prominin and nestin were quantified by qRT-PCR. **(h)** Representative H&E-stained brain sections from mice implanted with GBM1A neurosphere cells transduced with retrovirus expressing either miR-con or miR-148a (left panel). Table shows number of GBM1A-implanted animals with histopathologically detectable tumor formation. Right panel shows volumes of measurable brain tumors from mice implanted with A172-iGSCs transduced with retrovirus expressing either miR-con or miR-148a as quantified from H&E-stained serial brain sections. **P* < 0.05.

stem-like phenotype and tumor propagation. It is likely that Oct4 and Sox2 contribute, at least in part, to the oncogenic effects of multiple cancer-associated autocrine/paracrine pathways and their capacity to induce cancer stem-like tumor-propagating phenotypes.³

It is becoming increasingly evident that miRNAs have crucial roles in somatic cell reprogramming, self-renewal and differentiation.¹⁴ miRNAs have been extensively investigated in many cancers, including brain tumors; however, the molecular

mechanisms responsible for miRNA dysregulation and the contributions of specific miRNAs to cancer stem cell regulation remain only partially defined. Oct4, Sox2 and Nanog regulate ES cell miRNAs such as miR-290 cluster; miR-302/367; miR-106a cluster; and miR-200 family through promoter occupancy and gene transactivation.^{13,27,28} We now demonstrate using complementary pharmacologic, genome screening and bisulfite sequencing approaches that Oct4 and Sox2 silence a subset of miRNAs in GBM SCs via a mechanism involving DNMT upregulation and

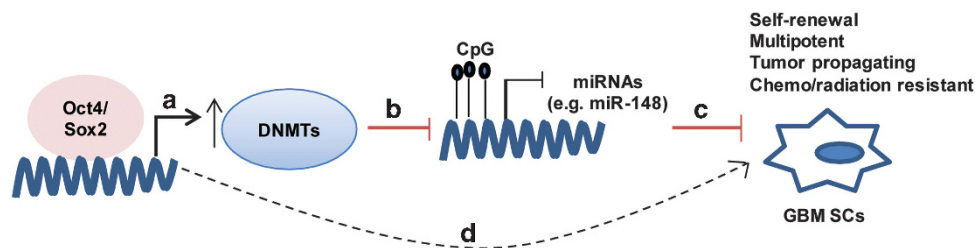


Figure 7. Proposed model shows GBM stem-like phenotype regulation by Oct4/Sox2. (a) Oct4 and Sox2 transcriptionally induce *Dnmt* gene expression by promoter binding. (b) DNMT expression induces promoter methylation events that inactivate expression of multiple miRNAs including miR-148a. (c) miR-148a expression inhibits GBM stem-like phenotype. (d) Oct4 and Sox2 also regulate GBM stem-like phenotype through other mechanisms that drive neoplastic cell stemness, self-renewal and tumor-propagating potential.

promoter hypermethylation. Some of the miRNAs found to be downregulated in our study (that is, miR-124, miR-200, miR-217 and miR-148a) have been found by others to be frequently downregulated in human cancers.^{29–32} DNMT-dependent DNA hypermethylation induced by Oct4 and Sox2 represents a previously unknown mechanism through which potential tumor-suppressive miRNAs can be dysregulated in cancer and cancer SCs.

DNA methylation changes occur with oncogenesis and hypermethylation of tumor suppressor genes, and demethylation of oncogenes drive tumor initiation and progression.³³ Subgroups of glioma have been shown to possess a hypermethylated phenotype (glioma-CpG island methylator phenotype, G-CIMP) that predicts tumor pathogenicity. This hypermethylator phenotype associates with mutant isocitrate dehydrogenase 1 and 2 that generate 2-hydroxyglutarate and inhibit α -ketoglutarate-dependent dioxygenases and tumor demethylation.³⁴ Studies in glioma have also found that elevated DNMT levels associate with tumor suppressor gene hypermethylation and SC subsets, linking DNMT activity with tumor-propagating cell populations.³⁵ We now show that Oct4 and Sox2 strongly correlate with *Dnmt1* and *Dnmt3b* expression in primary GBM neurosphere isolates and that Oct4 and Sox2 directly transactivate *Dnmt* genes and induce glioma cell hypermethylation. Moreover, we show that DNMTs are enriched in GBM SC subsets and that pharmacologic inhibition of DNMTs results in loss of capacity to self-renew as neurospheres. These results suggest that DNMT induction by Oct4 and Sox2 might cooperate with other hypermethylating mechanisms driven by isocitrate dehydrogenases 1 and 2 mutation and Tet protein dysregulation to induce tumor propagating potential.^{34,37} We caution readers against overinterpreting our results within the context of how global methylation status and glioma subtypes affect patient prognosis as our study focuses specifically on stem-like tumor-propagating cell subsets and their regulation by a methylation-dependent transcriptional circuit.

