A Tumorigenic MLL-Homeobox Network in Human Glioblastoma Stem Cells

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Abstract

Glioblastoma growth is driven by cancer cells that have stem cell properties, but molecular determinants of their tumorigenic behavior are poorly defined. In cancer, altered activity of the epigenetic modifiers Polycomb and Trithorax complexes may contribute to the neoplastic phenotype. Here, we provide the first mechanistic insights into the role of the Trithorax protein mixed lineage leukemia (MLL) in maintaining cancer stem cell characteristics in human glioblastoma. We found that MLL directly activates the Homeobox gene HOXA10. In turn, HOXA10 activates a downstream Homeobox network and other genes previously characterized for their role in tumorigenesis. The MLL–Homeobox axis we identified significantly contributes to the tumorigenic potential of glioblastoma stem cells. Our studies suggest a role for MLL in contributing to the epigenetic heterogeneity between tumor-initiating and non-tumor-initiating cells in glioblastoma. Cancer Res; 73(1); 1–11. ©2012 AACR

Introduction

Epigenetic regulators play a pivotal role in the etiology of multiple malignancies. Altered epigenomic landscapes have been identified in cancer cells compared with normal tissue (1–4), and add a layer of intricacy to the study of diseases that also tend to be mutationally unstable (5). The contribution of epigenetic factors to cellular heterogeneity in tumors has been poorly addressed and few mechanistic insights have been resolved, especially in human gliomas (6–10). In particular, the role of epigenetic factors in subpopulations of cells that drive tumorigenesis has only been partially addressed, and studies of glioma have mostly focused on proteins belonging to the Polycomb group (11, 12). Elucidation of aberrant epigenetic mechanisms in the tumor-initiating subpopulations holds promise for the identification of novel and targetable pathways.

The Trithorax group (TrxG) homolog mixed lineage leukemia (MLL) is an important epigenetic regulator during development and its role is especially well defined in hematopoiesis (13–15). MLL is a histone methyltransferase that catalyzes the methylation of histone 3 at lysine 4 (H3K4; ref. 16), thereby inducing a complex series of epigenetic modifications that result in positive regulation of downstream genes that are involved in the complex interplay of self-renewal and differentiation (16–19). MLL is also a driver of leukemogenesis, in a process that involves aberrant regulation of Homeobox (HOX) transcription factors, especially HOXA9 and HOXA10 (20–27). Therefore, MLL contributes to both normal and malignant stem cell function.

Alvarez-Buylla and colleagues recently showed that MLL is expressed in neural stem cells in the mouse subventricular zone and that MLL is required for Dlx2 expression and neurogenesis (28, 29). In GBM cell lines, MLL was found to be expressed in hypoxic conditions (7). However, although mutations in MLL2 and MLL3 have been identified in human medulloblastoma (30), a specific functional role for these Trithorax proteins and MLL in malignancies of the brain has not been established.

Glioblastoma (GBM) is the most common primary brain tumor, characterized by dismal prognosis (median survival, 15 months) even when aggressive therapies based on tumor resection and concomitant chemotherapy and radiotherapy are implemented (31). Previous studies identified a HOX signature in GBM bulk samples (32–34). Specifically, it was shown that high expression of HOXA9 (32) or HOXA10 (33) were predictive of poor patient survival. HOXA10 expression was also previously detected in neospaces cultures derived from GBM samples (33, 35). However, the mechanism controlling HOXA10 expression and the molecular role of HOXA10 in either bulk or GBM stem cells have not been addressed. Given the negative association between HOXA10 expression and patient survival, a more complete understanding of the pathways upstream and downstream of this transcription factor are required. Furthermore, nothing is currently known on the specific role of HOX genes in the GBM stem cell fraction.

In this report, we present data that highlight an important contribution of MLL in directly activating HOXA10 in GBM
stem cells. HOXA10 then activates other Homebox transcription factors, which also contribute to a Homebox transcriptional program in GBM. Our data represent the first mechanistic description of an MLL-dependent epigenetic program in populations of GBM cells with stem-like characteristics.

