Microarray-based Transcriptional and Epigenetic Profiling of Matrix Metalloproteinases, Collagens, and Related Genes in Cancer

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Epigenetic parameters (DNA methylation, histone modifications, and miRNAs) play a significant role in cancer. To identify the common epigenetic signatures of both the individual matrix metalloproteinases (MMPs) and the additional genes, the function of which is also linked to proteolysis, migration, and tumorigenesis, we performed epigenetic profiling of 486 selected genes in unrelated non-migratory MCF-7 breast carcinoma and highly migratory U251 glioma cells. Genome-wide transcriptional profiling, quantitative reverse transcription-PCR, and microRNA analyses were used to support the results of our epigenetic studies. Transcriptional silencing in both glioma and breast carcinoma cells predominantly involved the repressive histone H3 Lys-27 trimethylation (H3K27me3) mark. In turn, epigenetic stimulation was primarily performed through a gain of the histone H3 Lys-4 dimethylation (H3K4me2) and H3 hyperacetylation and by a global reduction of H3K27me3. Inactive pro-invasive genes in MCF-7 cells but not in U251 cells frequently exhibited a stem cell-like bivalent mark (enrichment in both H3K27me3 and H3K4me2), a characteristic of developmental genes. In contrast with other MMPs, MMP-8 was epigenetically silenced in both cell types, thus providing evidence for the strict epigenetic control of this anti-tumorigenic proteinase in cancer. Epigenetic stimulation of multiple collagen genes observed in cultured glioma cells was then directly confirmed using orthotopic xenografts and tumor specimens. We suggest that the epigenetic mechanisms allow gliomas to deposit an invasion-promoting collagen-enriched matrix and then to use this matrix to accomplish their rapid migration through the brain tissue.

Cell locomotion is a well orchestrated, multi-component, molecular and cellular process that involves multiple and complex regulatory pathways. Proteolysis and proteinases including MMPs, a family of 24 individual enzymes in humans, play an important role in cell function and especially in the processes of cell migration and invasion (1–3). In addition to MMPs, multiple genes and respective cellular proteins, including extracellular matrix (ECM) proteins, integrins, and other adhesion signaling receptors, are also directly involved in cell locomotion.

Gene products involved in cell locomotion, angiogenesis, tumor progression, and survival are all potential targets of epigenetic regulation via DNA methylation and histone modifications (4) and by micro-RNA (miRNA) mechanisms (5, 6). In malignancies, DNA methylation is frequently dysregulated. By interfering with the transcription initiation, methylation of the CpG islands (CpGi) inhibits transcription and represses tumor suppressor genes. Acetylation of the core histones H3, H4, H2A, and H2B is normally associated with the activation of gene transcription (7). Acetyl groups are added by a family of histone acetyltransferases and are removed by histone deacetylases. In turn, methylation of the lysine residues in the histone tails may lead to either transcriptional activation or repression. Methylation of histone lysines occurs in a form of mono-, di-, and trimethylation and is reversed by enzymatic demethylation (8, 9). Methylation of the H3K4, H3K36, and H3K79 residues attracts the polymerase II complex and, as a result, up-regulates gene expression (10, 11). In contrast, methylation of the H3K9, H3K27, and H4K20 lysine residues promotes the interaction of the modified histones with the heterochromatin protein 1 (HP1) or its homologues, and these events lead to gene silencing (12–15). In stem cells and also in T cells, H3K4 and H3K27 methylation frequently occurs in histones that are associated with the temporarily silenced developmental genes, the rapid activation of which is initiated by developmental stimuli (16–18).

In the miRNA mechanism, a primary miRNA transcript (pri-miRNA) is encoded by the genome. A pri-miRNA is cleaved by DROSHA to a pre-miRNA. A pre-miRNA is transported from the nucleus to the cytoplasm by exportin-5 and next cleaved by Dicer to generate the mature miRNA duplexes. After a strand separation, the mature miRNA species binds an RNA-induced silencing complex and then represses protein synthesis either by blocking translation or by causing transcript degradation.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3 and Tables S1–S5.

The ChIP-on-Chip, gene expression, and miRNA profiling data have been deposited in the Gene Expression Omnibus (GEO) database with the accession number GSE18899.

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2. The abbreviations used are: MMP, matrix metalloproteinase; MT-MMP, membrane type MMP; GBM, glioblastoma multiforme; ChIP, chromatin immunoprecipitation; CpGi, CpG island; ECM, extracellular matrix; H3K4me2, dimethylated Lys-4 of histone H3; H3K27me3, trimethylated Lys-27 of histone H3; H3ac, pan-acetylated histone H3; IP, immunoprecipitation; miRNA, microRNA; MMP, matrix metalloproteinase; POL2, polymerase II Δ subunit; TIMP, tissue inhibitor of matrix metalloproteinases; WCE, whole cell extract; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TGF, transforming growth factor.
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miRNAs, which potentially can regulate ~30% of human genes (20), are deregulated in cancers by different mechanisms, including epigenetic activation/repression (5, 6).