miR-148a was found to be one of the more significantly downregulated miRNAs in response to Oct4 and Sox2 co-expression. miR-148a has been previously reported to have either tumor-promoting or tumor-suppressing actions.^{32,38,39} Consistent with a tumor-suppressive function, miR-148a has been shown to inhibit gastric cancer cell invasion and metastasis by targeting ROCK1, to suppress hepatoma cell epithelial–mesenchymal transition and metastasis by targeting c-Met and Snail signaling⁴⁰ and to promote apoptosis by targeting Bcl2 in colorectal cancer. Interestingly, miR-148a has been recently shown to have a critical role in hepatocyte differentiation and act as a tumor suppressor in hepatocellular carcinoma cell lines.⁴¹ Conversely, miR-148a has also been shown to promote cell proliferation by targeting p27⁴² and regulating EGF receptor function.³⁹ We conclude that miR-148a expression inhibits Oct4/Sox2-driven stem-like tumor-propagating phenotypes in GBM based on multiple complementary cell responses, including the inhibition of sphere-forming capacity *in vitro*, inhibition of stem

cell marker expression along with induction of differentiation markers *in vitro* and, most importantly, the inhibition of tumor-propagating capacity *in vivo*.

The transcripts targeted by miR-148a that mediate its effects on the GBM stem-like phenotype have yet to be defined. TargetScan analysis (<http://www.targetscan.org>) identifies multiple miR-148a targets of potential interest within the context of cancer stem cell regulation (see Supplementary Figure S8C). These include transcripts coding for the Ras exchange factor SOS2,⁴⁰ the chromatin remodeling enzyme INO80,⁴³ the homeobox transcription factor Meox2⁴⁴ and the tumor promoter MafB.⁴⁵ Dnmt1 was also among the predicted high confidence ($\geq 50\%$ probability) targets and it has been reported that miR-148a inhibits Dnmt1 expression in malignant cholangiocytes by targeting 3'-untranslated sequences⁴⁶ and inhibits Dnmt3b isoforms (3b1, 3b2 and 3b4 but not 3b3) in HELA cells by targeting coding sequences.⁴⁷ Preliminary data suggests that forced expression of miR-148a can downregulate Dnmt1 and Dnmt3b in glioma cells (Supplementary Figure S8D), raising the possibility that Oct4 and Sox2 induce DNMTs and downstream methylation events through the combined effects of direct DNMT promoter transactivation and methylation-dependent repression of miR-148a.

In conclusion, this study describes a novel molecular circuit by which the core reprogramming transcription factors Oct4 and Sox2 regulate stem-like phenotypes and tumor-propagating capacity in GBM through DNMT-dependent regulation of microRNA networks. We specifically identify miR-148a as an inhibitor of GBM cell tumor-propagating capacity and identify other Oct4/Sox2-regulated microRNAs with potential regulatory functions in GBM. These findings identify methylation- and microRNA-based strategies for inhibiting the GBM SCs and their contributions to tumor growth and recurrence.