Materials and Methods

Cell culture and primary tumor cells

Glioblastoma neural stem (GNS) and human fetal neural stem (NS) cells were grown in culture as previously described (36, 37). Briefly, flasks or 10-cm cell culture dishes were coated with poly-D-ornithine and laminin (both from Sigma). Cells were grown as adherent monolayers in serum-free Neurocult NS-A Basal (Stemcell Technologies) media, supplemented with 2 mmol/L L-glutamine, N2 and B27 supplements, 75 μg/ml bovine serum albumin, 10 ng/ml recombinant human EGF (rhEGF), 10 ng/ml basic fibroblast growth factor (bFGF), and 2 μg/ml heparin. Cells were harvested by mild accutase (Sigma) treatment. All NS cell cultures expressed NS cell markers and responded to differentiation cues by expressing lineage markers (data not shown).

Primary cells from GBMs were acutely dissociated in artificial cerebrospinal fluid (38) by treatment with an enzyme cocktail at 37°C, as previously described (39, 40). Cells were sorted for the cell surface marker CD15 (41) in either Aria-SC or Aria-CFI cell sorters.

Mouse husbandry

All mouse procedures were approved by The Hospital for Sick Children’s Animal Care Committee. For orthotopic transplantations, mice were anesthetized with ketamine/xylazine. Thereafter, 200,000 GliNS1 cells were suspended in PBS and injected in the forebrain of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice with a G27 needle. The coordinates for the injection were 1 mm anterior of the bregma, 2 mm to the right of the midline, and 3 mm deep. Mouse brain fixation and histopathology were done as previously described (40).

Western blots and immunohistochemistry

Western blots were carried out with the following antibodies: anti-HOXA10 (1:1,000; sc-17159; Santa Cruz Biotechnology); anti-Actβ (1:5,000; A5441; Sigma-Aldrich); anti-MLLC (1:1,000; 05-765; Millipore). Secondary antibodies: anti-goat::HRP (1:5,000; 805-035-180; Jackson ImmunoResearch Lab); anti-mouse::HRP (1:10,000–20,000; A9044; Sigma-Aldrich).

Immunohistochemical analysis was conducted on frozen or paraffin-embedded primary tumor sections. Antibodies used: rabbit anti-SOX2 (1:500; ab97795; Abcam); mouse anti-MLLC (1:500 and 1:1,000). Secondary antibodies used: anti-rabbit::A568 (1:500; A11036; Invitrogen/Molecular Probes); anti-mouse::A488 (1:1,000; A11029; Invitrogen/Molecular Probes). Images were acquired using a Quorum WaveFX spinning disk confocal system (Zeiss Axiovert 200M; Quorum Technologies Inc.).

Quantitative real-time PCR, MLL chromatin immunoprecipitation, DNA quantitative PCR, and statistical analysis

RNA was extracted with the RNeasy Mini Kit (74106; Qiagen) in combination with QIASHredder columns (79656; Qiagen) according to the manufacturer’s instructions. cDNA synthesis was done with the Transcriptor Reverse Transcriptase system (03531287001; Roche) according to the manufacturer’s protocol. Chromatin immunoprecipitation (ChIP) was carried out with anti-MLL (1:50 dilution; 05-765; Millipore/Upstate) and ChIP Kit (ab500; Abcam) according to the manufacturer’s protocol. Quantitative PCR (qPCR) was conducted with a Chromo4 DNA Engine gradient cycler (PTC-200; MJ Research) and analyzed with Opticon Monitor 3 software. Statistical analysis was done with Prism 5.0d. ChIP enrichment analysis was conducted as previously described (42).

Gene expression arrays, HOXA10 ChIP studies, and bioinformatics

ChIP studies to identify HOXA10 targets were carried out with the α-HOXA10 A20 (sc-17159) and N20 antibodies (sc-17158; both from Santa Cruz Biotechnology). ChIP was carried out according to the manufacturer’s protocol specified with the ChIP Kit (ab500; Abcam). Briefly, cells were fixed, lysed, and the resulting chromatin sonicated to produce fragments in the 300 to 1500 bp range. Following immunoprecipitation with the appropriate antibodies, DNA was purified according to the manufacturer’s protocol. Resulting DNA was amplified with Sequenase (70775Y; US Biologicals), with some minor modifications to the manufacturer’s protocol. Following quality control, the samples were used for analysis with the Affymetrix HuP1.0R platform at the TCAG microarray facility (The Hospital for Sick Children, Toronto, Ontario). Putative HOXA10 targets were identified by grouping the A20 and N20 results against a negative control obtained with no primary antibodies. Statistical significance of putative targets was determined by calculating MAT scores on T-statistics with the analysis software Partek version 6.6beta. Target validation was carried out with qPCR as described earlier.