It is still unclear how pro-migratory genes, including MMPs, tissue inhibitors of metalloproteinases (TIMPs), ECM proteins, and cell adhesion and signaling receptors, are controlled epigenetically (21–25). Genome-wide transcriptional profiling alone (26–28) estimates the levels of the individual mRNAs in the cell sample, but it does not determine the transcriptional state of the individual genes.

To shed more light on the specific mechanisms of epigenetic control of pro-migratory genes in cancer, we used a multi-level microarray-based, proof-of-principal approach. For our epigenetic studies, we specifically selected non-migratory MCF7 breast carcinoma and super-migratory U251 glioma cells. As a result, we now determined for the first time the epigenetic profile of selected 486 pro-migratory genes that are independent of tissue type in highly migratory and non-migratory cancer cell lines. The genes we selected included MMPs themselves and also the genes that, according to the data mining in the bioinformatics databases, are functionally linked to MMPs. We also determined the profile of the 799 individual miRNAs in the cells. We believe that the approach we have developed here can and will be used in multiple cancer types and that it will lead to the identification of novel and promising drug targets.

MATERIALS AND METHODS

Reagents and Antibodies—All reagents were purchased from Sigma unless indicated otherwise. Rabbit polyclonal antibodies to H3ac, H3K4me2, and H3K27me3 histones were purchased from Millipore. A murine monoclonal antibody against POL2 was from Covance. A rabbit MT1-MMP Ab815 antibody was from Chemicon. The antibodies to human nucleobindin 1, type I collagen, glial fibrillary acidic protein, βIII-tubulin, and Ki67 were purchased from Millipore, Novus Biologicals, DAKO, Covance, and Abcam, respectively.

Cell Culture—Human glioma/glioblastoma (GBM) U251 and breast carcinoma MCF-7 cells (60–80% confluent) grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum were used in our experiments. Both cell lines were purchased from ATCC (Manassas, VA).

Microarray Design for Chromatin Immunoprecipitation (ChIP)-on-Chip—Microarray probes were designed using eArray software (Agilent). Unique DNA probes specific to 486 human genes from the high density ChIP Data base (Agilent) were included in each microarray. Microarray, 8 × 15K format, slides were custom manufactured by Agilent. Additional hybridization control probes (universal control grid LAS77) were included in the arrays (Agilent). Each individual probe in the microarray was replicated 3–10 times.

Chromatin Preparation—Cells were treated for 10 min with 1% p-formaldehyde and then with sodium glycine (100 mM) to inactivate p-formaldehyde. Cells were next washed twice with ice-cold phosphate-buffered saline, detached, and collected by centrifugation. The pellet was re-suspended in 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 3 mM CaCl₂, 0.2% Triton X-100, and the Complete Proteinase Inhibitor Mixture (Roche Applied Science). Chromatin DNA was digested at 37 °C for 10 min using micrococcal nuclease (Roche Applied Science) to generate ~80% mono-nucleosomal and 20% di-nucleosomal DNA fragments. The reactions were stopped using 5 mM EDTA. Digested chromatin was solubilized for 15 min on ice using 0.2% SDS. The pellet was removed by centrifugation (10,000 × g, 20 min). For each chromatin sample a corresponding control fraction of the input DNA was prepared as follows. Genomic DNA samples (50 μg each) were incubated for 16 h at 65 °C with 0.5 NaCl. RNA and proteins were digested using 10 μg/ml RNase A (37 °C; 15 min) and 1 mg/ml Proteinase K (50 °C; 1 h), respectively. DNA was purified using the DNA GenElute PCR Clean-up System. DNA fragmentation was confirmed using 2% agarose gel-electrophoresis. DNA concentration was measured at 260 nm.

ChIP and DNA Purification—Chromatin samples (100 μg each) were diluted in 500 μl of ChIP buffer (50 mM HEPE, pH 7.5, containing 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 0.1% sodium deoxycholate, 0.1% SDS, and the Complete Proteinase Inhibitor Mixture). The samples were pre-cleared by incubating for 1 h at 4 °C with 50 μl of Protein G-coated magnetic Dynabeads suspension (Invitrogen). The H3ac, H3K4me2, H3K4me3, or H3K27me3 antibodies (10 μg each) and 50 μl of Protein G-coated magnetic Dynabeads suspension were then added to the pre-cleared samples. Non-immune rabbit IgG was used as a control. To reduce nonspecific binding, the beads were pretreated for 1 h with the sonicated salmon sperm DNA (10 mg/ml). The final volume of the samples was made 600 μl using the ChIP buffer. ChIP samples were prepared in triplicate. The samples that did not contain the antibody were used as a control. The samples were then incubated at 4 °C for 12 h on a rotating platform. The beads were captured using a magnetic rack (Qiagen) and washed 4 times in the ChIP buffer and twice in the ChIP buffer containing 0.5 mM NaCl. The bound material was eluted from the beads at 37 °C for 30 min in 100 μl of 0.1% NaHCO₃, 1% SDS. The soluble fraction was separated from the beads by centrifugation (10,000 × g, 5 min). The soluble material was incubated at 68 °C for 4 h in the presence of 0.5 mM NaCl. RNA and proteins were digested using 10 μg/ml RNase A (37 °C; 15 min) and 0.25 mg/ml Proteinase K (50 °C; 15 min), respectively. DNA was purified using the DNA GenElute PCR Clean-up System. DNA concentration was measured at 260 nm.