MATERIALS AND METHODS

Cell lines, transfection and transduction

GBM neurosphere, neurosphere growth assay and differentiation assay. GBM-derived neurosphere lines (GBM1A and GBM1B) were originally derived and characterized by Vescovi and co-workers.² The GBM-KK neurosphere line was derived from a single GBM patient and kindly provided by Dr Jaroslaw Maciacyk (University of Freiburg, Freiburg im Breisgau, Germany). Low-passage primary neurospheres were derived directly from human GBM clinical specimens and from patient-derived xenografts obtained from pathologic GBM specimens obtained during clinically indicated surgeries at Johns Hopkins Hospital (Baltimore, MD, USA) using established methods.² Neurospheres were cultured in serum-free medium containing Dulbecco's modified Eagle's medium/F-12 (Invitrogen, Carlsbad, CA, USA), supplemented with 1% bovine serum albumin, 20 ng/ml EGF and 10 ng/ml FGF.

For neurosphere growth, cells were dissociated into single cells and cultured in ultra-low attachment flasks (2.5×10^4 cells/ml). After 7 days, GBM neurospheres were embedded in 1% agarose and stained with 0.1% Wright stain solution for 1–2 h at 37 °C. Cells were washed four times with phosphate-buffered saline and incubated at 4 °C overnight (in phosphate-

buffered saline) before quantification. Spheres larger than 100 μm were quantified using computer-assisted image analysis.²² Forced differentiation was performed according to the method of Galli and co-workers⁴⁸ with some modifications. Briefly, the neurosphere cells were plated onto Matrigel in FGF-containing neurosphere medium (no EGF) for 2 days and subsequently grown in 1% FBS without EGF/FGF for 5 days. Human glioma cell lines A172 were originally obtained from ATCC (Manassas, VA, USA).

To generate lentivirus, 293T cells were co-transfected with plasmids pLM-mCitrine-Sox2 or pLM-vexGFP-Oct4 (Addgene, Cambridge, MA, USA) and packaging mix plasmids (Open Biosystems, Waltham, MA, USA) using Fugene HD (Promega, Madison, WI, USA). To generate retrovirus, the packaging cell line 293GP (generously provided by Dr Hongjun Song, Johns Hopkins University) was co-transfected with p-miR-148a or p-miR-con (kindly provided by Dr Reuven Agami, The Netherlands Cancer Institute, Amsterdam, The Netherlands)⁴⁷ and p-*VSvG* (kindly provided by Dr Xu Luo at the University of Nebraska Medical Center, Omaha, NE, USA). High-titer virus was collected at 48–72 h and used to infect cells.

To induce somatic cell reprogramming, A172 glioma cells were transduced with vexGFP-Oct4 and mCitrine-Sox2. The cells expressing both Oct4 and Sox2 were then selected by flow cytometry and grown in the mTeSR1 medium (STEMCELL Technologies Inc., Vancouver, BC, Canada). Single ES-like colonies were picked 5 weeks after transduction and maintained in neurosphere media supplemented with EGF and FGF.

Immunoblotting

Western blot was performed using quantitative Western blot system (LI-COR Bioscience, Lincoln, NE, USA) following the manufacturer's instructions. Membranes were blotted with Oct4, Sox2, cleaved caspase-3, cleaved PARP, Bmi1, Dnmt3a, Dnmt1 (all from Cell Signaling, Danvers, MA, USA), GFAP, Tuj1 (Millipore, Billerica, MA, USA), β -actin (Sigma, St Louis, MO, USA). Secondary antibodies were labeled with IRDye infrared dyes (LI-COR Biosciences, Lincoln, NE, USA) and protein levels were quantified using the Odyssey Infrared Imager (LI-COR Biosciences).

Immunofluorescence

A172-derived iGSC neurospheres were embedded and sectioned on a cryostat at 5 μm as described previously.⁹ Sections were immunostained with anti-SSEA-1 and anti-SSEA-4 antibodies (Millipore). For neurosphere differentiation analysis, A172-derived iGSCs neurospheres were forced to differentiate according to our published protocol.⁸ Differentiated cells were fixed with 4% paraformaldehyde and immunostained with anti-GFAP, anti-neurofilament M⁺, O4 and O1 antibodies (Millipore) according to the manufacturer's protocols. Secondary antibodies were conjugated with Cy3. Coverslips were fixed using Vectashield antifade solution containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). Immunofluorescent images were taken and analyzed using Axiovision software (Zeiss, Oberkochen, Germany).