For microarray studies, total RNA was isolated from GNS and human fetal NS cells as outlined in the above section. RNA was used for gene expression profiling at the TCAG microarray facility using the Affymetrix HuP1.0R platform with the Affymetrix U133 or Gene 1.0 ST chip platforms. Statistical analysis was conducted on the Partek Genomic Suite. Some heatmaps were generated with Spotfire Enrichment analysis was conducted with the Database for Annotation, Visualization and Integrated Discovery (DAVID) online tools (43, 44).

Short hairpin RNA constructs and transfections

5 μg of shHOXA10, the short hairpin RNA (shRNA) constructs targeting HOXA10 (RHS4533-NM_018951; Open Biosystems), or control shRNA constructs targeting GFP (shGFP), were transfected in GNS or NS cells using the Amaxa Nucleofector Kit (VPG-1004) and a Nucleofector II electroporator (both from Amaxa Biosystems), according to the manufacturer’s instructions. Approximately 1 million cells were transfected for each technical replicate. MLL was similarly targeted.
with shRNA constructs (RMM3981; Open Biosystems). In this case, 2 μg of shMLL or empty plKO.1 vector were transfected for each technical replicate.

**MTT assays**

MTT assays were conducted with the Cell Proliferation Kit 1 (11465007001; Roche) according to the manufacturer’s protocol.

**Results**

**A HOX signature in patient-derived GBM stem cells**

Our group has developed methods for culturing precursor, or stem-like, cells from resected specimens of human high-grade gliomas in serum-free conditions, with EGF and FGF, adherently on laminin-coated plates (36, 45). Laminin has been suggested to be an important niche factor for GBM stem cells *in vitro* (46). We define the resulting primary cultures of GBM stem cells as GNS cells. GNS cells are genetically stable, they express neural precursor markers such as Nestin and SOX2 and, when transplanted in mice, they produce xenografts histologically similar to the primary tumors they originate from (ref. 36; Supplementary Table S1). For 21 of these GNS cultures, single-nucleotide polymorphism (SNP) arrays were carried out to assess their genotype (at passage number 5–7). Copy number variation data for salient GBM genes are available in Supplementary Fig. S1 and show characteristic molecular alterations seen in primary patient specimens.

In order to identify GNS-specific gene signatures, we studied gene expression profiles in GNS cells with 2 different array platforms. On a first platform (Affymetrix U133.2), we compared gene expression in 6 GNS primary cultures, 2 normal human fetal NS cell primary cultures and 6 non-neoplastic brain cortical resections (36). Gestational ages for NS cell donors are provided in Supplementary Table S2. We identified a gene expression signature consisting of HOX genes in GNS cells. (We hereby define the Homeobox-encoding genes in the clusters HOXA to HOXD as HOX genes, whereas we define the nonclustered, Homeobox-encoding genes found throughout the genome as Homebox genes.) Interestingly, hierarchical clustering of GNS cells, NS cells, and cortical resections on the basis of expression of HOX genes alone segregated all samples into 2 main groups: 1 cluster populated by GNS cells and 1 including NS cells and non-neoplastic brain (Fig. 1A). This dataset suggested that HOX genes might play a role in the malignant behavior of patient-derived GBM stem cells and deserved further investigation. Furthermore, this clustering confirms that, at least with regard to HOX genes, normal NS cells seem to be an appropriate control for experiments involving GNS cells.

We used a second array platform (Affymetrix Gene 1.0 ST) to compare gene expression in a larger set of 27 GNS and 12 primary fetal NS cell cultures. This array platform offered a higher number of probes per gene and, therefore, higher sensitivity. In Fig. 1B, we show probe intensities for all HOX genes. This heatmap was derived by normalizing probe intensities for each GNS primary culture to the average of 12 primary NS cultures. Analysis of this larger collection of GNS cells allowed the definition of 2 groups: some GNS cells are characterized by high expression of HOX genes (HOXhigh GNS cells), while others have more moderate expression of HOX genes (HOXlow GNS cells). On the basis of the results of 2 independent array platforms, we show that 19 out of 32 primary GNS cultures are HOXhigh.