ChIP-on-Chip—The immunoprecipitated DNA samples (10 ng each) were amplified using the WGA2 whole genome amplification system. Amplification products were purified using the DNA GenElute PCR Clean-up System. DNA concentration was measured at 260 nm. The quality of the amplified DNA samples was confirmed using 2% agarose gel-electrophoresis. The amplified IP DNA and WCE DNA samples (1 μg) were labeled with Cy5-DUTP and Cy3-DUTP, respectively, using the Genomic DNA Enzymatic Labeling kit (Agilent). Labeled DNAs were hybridized with custom microarrays for 40 h at 65 °C and then washed according to the manufacturer’s instructions. Hybridization images were obtained using an Agilent DNA microarray scanner, and intensity data were extracted using Feature Extraction software Version 10.5 (Agilent). Genomic regions enriched by ChIP were identified with a
**Gene Expression Profiling**—Total RNA was extracted from cultured cells (1 × 10^5) using TRIzol reagent (Invitrogen) and dissolved in diethyl pyrocarbonate-treated H2O to a final concentration 500 ng/μL. RNA integrity was validated using Experion automated electrophoresis system (Bio-Rad). Samples of total RNAs (0.5 μg) were labeled and hybridized to a HumanWG-6 BeadChip Version 3.0 (Illumina) according to manufacturer’s protocol. Data were collected, and the initial analysis was performed using GeneSpring software (Agilent). Only the statistically significant data (p < 0.05) were analyzed further.

**miRNA Expression Profiling**—Total RNA was purified from cells using the miRNA Mini system (Qiagen). The quality of the purified RNA samples was assessed using the Bio-Rad Experion system and by measuring the 260/280 nm and the 260/230 nm ratios. Total RNA (100 ng) was labeled with a cyanine 3-pCp reagent using the miRNA Complete Labeling and Hybridization HybKit (Agilent). The labeled samples were then hybridized for 20 h at 55 °C with Agilent Human miRNA Microarray Version 2 designed using Sanger Data base Version 10.1. Microarray slides were washed according to the manufacturer’s protocol and scanned using a DNA microarray scanner G2565CA (Agilent). The raw data were processed using Feature Extraction software Version 10.5 and normalized using DNA Analytics and GeneSpring 10 software (Agilent). Differentially expressed miRNAs with signal intensities higher than 2-fold than the background standard deviation were filtered by t test with p < 0.05. The heatmap charts were generated using GenePattern software (29).

**RT-PCR Analysis of the Selected Genes**—Specific oligonucleotide primers (Eurogentec) for the RT-PCR analyses were designed using Primer 3 software (30) (supplemental Table S4). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. RT-PCR analysis was performed using the OneStep RT-PCR system (Qiagen). The 25-μl reactions each included total RNA (40 ng) and the respective primers (0.6 μM) designed to amplify the cDNA. After the completion of the first strand synthesis, RT-PCR reactions (35 cycles) were performed using denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and elongation at 72 °C for 1 min. The products were separated by 2% agarose gel-electrophoresis.

**Glioma Xenograft Models in Immunodeficient Mice**—To generate the orthotopic glioma tumor xenografts, U251 cells (4 × 10^5) were microinjected into both cerebral ventricles of cryoanesthetized newborn (P0) SCID mice. In 4 weeks the mice were sacrificed according to the National Institutes of Health guidelines. The brains were frozen-sectioned. The sections were stained with the antibodies to human nucleobindin 1, type I collagen, glial fibrillary acidic protein, βIII-tubulin, and Ki67 followed by the Alexa 488 (green) - and Alexa-594 (red)-conjugated species-specific secondary antibodies. Images were acquired using a BX51 fluorescent microscope equipped with a MagnaFIRE digital camera (Olympus). Images were processed with MagnaFIRE 2.1C software (Olympus).

To generate the non-orthotopic glioma xenografts, U251 cells were xenografted subcutaneously into 4-week-old athymic female BALB/c nu/nu immunodeficient mice (Benton & Kingman) as described earlier (31). At 45–55 days after the cell injection, tumors (~1000 mm^3 in size) were excised free of connective tissue, washed in ice-cold phosphate-buffered saline, cut, and flash-frozen in liquid nitrogen. At the end of the experiments, mice were sacrificed according to the National Institutes of Health guidelines.

**Quantitative-PCR Analysis of Glioma Samples**—Frozen tumor samples (10–100 mg) were homogenized, and total RNA was extracted in 1 ml of TRIzol according to the manufacturer’s instructions. cDNA was synthesized using RNA (500 ng), random hexamer primers (100 ng), and the SuperScript First-strand synthesis system (Invitrogen). Q-RT-PCR reactions (25 μl each) contained a Power SYBR green PCR mix (12.5 μl; Applied Biosystems), specific primers (10 pmol each, supplemental Table S4), and cDNA (2.5 ng). Each cDNA sample was analyzed in triplicate. Human GAPDH was used as a control. The PCR amplification was performed using an MX3000P thermocycler (Stratagene). ROX dye was used as an internal standard. Specific product amplification was monitored by measuring a melting curve of the PCR product. The amplified products were also analyzed by agarose gel electrophoresis. The data were processed using MxPro software (Stratagene) to calculate the mean cycle threshold (ΔCt) values and p values.