Bisulfite sequencing and global DNA methylation analysis

Genomic DNA was isolated using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) and DNA was subjected to bisulfite treatment using EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA). The bisulfite-converted DNA was amplified using primers described in Supplementary Table 1. The PCR products were cloned into pCR II TA vectors using the TOPO-TA Cloning Kit (Invitrogen, Grand Island, NY, USA) and sequenced using Sanger sequencing. The sequencing data was analyzed using the BISMA software (<http://services.ibc.uni-stuttgart.de/BDPC/BISMA/>).⁴⁹ For global DNA methylation, genomic DNA was immobilized on wells coated with DNA affinity substrate. The methylated fraction of DNA was recognized by an antibody directed against 5-methylcytosine and quantified through an enzyme-linked immunosorbent assay-like colorimetric reaction (Epigentek, Farmingdale, NY, USA).

Chromatin immunoprecipitation

Chromatin immunoprecipitation assays were performed on GBM1A neurosphere cells coexpressing Oct4 and Sox2 using the MAGnify Chromatin Immunoprecipitation system (Life Technologies, Grand Island, NY, USA). Immunoprecipitation was performed with anti-Oct4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Sox2 (R&D Systems, Minneapolis, MN, USA) or anti-IgG (Life Technologies). Specific regions were quantified by qRT-PCR using primers described in the Supplementary Table 2, and PCR products were visualized on agarose gels.

Luciferase reporter assay

The putative promoter regions containing Oct4 and/or Sox2 binding sites were amplified from BAC clones spanning the Dnmt1 (RP11-298C17; Life Technologies) and Dnmt3b (RP11-713N22; Life Technologies) promoters using primers described in Supplementary Table S2. PCR products were cloned into the *XhoI* and *BglII* sites of the pGL4.2 vector (Promega) and verified using Sanger sequencing. The 293T cells were transfected with the indicated reporter constructs 24 h before transfection with Oct4 mRNA and/or Sox2 mRNA. GFP mRNA was used as negative control. The 293T transfected cells were collected 24 h after mRNA transfection and luciferase activity was measured using a Luciferase Assay Kit (Promega).

QRT-PCR, miRNA expression and miRNA microarray analysis

Total RNA was extracted using RNeasy Mini Kit (Qiagen) and qRT-PCR was performed as described previously.⁸ Relative expression of each gene was normalized to 18S RNA. Primer sequences are listed in the Supplementary Table 3. For miRNA analysis, total RNA including small RNA was extracted using miRNeasy Kit (Qiagen). Pre-miRNA expression was analyzed as described previously.⁵⁰ Mature miR-148a expression was detected using miScript SYBR Green PCR Kit (Qiagen) and probes for RNU6 (Qiagen) and miR-148a (Qiagen). The cDNA was synthesized using miScript II RT Kit (Qiagen) and was used to perform microarray analysis using a brain cancer miRNA PCR Array (SABiosciences, Frederick, MD, USA) according to the manufacturer's instructions. Array data were analyzed using miScript miRNA PCR array data analysis tools (Qiagen and SABiosciences).

Tumor formation *in vivo*

Tumor-propagating capacity of A172-iGSCs and GBM1A neurospheres was tested in subcutaneous and intracranial animal models according to our published protocol.⁵¹

Flow cytometry

The percentages of cells expressing CD133 and SSEA-1 were determined following the manufacturer's specifications. Single-cell suspensions were labeled with phycoerythrin-conjugated anti-CD133 antibody (clone 293C3; Miltenyi Biotec, Auburn, CA, USA) or with anti-SSEA-1 FITC (BD Biosciences San Jose, CA, USA). The stained cells were then sorted using the FACS Vantage SE flow cytometer (BD Biosciences).

Statistical analysis

Two group comparisons were analyzed by *t*-test and *P*-values were calculated. *P* < 0.05 was considered significant and symbolized by an asterisk in the graphs. All data shown are mean \pm s.e.m., unless otherwise specified. When normalizing mRNA (or miRNA) expression, we set the mean expression of controls as '1' and the error reflects the deviation from the mean of at least triplicate readings. Correlation calculations between *Dnmt* expression with Oct4 and Sox2 were performed by simple linear or multiple linear regression analysis using SigmaPlot 12.0 software.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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