In order to investigate the notion of a HOX signature in a subgroup of patient-derived GNS cells further, we compared genome-wide expression data of the HOXhigh GNS cells (13 lines) and HOXlow GNS cells (14 lines) as defined earlier (Fig. 1B). We identified a set of 486 differentially expressed genes between the 2 groups, setting a greater than 2-fold enrichment and ANOVA P less than 0.05 as selection criteria. Of these, 317 were significantly upregulated in HOXhigh GNS cells compared with HOXlow GNS cells and were examined for enrichment of specific gene classes with DAVID. This analysis identified a significantly enriched Homeobox cluster (Fig. 2A) comprised of 18 genes (listed in Fig. 2B), including HOX genes found in the 4 clusters (HOXA to HOXD) and other Homeobox genes.

Bulk tumor tissue was available from 6 high-grade gliomas that gave rise to HOXhigh GNS cells and 7 bulk tumor samples that gave rise to HOXlow GNS cells. These bulk tumors were used for gene expression array analysis. Unsupervised hierarchical clustering of the tumor samples was carried out on the basis of a gene set solely including HOX genes. Interestingly, 5 out of 6 bulk samples that gave rise to HOXhigh GNS cells clustered in the same group (Group 2 in Fig. 2C). We identified differentially expressed genes in Group 1 and Group 2 bulk GBMs. Among the most highly upregulated genes in Group 2, we identified most of the HOX genes that also characterized HOXhigh GNS cells (Supplementary Fig. S10). These results suggest that the HOX signature identified in HOXhigh GNS cultures aligns with a gene expression profile that also characterizes the bulk patient tumor specimen. This finding suggests that a cancer stem cell signature may be identifiable in bulk GBM samples and is in line with recent findings in leukemia, consistent with an idea that at least some genes expressed in the tumorigenic stem cell programs also persist in tumor bulk (47).

Although some anterior HOX genes are expressed in the brain (like HOXB1; ref. 48), we found it interesting that most developmentally posterior HOX genes (for instance, HOXA9, HOXA10, HOXC10, and HOXD9) are also robustly expressed in GNS cells. Considering the established role of MLL in activation of HOXA10 transcription in hematopoiesis and leukemic cells, we decided to further explore the existence of a Trithorax–Homeobox axis in GNS cells.

To this end, we next investigated expression of HOXA10 by quantitative real-time PCR (qRT-PCR; Fig. 3A) in a subset of NS cells and GNS cells. In all cases, HOXA10 was strongly expressed in GNS cells, compared with NS cells. Western blots detected HOXA10 protein in HOXhigh GNS cells, as predicted. HOXA10 was not detectable in HOXlow GNS cells and in NS cells. MLL protein was detected in both NS cells and GNS cells, but its levels were higher in GNS cells (Fig. 3B).

In order to rule out the possibility that expression of MLL and HOXA10 was an artifact of our cell culture system, we...
carried out Western blotting with cells freshly sorted for the glioma stem cell marker CD15/SSEA-1 (41) from resected GBM specimens. In all 3 cases, we observed strong MLL expression in the CD15⁺ fraction (Fig. 3C and D). In 1 case, we also detected enrichment of HOXA10 in the CD15⁺ fraction (Fig. 3D).

HOXA10 was not detected in GBM481 and GBM482 (Fig. 3C). Interestingly, these primary tumors gave rise to GNS cells (G481NS and G482NS) that were classified as HOXlow by hierarchical clustering of microarray data (Fig. 1B). Furthermore, G481NS, the GNS cells derived from GBM481, also had HOXA10 levels that were undetectable by Western blot (Fig. 3B).
We stained frozen sections of 1 primary GBM (GBM498) and 1 primary astrocytoma (A494) for MLL and SOX2, a marker of stemness properties. We observed strong colocalization of MLL and SOX2, suggesting expression in a precursor pool, in a subpopulation of approximately 10% of cells in both samples (n = 356; Fig. 3E; Supplementary Fig. S9). Single positive cells were also observed (5.6% MLL\(^{+}\) and 46.2% SOX2\(^{+}\)). Similar results were obtained for 3 other paraffin-embedded primary GBM samples, where a significant fraction of SOX2\(^{+}\) cells were also MLL\(^{+}\) (Supplementary Fig. S9). Taken together, data from 8 primary brain tumors suggest that MLL is robustly expressed in the stem-like fraction of high-grade gliomas (marked by CD15 or SOX2) and confirms that HOXA10 is activated in GNS cells with a HOX signature.