**RESULTS**

**Design of a Custom ChIP-on-Chip Microarray**—The list of 486 human genes of our custom-built ChIP-on-Chip microarray is shown in supplemental Table S1. The Agilent HD ChIP probe data base was used to select the individual hybridization probes. These probes were complimentary to the −5 to −3-kb regions of the respective gene promoter sequence (numbering starts from the transcription initiation site of the gene). As a result, 15,744 individual probes were arranged in an 8 × 14K microarray format. Each individual probe was present at least in triplicate in the microarray. Ten copies of the individual probes were included for each of the individual MMPs.

Our custom microarray permits a simultaneous determination of the levels of the polymerase II Δ subunit (POL2) and the three histone modifications (H3K27me3, H3ac, and H3K4me2) under the same experimental conditions. We have specifically selected these three histone modifications for our analysis because according to our studies and those by others (25, 32, 33) they represent the most reliable measure of the transcriptional activity of the chromatin. POL2 was included in the microarray as a reliable indicator of the transcriptionally active genes.

The microarray we designed allowed us to profile the genes encoding 23 MMPs and their natural protein inhibitors (four TIMPs (TIMP-1, -2, -3, and -4) and RECK (reversion-inducing cysteine-rich protein with Kazal motifs)), and FUR (furin, a common activator of the multiple individual MMPs) and additional 416 genes. According to the data mining in the bioinformatics databases, including Ingenuity, NextBio, and AmiGO, these 445 genes were linked to cell migration, invasion, tumorigenesis, and angiogenesis (supplemental Table S1). Additional 41 genes we included in the array represented the known human histone acetyltransferases, histone deacetylases, histone
methyltransferases, and histone demethylases. There were also a number of the required control loci with the previously determined epigenetic characteristics, including GAPDH, in the array. As a result of this custom design, our microarray allowed us to accomplish the proof-of-principle profiling of 486 individual genes in non-migratory breast carcinoma MCF-7 and highly migratory U251 glioma cells and obtain volumes of statistically significant information.

**Gene Expression Profiling**—As a first step, we performed gene expression profiling of the cells. The results for 486 genes included in our ChIP-on-Chip array were analyzed further (Fig. 1A; supplemental Tables S2 and S3). From these 486 genes, the expression of 57 genes was especially high in U251 cells. These genes included the ECM components (tenascin C (TNC), osteopontin (SPP1), osteonectin (SPARC), laminin B1 (LAMB1), versican (VCAN), thrombospondin-2 (THBS2), angiopoietin 1 (ANGPT1), and collagens (COL)), proteinases and their inhibitors (urokinase (PLAU), furin (FUR), MMPs, and...
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TIMPs), growth factors and cytokines (CCL2, CTGF, TGFBI, TGBF1, CXCL14, IL8, FGFB2, TRAIL, neuregulin 1 (NRG1), and BDNF), adhesion and signaling receptors (neuronal cadherins-2 (CDH2) and -6 (CDH6), sarcoglycan (SGCE), connexins-43 (GJA1) and -26 (GJB2), VCAM1, CD44, and integrin α4 (ITGA4)), metabolic enzymes (cytosolic aldehyde dehydrogenases A1 and 2), and transcription regulators (PAX6, RORB and HEY1).

Conversely, from 489 genes that were included in the ChIP-on-Chip array, the expression of 14 genes was especially high in MCF-7 cells. These genes included macrophage inhibitory factor (MIF), chemokine (CX3C motif) ligand 12 (CXCL12), MMP-9, TIMP-3, bone morphogenic protein BMP4, epithelial cadherin CDH1, PDZ-domain protein gene PARD6B, breast cancer-specific chemokine receptor CXCR4, metastasis suppressors MTSS1 and SYK, and transcription regulators ASCLI, ASCLI2, HEY2, and HEY1 (Fig. 1A).

Most strikingly, multiple collagen genes (COL11A1, COL7A1, COL6A1, COL8A1, COL5A1, COL6A2, COL4A2, COL1A1, COL12A1, COL18A1, and COL14A1) were transcriptionally activated in U251 cells. Thus, the RT-PCR analysis of the selected collagen genes corroborated the gene expression profiling results (except COL18A1, the expression of which, according to the RT-PCR analysis, was similar in MCF-7 and U251 cells). RT-PCR confirmed high expression levels of COL11A1, COL7A1, COL8A1, and COL5A1, MMPs (MMP-2, MMP-7, MT1-MMP, and MT3-MMP), and TIMP-4 in U251 cells (Fig. 1B). The levels of MT2-MMP, MT4-MMP, and MT6-MMP were comparable in MCF-7 and U251 cells. Consistent with the microarray data, the results of RT-PCR showed that MCF-7 cells exhibited high levels of MIF.