**MLL directly regulates HOXA10 activation in GNS cells**

Our data strongly suggest that HOXA10 might play an important biological role for brain tumor stem cell function. Intrigued by the possibility of a functional MLL–HOXA10 axis in a solid tumor, we tested whether MLL physically interacts with the HOXA10 promoter by carrying out a ChIP-PCR (ChIP followed by PCR) experiment in patient-derived GNS cells. We designed primer sets to amplify 3 different segments in the 5' region of HOXA10 spanning a total of approximately 2,800 bp (Fig. 4A). We carried out ChIP with an \(\alpha\)-MLL antibody targeting the endogenous protein and found that MLL interacts with the promoter region of HOXA10, specifically with sequences approximately 1,380 bp upstream of the HOXA10 translational start site (region B in Fig. 4A) in G179NS (Fig. 4B) and G411NS cells (Fig. 4C; Supplementary Fig. S8). \(\alpha\)-H3 (an antibody against histone 3, a ubiquitous histone) was used as a positive control (Supplementary Fig. S2).

Next, in order to independently confirm a direct functional interaction between MLL and HOXA10, we carried out shRNA MLL-knockdown in G411NS cells. Forced reduction of MLL protein levels in these GNS cells resulted in a proportional decrease of HOXA10 protein levels (Fig. 4D; \(P = 0.0066\) by 2-way ANOVA, based on 2 biologic replicates). Together, both the ChIP-PCR and knockdown data show a direct relationship between MLL and HOXA10 in patient-derived GNS cells.

**HOXA10 confers a proliferative advantage to GNS cells**

We next investigated the possibility that high levels of HOXA10 might confer a selective advantage to GNS cells by affecting their proliferation rate. We tested this possibility \textit{in vitro} by conducting MTT assays. We transiently transfected the human GNS cells GliNS1 (Fig. 5A) and G144NS (Fig. 5B) with either a control shRNA construct targeting GFP (shGFP) or 2 shRNA constructs targeting HOXA10 (shHOXA10-1 and shHOXA10-2; Supplementary Fig. S3). Downregulation of
HOXA10 caused a robust and reproducible decrease in cell proliferation in both GliNS1 (Fig. 5A, representative of 2 independent experiments) and G144NS (Fig. 5B, representative of 2 independent experiments) cells.

As HOXA10 had an effect on GNS proliferation in vitro, we investigated its possible role on tumor progression in a mouse xenograft model. GliNS1 cells (Supplementary Fig. S8) carrying HOXA10-targeting shRNA constructs were stereotactically injected into the forebrains of NOD/SCID mice (n = 6). GliNS1 cells transfected with shGFP constructs (n = 2) or untransfected (n = 6) were similarly injected into mice as controls. The injected mice were monitored for the development of neurologic symptoms and the dome-head appearance conferred by brain tumors. We found that knocking down HOXA10 in GliNS1 xenotransplants significantly increased median and maximum mouse survival compared with control (shGFP) animals (Fig. 5C, Mantel–Cox log-rank P = 0.0039; for full statistics see Fig. 5D). Furthermore, we attempted to knock down HOXA10 in the 2 GNS cell cultures that have the highest level of HOXA10 expression, namely G411NS and G432NS. We made 2 separate attempts at generating stable HOXA10 knock-down lines from these GNS cells. In both cases, cells carrying the empty vectors were able to grow. Conversely, both G411NS and G432NS cells carrying knockdown vectors failed to grow in vitro. The outcomes of these experiments attest to the importance of HOXA10 for continued GNS cell proliferation. Taken together, the results of our in vitro MTT assays and mouse survival in our xenotransplant model provide strong evidence that HOXA10 importantly contributes to proliferative and growth advantages of GNS cells.
The MLL–HOXA10 axis maintains expression of a Homeobox gene network

Scant information on HOXA10 genome-wide target specificity exists. We therefore decided to identify putative HOXA10 targets in our GNS cells with a ChIP-chip (combining ChIP with microarray technology) strategy. Conditions were optimized to immunoprecipitate the endogenous HOXA10 protein with 2 independent antibodies (a third antibody proved less optimal for IP; see Supplementary Fig. S4 for antibody specificity) in G411NS cells. Stringent bioinformatic analysis was conducted (see Materials and Methods) and led to the discovery of 261 putative HOXA10 targets. Gene ontology analysis highlighted enrichment for the terms growth and synapse (Supplementary Fig. S5), terms consistent with a potential role of HOXA10 in malignancies of the brain. Further analysis with DAVID software found significant enrichment (P < 0.05) of genes in the clusters Growth factor activity, Homeobox, CHROMO domain, Cytokine, and Actin cytoskeleton organization, and an interesting, albeit not statistically significant, Chromosome organization cluster (Fig. 6A). Interestingly, some of the HOX genes identified in our ChIP-chip studies, namely HOXA7 and HOXC10, were also part of the HOX signature that characterized HOXhigh GNS cells as compared with HOXlow GNS cells (Fig. 2B).

As a first attempt at validating the putative HOXA10 targets, we conducted genomic qPCR on chromatin immunoprecipitated from G411NS. We validated 7 out of 7 targets using this method (Fig. 6B). Some of the identified genes with established roles in tumorigenesis and cancer stem cell biology, notably TERT, FGF17, JAG2, and NODAL, were validated as HOXA10 targets.

Evidence suggests that HOXA10 can function both as an activator and a repressor of gene expression. To gain better insights into the role of HOXA10 in GNS cell function, we looked at the expression levels of the putative targets that were enriched in the clusters identified by DAVID in our collection of GNS cells. We produced a heatmap comprising probe intensities for all genes found in the enriched DAVID clusters (Fig. 6A). Interestingly, unsupervised hierarchical clustering of GNS cells based on the expression levels of HOXA10 target genes closely recapitulated their clustering based on HOX gene expression (Fig. 6C). Of particular interest was the finding that HOXhigh GNS cells had reduced levels of IRX3 expression.
compared with HOX\textsubscript{high} GNS cells. The IRX3 locus is known to be hypermethylated and silenced in brain tumors (49), but it appears to be further silenced in HOX\textsubscript{high} cells.

In addition, we noticed that 36 of the 261 putative HOXA10 targets have an established role in leukemia, based on published evidence (Supplementary Table S3). Unsupervised hierarchical clustering was carried out on the basis of the expression profiles of these genes. As seen in the dendrogram in Supplementary Fig. S7, GNS cultures with a HOX signature tend to cluster together, suggesting that expression of these genes might truly be controlled by HOXA10.

In addition, we validated HOXA10 binding to HOXA7, HOXC10, and the chromobox-encoding CBX6 promoters by genomic qPCR (Fig. 7A). In order to further assess the functionality of this MLL–Homeobox axis we identified, we decided to perturb the axis upstream and interrogate the downstream effects. We, therefore, knocked down MLL in G411NS, G432NS, and G511NS and evaluated its effects on expression of HOXA10 and the HOXA10 targets HOXA7, HOXC10, and CBX6. Our results indicate that expression of HOXA10 and its targets is dependent on MLL expression (Fig. 7B–D).

Discussion

In the present study, we provide evidence that GBMs are characterized by heterogeneous populations of cells with respect to their epigenetic properties. Specifically, we found enrichment of MLL in the CD15\textsuperscript{+} fraction of cells sorted from freshly resected GBM samples and in the SOX2\textsuperscript{+} fraction in high-grade glioma samples. In addition, we found robust expression of Homeobox genes in about half the GNS cells we profiled. Interestingly, the epigenetic modifier MLL was detected at the protein level in all the GNS cells we examined, and at higher levels than in normal NS cells. The different behavior of MLL in cells with high HOXA10 expression and low HOXA10 expression cannot be merely attributed to levels of...
regulator of which an important epigenetic factor, MLL, functions in GBM also like to emphasize the physiologic relevance of our and between HOXA10 and its downstream targets. We would this TrxG protein, as shown by our expression studies. We believe other epigenetic mechanisms regulating MLL function are at play and we are currently pursuing them actively.