Epigenetic Signature of the Transcriptionally Active Genes—To elucidate the role the epigenetic mechanisms play in the regulation of pro-invasive genes, especially MMPs and collagens, the chromatin-associated DNA samples were isolated from U251 and MCF-7 cells and immunoprecipitated using the H3K27me3, H3K4me2, H3ac, and POL2 antibodies (25). The antibody-bound fractions were purified and then used for hybridization to the ChIP-on-Chip microarray. Quantitative-PCR with the MMP-2 specific primers was used to test the quality of immunoprecipitated samples (25). The hybridization signal was recorded, and the statistically significant data (p < 0.05) were analyzed further to calculate the enrichment value. The ratio of the immunoprecipitated DNA (IP) to the whole cell extract (WCE) DNA is shown in Fig. 2. The distribution parameters of the specifically precipitated DNA material relative to the whole cell DNA confirmed the specificity of the antibodies and the overall high quality of the samples we used in our further analysis.

According to the results of our ChIP-on-Chip profiling, the multiple pro-invasive genes, the expression of which was up-regulated in high migratory U251 cells, exhibited a number of the characteristic epigenetic parameters. These parameters included the global reduction of the repressive H3K27me3 mark and hyperacetylation of histone H3 (supplemental Figs. S1 and S2). The epigenetic parameters of 10 selected genes that are highly expressed and are silenced in U251 and MCF-7 cells, respectively, are shown in Fig. 3. Thus, high levels of the repressive H3K27me3 mark (2–8-fold higher relative to the median level) were a dominant feature of the silent loci in MCF-7 cells including CCL2, CTGF, TNC, SPARC, CDH2, LAMB1, CDH6, VCAN, SPP1, and ITGA4. Conversely, low levels of the repressive H3K27me3 mark correlated well with the enhanced transcriptional activity of the invasion-related genes in U251 cells. On average, there also was a 16-fold enrichment...
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The expression levels of the 14 transcriptionally active genes in MCF-7 cells and epigenetic profiling of the selected ten genes are shown in supplemental Table S3 and in Fig. 4, respectively. These transcriptionally active genes also exhibited high levels of H3K4me2 and, in addition, histone H3 hyperacetylation. Conversely, low levels of H3K4me2 and H3ac resulted in the transcriptional silencing of these genes in U251 cells.

In addition to the presence of the H3K27me3 repressive mark, multiple inactive pro-invasive genes in MCF-7 cells also exhibited high levels of H3K4me2 (e.g. CTGF, CDH2, LAMB1, CDH6, and VCAN). The co-existence of H3K27me3 and H3K4me2 constitutes a stem cell-like bivalent mark, a specific characteristic of developmental genes (16, 17). The presence of this bivalent mark was not detected in U251 cells (Fig. 3).

Differential Epigenetic Regulation of TIMPs—Because TIMP-1, -2, -3, and -4 are potent regulators of MMPs, we analyzed the TIMP genes in more detail. According to both the gene expression microarray and the RT-PCR analysis, there are similar levels of TIMP-1 and TIMP-2 in U251 and MCF-7 cells (Fig. 5A). The TIMP-3 mRNA level was high in MCF-7 cells alone. In turn, the TIMP-4 gene was up-regulated in U251 cells. We determined high levels of both H3ac and H3K4me2 and low levels of H3K27me3 in the 12-kb-long, −8.0 to +4.0-kb, region of the TIMP-1 gene promoter in both U251 and MCF-7 cells (numbering starts from the transcription initiation site of the gene). The enrichment of POL2 was observed in the region that was proximal to the transcription initiation site (Fig. 5B). These epigenetic characteristics correlate well with the active status of the TIMP-1 gene in both cell types.

There is a single CpGi in the TIMP-1, TIMP-3, and TIMP-4 promoters. In addition to the unmethylated first CpGi, there is a hypermethylated second CpGi upstream of the TIMP-2 gene promoter (25). There were high levels of POL2, H3ac, and H3K4me2 and low levels of H3K27me3 in the first CpGi region of TIMP-2. In contrast, H3ac, H3K4me2, and H3K27me3 were low in the second CpGi region (Fig. 5B). As a result, we conclude that the first CpGi of TIMP-2 exhibits the repressive epigenetic features, whereas the second CpGi region displays the “active-state” chromatin parameters, thus providing an opportunity for a fine epigenetic balance of TIMP-2 expression in cancer.

Similarly, there were high levels of POL2, H3ac, and H3K4me2 in the region that was downstream of the TIMP-3 transcription initiation site in the 5.0-kb-long sequence of the TIMP-3 promoter in MCF-7 cells, indicative of the transcriptional activity (Fig. 5B). Conversely, the repressive H3K27me3 mark was elevated in the same region in U251 cells, in which TIMP-3 expression was down-regulated.

The epigenetic regulatory parameters were opposite for TIMP-4. Thus, POL2, H3ac, and H3K4me2 were up-regulated in the CpGi region of the 11-kb-long, −8.0 to +3.0-kb, chromosomal region of the TIMP-4 promoter in U251 cells (Fig. 5B). Overall, it is likely that the epigenetic modifications play a significant role in the regulation of all four known species of TIMPs.

Epigenetic Regulation of MMPs—Nine MMP genes (MMP-7, MMP-20, MMP-27, MMP-8, MMP-10, MMP-1, MMP-3, MMP-12, and MMP-13) are clustered in a 430-kb long, 11q22.3 region of the long arm of chromosome 11 (34, 35). Other individual MMPs are expressed in the different chromosomal
regions distinct from c11q22.3. The epigenetic profiling of the 11q22.3 MMP cluster clearly determined the elevated levels of the active-state POL2, H3ac, and H3K4me2 marks, and the existence of the actively transcribed MMPs, especially the MMP-7, MMP-10, MMP-1, MMP-3, MMP-12, and MMP-13 from the 430-kb-long 11q22.3 region. The solid line represents the median IP/WCE ratio of the respective mark across the array. Enrichment of the mark is above the line. The data are presented in a log2 format. The gene promoters are schematically shown at the bottom of the panels. U251 and MCF-7 cells are in blue and red, respectively. The arrows show the position of the respective gene in the cluster and the direction of transcription.

Epigenetic Control of Collagens—Our ChIP-on-Chip assay readily recorded the presence of elevated levels of the stimulatory H3K4me2 and H3ac marks in the collagen genes in U251 cells including collagens COL1A1, COL6A1, COL7A1, COL4A1, COL5A1, COL18A1, COL8A1, COL12A1, COL1A1, COL4A2, and COL14A1 (Fig. 9). The high levels of H3K4me2 and H3ac directly correlated with the transcriptional activity of these collagen genes in U251 cells (Fig. 1). In turn, the presence of the repressive H3K27me3 mark was a predominant feature of the transcriptionally inactive collagen genes in MCF-7 cells. Overall, our results indicate that the balance between the stimulatory and repressive epigenetic marks plays a significant role in the regulation of MMPs in cancer cells.

Furin, a subtilisin-like proprotein convertase that activates multiple MMPs including MT-MMPs (36), was also up-regulated in U251 cells. In agreement, the FUR gene displayed the gain of H3ac, H3K4me2, and POL2 active chromatin marks (Fig. 8). In MCF-7 cells the expression of furin is low because of the significant level of the H3K27me3 repressive mark, especially in the region that is downstream of the FUR promoter (Figs. 1A and 8).

Glycosylphosphatidylinositol-linked, lipid raft-associated MT4-MMP and MT6-MMP do not contribute to cell migration (37), and MMP-9 is normally secreted as a latent zymogen, which is not efficiently activated (38, 39). As a result, the elevated expression of these individual MMPs cannot contribute significantly to the locomotion of non-migratory MCF-7 cells. In turn, the presence of pro-invasive MMP-2, MMP-7, MT1-MMP, and MT3-MMP can significantly stimulate the migration of U251 cells. Overall, our results indicate that the balance between the stimulatory and repressive epigenetic marks plays a significant role in the regulation of MMPs in cancer cells.

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FIGURE 5. Epigenetic control of TIMPs in U251 and MCF-7 cells. A, the TIMP-1, -2, -3, and -4 mRNA levels (±S.E.) in the cells are shown. The data are derived from the gene expression analysis. The cut-off level (50 intensity units) is shown as a dotted line. B, shown is the epigenetic signature of TIMPs in U251 and MCF-7 cells. The solid line represents the median IP/WCE ratio of the respective mark across the array. Enrichment of the mark is above the line. The data are presented in a log2 format. The positions of the respective probes are mapped relative to the transcription initiation start of the gene promoter (shown at the bottom of the panels). U251 and MCF-7 cells are in blue and red, respectively. Black bars correspond to the first exon. The arrows show the direction of transcription. Gray bars show the CpGi regions. Thin solid bars correspond to the 1-kb-size genomic fragments.

FIGURE 6. Epigenetic control of MMP-7, MMP-20, MMP-27, MMP-8, MMP-10, MMP-1, MMP-3, MMP-12, and MMP-13 from the 430-kb-long 11q22.3 region. The solid line represents the median IP/WCE ratio of the respective mark across the array. Enrichment of the mark is above the line. The data are presented in a log2 format. The gene promoters are schematically shown at the bottom of the panels. U251 and MCF-7 cells are in blue and red, respectively. The arrows show the position of the respective gene in the cluster and the direction of transcription, respectively.
others (Ref. 40 and ONCOMINE) and that epigenetic parameters play a significant role in stimulating the expression of several collagen types in glioma cells.

Because the levels of collagen are exceedingly low in the normal brain, it is tempting to hypothesize that the synthesis and then deposition of the collagen-enriched matrix by glioma cells contributes to the rapid spread of the infiltrating malignant cells through the brain tissue. To test this hypothesis, we have established and analyzed the orthotopic human glioma xenografts in the mouse brain.