We provide strong evidence in support of a functional MLL–HOXA10 axis in glioma stem-like cells. This finding is surprising because HOXA10 was not known to be expressed in the brain or other anterior structures, suggesting that this represents aberrant activation of a developmental program in cancer cells. Frequent duplications of chromosome 7, the site of the HOXA10 locus, are found in GBM. However, the strong expression of HOXA10 we detected in GNS cells was from several to many orders of magnitude higher than in normal NS cells, thus suggesting that high HOXA10 expression is a specific and biologically relevant feature linked to GBM stem cell properties and not simply due to chromosome 7 duplication. As further evidence, not all of the HOXA cluster genes are overexpressed in GNS cells (see, for instance, HOXA3 and HOXA6 in Fig. 1A). Data mining of 138 GBM bulk samples from The Cancer Genome Atlas (5, 50, 51) confirms the absence of correlation between copy number variation at the HOXA10 locus and HOXA10 mRNA levels (Supplementary Fig. S6). Furthermore, the increased HOXA10 expression was not an artifact of cell culture. First, control NS cells were grown in the same conditions without upregulation of HOXA10. Second, we detected increased levels of HOXA10 protein in freshly sorted cells from 1 out of 3 primary GBMs.

The present report is the first to describe the mechanism by which an important epigenetic factor, MLL, functions in GBM stem cells. A recent publication identified hypoxia as a positive regulator of MLL in glioma cells (7). However, that report failed to identify direct MLL targets and simply focused on the coordinated expression of MLL and hypoxia-inducible factors under hypoxic conditions, in both GBM stem and non-stem cells. We, therefore, believe that our work provides an important conceptual advancement in the glioma stem cell field, by establishing the direct interaction between MLL and HOXA10 and between HOXA10 and its downstream targets. We would also like to emphasize the physiologic relevance of our findings, because all the direct regulatory interactions were established by studying the endogenous proteins.

Some of the validated HOXA10 targets we identified, TERT, FGF17, JAG2, and NODAL, are of particular interest because they were previously identified as either having a role in tumorigenesis or being overexpressed in tumors with high HOX expression. Work in leukemia showed that the MLL–AF4 fusion protein upregulated TERT expression through direct regulation of HOXA7 (52). Furthermore, a correlation between high FGF17 and HOXA10 expression was identified in lung cancer (53). JAG2 was shown to be activated by a NUP98–HOXA10 fusion protein with hematopoietic stem-cell-transforming function (54). Recently, Nodal has been shown to be required for self-renewal and tumorigenicity of pancreatic stem cells (55). Furthermore, 1 of the most highly ranked HOXA10 targets is CDX4, which is a bona fide HOXA10 target in myeloid cells (56), thereby further corroborating the strength of our ChIP assay for HOXA10.

Another interesting observation was the direct regulation of other Homeobox genes by HOXA10. Some of these targets were validated 4-fold by ChIP-array; by genomic qPCR; these genes were usually highly expressed in HOXA10-high GNS cells; and their expression decreased when the MLL–HOXA10 axis was perturbed by knocking down MLL. These data show that a network of Homeobox genes is activated by HOXA10, suggesting potential cooperative contributions of these transcription factors to the tumorigenic phenotype.

It is worth noting that whether we carried out hierarchical clustering of our GNS cells on the basis of expression of HOX genes or expression of the HOXA10 targets we identified by ChIP array, we obtained the same grouping of GNS cells. This observation suggests that HOXA10 might function as a core transcriptional node in GNS cells and reinforces the importance of the MLL–HOXA10 axis in GBM stem cell biology.

Our work and that of other researchers point to an important role of TrxG proteins in brain malignancies. A recent paper showed that 16% of medulloblastoma patients have mutations in MLL2 or MLL3 (30). Therefore, in addition to a contribution...
by Polycomb proteins to tumorigenicity, our data and that of other researchers indicate that TrxG proteins and their targets also make a contribution. We propose that loss of an appropriate balance between TrxG-mediated activation and Polycomb-mediated repression of gene expression contributes to the neoplastic phenotype in GBM stem cells. The role of other TrxG proteins in GBM will be the focus of further studies. In conclusion, the identification of a functional MLL-Homeobox network might help in the stratification of GBM patients. Fully understanding the contribution of TrxG proteins to the establishment of a glioma epigenome will be an important step to fully understand the complex biology of this disease.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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