**Collagen Immunoreactivity of the Orthotopic Human Glioma Xenografts in the Mouse Brain**—The U251 orthotopic glioma xenograft model was established in the SCID (P0, embryo) mouse brain. The presence of human U251 gliomas in the mouse brain was unambiguously identified using the glial fibrillary acidic protein (an astrocyte marker), βIII-tubulin (an early neuronal marker), human nucleobindin 1 (a ubiquitous Ca²⁺-binding protein), and Ki67 (a cell cycle-related nuclear protein and an active proliferation marker) antibodies. The immunostaining data demonstrated the rapidly growing loci of human U251 glioma tumor in the mouse brain. These multiple tumor loci in the brain resulted because of the spread of U251 cells from the injection site (cerebral ventricles) to the multiple locations in the mouse brain. Immunostaining of the brain sections with the type I collagen antibody revealed immunopositivity of the U251 tumor loci. The intact regions of the brain were clearly negative (Fig. 9C). These results agree well with our epigenetic profiling and RT-PCR results. The staining also demonstrated the presence of the collagen immunopositivity in the adjacent regions of the brain, which were outside of the tumor. It appears that in addition to producing collagen themselves, U251 cells stimulated the synthesis of collagen by the neural murine brain cells (human nucleobindin 1-negative), which were in a close proximity to tumor loci.

**Expression of Collagens in GBM Samples**—To test if collagens are synthesized by glioma cells, we measured the expression of selected collagen genes in cultured U251 cells, non-orthotopic glioma xenografts in mice, and GBM grade IV specimens from several cancer patients (Fig. 10). Our quantitative-PCR analysis clearly demonstrated the high level of synthesis of collagens by glioma samples, thus supporting our gene microarray and Chip-on-Chip results and also our observations in the orthotopic glioma xenografts (Figs. 1A and 9).
Our data are consistent with the results of others. Thus, the microarray data of the ONCOMINE data base show that the genes coding for the individual collagen chains are within the top up-regulated genes in gliomas/GBMs as compared with the normal brain. The colored clustergram shows the expression pattern of the several representative collagen types in 23 human normal brain samples and 81 GBM specimens that are included in the ONCOMINE data base, Sun brain data subset (40) (Fig. 10B). Overall, the data clearly indicate that GBMs produce high levels of collagen species in the human brain.

### miRNA Profiling in U251 and MCF-7 Cells
To determine the potential role of miRNAs in the epigenetic control, we also performed global miRNA profiling in U251 and MCF-7 cells. For this purpose, we used the human miRNA microarray Version 2 (Agilent) containing 723 human and 76 human viral miRNAs from the Sanger data base Version 10.1. To obtain statistically significant data, hybridization was performed in duplicate using two microarrays for U251 and MCF-7 cells samples each, and only the data with \( p < 0.05 \) were analyzed further (supplemental Table S5). We identified the miRNA species, which were dominant in both cell lines including hsa-miR-24, -27a, -23a, -923, -16, -21, -156, -107, -103, -106b, -25, -425, and -93 and hsa-let-7a,b,d,f,i. Hsa-miR-21 was the most dominant in both cells. The total number of the expressed miRNA species was ~3-fold higher in U251 cells relative to MCF-7 cells. More than 50 miRNA species were expressed exclusively in U251 cells, whereas only 17 miRNAs were expressed in MCF-7 cells alone (Fig. 11 and supplemental Fig. S3 and Table S5) suggesting, as a result, a significant role the individual miRNAs play in the epigenetic control in gliomas.

### DISCUSSION
Our goal was to gain an additional knowledge of the epigenetic modifications that are involved in the regulation of the MMP functionality in cancer cell migration and invasion. For this purpose we determined the epigenetic signature of the selected 486 genes and the 799 individual miRNAs in genetically unrelated highly migratory U251 glioma and non-migratory MCF-7 breast carcinoma cancer cell lines. The selected genes included MMPs themselves and also the genes that, according to the data mining in the bioinformatics databases, are linked to the MMP functionality and cell migration. Overall, our results suggest that epigenetic parameters play an important role in the regulation of MMPs and multiple other genes involved in cancer cell locomotion, thus providing...
a foundation on which novel diagnostic tools and selective epigenetic drugs can be designed.

We determined that the active chromatin signature including high levels of POL2, hyperacetylation of histone H3, and the loss of the repressive H3K27me3 tri-methylation modification are a common feature of the promoter regions of the transcriptionally active pro-invasive genes in both MCF-7 and U251 cells. Conversely, hypoacetylation and high levels of H3K27me3 are the features of the transcriptionally silenced pro-invasive genes.

Global hyperacetylation of migration-associated genes explains why histone deacetylase inhibitors were inefficient in repressing migration and invasion of cancer cells. Based on our data, it becomes clear that inhibition of histone deacetylase activity will not result in transcriptional silencing of the pro-migratory genes including MMPs in cancer cells.

Inactive pro-invasive genes in MCF-7 cells but not in U251 cells frequently exhibited a stem cell-like bivalent mark (enrichment in both H3K27me3 and H3K4me2 epigenetic marks). Previously, this stem cell-like epigenetic signature was reported only for the developmental genes in stem cells and also in T cells in human (16, 17).

Transcriptional inactivation of anti-tumorigenic MMP-8 highlights the importance of epigenetic parameters in cancer. According to the results of others (41, 42), wild-type MMP-8 is a tumor protective factor. MMP-8 has the ability to inhibit melanoma progression and to reduce the metastatic potential of breast and lung cancer cells in both mice and humans. Based on our data, it is now highly likely that the epigenetic suppression, in addition to somatic mutations (42) is the primary mechanism that reduces the functional activity of MMP-8 in malignancy including genetically unrelated cancer types.

Furthermore, epigenetic profiling and the follow-on study allowed us to detect high levels of the expression of the genes coding for the individual collagen chains in glioma U251 cells. Collagens are the essential components of the ECM. Transcriptional activation of these collagen genes is clearly epigenetically controlled by hyperacetylation and H3K4me2 (trimethylated Lys-4 of histone H3) deposition in U251 cells. In MCF-7 cells, these collagen
genes are epigenetically silenced by the H3K27me3 repression, and as a result, they are transcriptionally inactive.

To corroborate these results, we established the orthotopic glioma tumor xenografts in the brain of the newborn immunodeficient mice. We then confirmed the presence of the significant level of type I collagen immunoreactivity in the multiple glioma loci we observed in the affected mouse brain. In contrast, the non-affected brain regions were clearly collagen-negative because normally there is no collagen in the brain (except that in the blood vessels and capillaries).

Intriguingly, normal neural brain cells, which were in the close proximity to the glioma loci, frequently exhibited a level of the collagen immunostaining. It is likely that glioma cells not only deposit collagens themselves but also stimulate collagen synthesis by the tumor microenvironment, potentially, through the TGFβ-SMAD pathway (43–46). In agreement with this suggestion, we recorded the abundant expression of TGFβ2 (Fig. 1A) and TGFβ3 (not shown) in U251 cells. The transcriptional activity of the collagen genes in U251 cells also correlated with the significant expression level of TGFBI, the gene that encodes an RGD-containing adhesion protein that binds to type I, II, and IV collagens and that plays an important role in cell-collagen interactions (47, 48). It appears that our data provide a mechanistic rationale for the on-going clinical trials of the selective TGFβ2 inhibitor AP 12009 (trabedersen) which targets mRNA encoding TGFβ2 in GBM patients (49).

The epigenetic stimulation leading to the enhanced expression of the collagen genes we recorded in cultured U251 cells was then directly confirmed using glioma specimens from cancer patients. Furthermore, the analysis of the microarray data available from the ONCOMINE data base also supports the elevated expression of collagens in GBMs, especially if compared with the exceed...

**FIGURE 10.** Expression of collagen genes in glioma samples. A, the quantitative-PCR analysis was used to measure the levels of mRNAs coding for selected collagens (COL11A1, COL7A1, COL8A1, COL5A1 and COL1A1) in U251 cells, U251 non-orthotopic xenograft in mice, and several GBM grade IV specimens. For simplicity, only the patient sample 2394 is shown in the panel. GAPDH was used as a control. Each sample was analyzed in triplicate. Relative mean cycle threshold (ΔCt) values are shown. B, the microarray data was derived from the ONCOMINE databases, Sun brain data subset. The subset includes 23 human normal brain samples and 81 GBM specimens. Red and blue colors denote high and low levels of expression.

**FIGURE 11.** MiRNA profiling in U251 and MCF-7 cells. The results are shown as a Venn diagram. U251 and MCF-7 cells are in blue and red, respectively. Numbers are the individual miRNA species that were expressed only by MCF-7 cells (red circle), by U251 cells (blue circle), and by both cell types (overlap). The identity of the individual miRNAs is shown in the respective panels. Only miRNA expressed in either or both cell lines and p < 0.05 are shown. The results of the profiling are summarized in supplemental Table S5 and Fig. S3.
ingly low levels of collagen in the normal brain. On a similar note, infiltrating GBM cells in peritumoral brain parenchyma express the elevated levels of fibronectin, an additional critical component of the remodeled ECM (50).

Excitingly, while our manuscript was under review, others also reported that fibrillar collagens were extensively deposited in GBMs, that the collagen internalization receptor Endo180/CD280/MRC2/UPARAP is both highly expressed in GBMs, and that it mediates the invasion of brain cancer cells through collagen-containing matrices (51). Intriguingly, Endo180 is also a regulator of MT1-MMP activity (52), which is a potent collagenase (53). Taking together, these studies suggest that glioma-derived collagen is an important constituent of the tumor microenvironment in the brain and that the synthesis and the deposition of ECM collagens play a role in facilitating glioma cell migration through the brain tissues. The experiments to link the ECM components including collagens to the rapid migration of infiltrating glioma cells in the brain in vivo are now in progress.

Our miRNA profiling confirmed a role miRNAs play in the regulation of cell function including migration. Multiple individual miRNA species that we recorded in U251 cells such as miRNA7, miRNA21, miRNA128, and miRNA221/222 have already been shown to play significant role in gliomas (54–60). Conversely, the individual miRNAs from a miRNA-200 family, which according to the results of our profiling studies were uniquely up-regulated in MCF-7 cells, have previously been reported to repress cell invasion by up-regulating E-cadherin (61). Based on these results, we believe that in addition to the conventional DNA methylation and histone post-translational modifications (25), glioma cells employ the miRNA mechanisms to impose an extra level of epigenetic control in cell migration processes.

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