

Primary Glioblastomas Express Mesenchymal Stem-Like Properties

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Abstract

Glioblastoma is the most common and aggressive primary brain cancer. Recent isolation and characterization of brain tumor-initiating cells supports the concept that transformed neural stem cells may seed glioblastoma. We previously identified a wide array of mesenchymal tissue transcripts overexpressed in a broad set of primary glioblastoma (*de novo*) tumors but not in secondary glioblastoma (derived from lower-grade) tumors, low-grade astrocytomas, or normal brain tissues. Here, we extend this observation and show that a subset of primary glioblastoma tumors and their derived tumor lines express cellular and molecular markers that are associated with mesenchymal stem cells (MSC) and that glioblastoma cell cultures can be induced to differentiate into multiple mesenchymal lineage-like cell types. These findings suggest either that a subset of primary glioblastomas derive from transformed stem cells containing MSC-like properties and retain partial phenotypic aspects of a MSC nature in tumors or that glioblastomas activate a series of genes that result in mesenchymal properties of the cancer cells to effect sustained tumor growth and malignant progression. (Mol Cancer Res 2006;4(9):607–19)

Introduction

Glioblastoma (WHO, grade IV) is the most common and aggressive form of adult brain tumor (1) and can derive either *de novo* with no prior evidence of a lower-grade tumor, called primary glioblastoma, or through malignant progression from a lower-grade malignancy, called secondary glioblastoma.

Genetic studies suggest that primary and secondary glioblastomas are two distinct clinical entities, each developing along distinct genetic pathways with characteristic genomic losses. Whereas secondary glioblastomas clearly develop slowly from within lower-grade astrocytoma and frequently contain mutations in the *p53* gene (~60%), primary glioblastomas develop rapidly and in general are thought to arise from astrocytic precursors and are genetically characterized by amplification and/or overexpression of epidermal growth factor receptor (EGFR; ~60%; refs. 1-3). The cell of origin of glioblastomas has been recently considered to be of the neural stem cell (NSC) lineage and this concept is supported by the recent experimental data generated from multiple laboratories showing that only CD133⁺ (a cell surface marker of normal human NSC) but not CD133⁻ cells isolated from glioblastoma tumors contain tumor-initiating cells (4-7). Additionally, these cells are multipotent in culture and can be induced to differentiate into neuronal, astroglial, or oligodendroglial cells. Therefore, glioblastoma may be a NSC disorder or transformed cells reactivate NSC properties (8, 9). For many years, it has been appreciated that, during *in vitro* cultivation, glioblastoma cells can express a variety of heterogeneous markers, including multiple mesenchymal markers, and were generally thought to be due to mesenchymal drift or cultures are overgrown by mesenchymal cells from the abnormal vascular proliferations of the original glioma (10, 11). Subsequently, a study showing that permanent glioblastoma cell lines formed tumors in nude mice failed to sustain evidence of glial differentiation but exhibited the characteristics of mesenchymal differentiation both *in vitro* and *in vivo* (12). Correspondingly, our recent study using whole genome gene expression analyses of 85 high-grade glial tumors also identified a subset of glioblastoma tumors overexpressing transcripts of extracellular matrix (ECM) components, which correlates with a set of genes typically expressed in mesenchyme-derived tissues (13). Follow-up studies further identified that a series of mesenchymal-associated transcripts that partially overlapped with previous study are only overexpressed in a subset of primary glioblastomas but not in secondary glioblastoma, low-grade astrocytoma, or normal brain (14). These observations thus support the possibility of a fundamentally distinct mechanism of oncogenesis and progression in at least a subset of primary glioblastomas.

Recent studies suggest that stem cell plasticity is a characteristic of NSC and that the differentiation of NSC into unanticipated cell types, including hematopoietic, muscle,

Received 1/9/06; revised 6/21/06; accepted 6/26/06.

Grant support: National Cancer Institute grant U01CA88173, Accelerate Brain Cancer Cure, the Henry Singleton Brain Tumor Program, and UCLA Jonsson Comprehensive Cancer Center seed grant.

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (<http://mcr.aacrjournals.org/>).

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doi:10.1158/1541-7786.MCR-06-0005

Table 1. Top 100 Primary Glioblastoma-Associated Genes Expressed at Higher Levels Compared with Normal Brain Tissue

Gene	Symbol	Fold change	P	Gene	Symbol	Fold change	P
Collagen type III, $\alpha 1$	COL3A1	18.47	0.000237	Sushi-repeat-containing protein, X chromosome	SRPX	4.16	0
Carbonic anhydrase III, muscle specific	CA3	13.66	0.006805	Vascular endothelial growth factor	VEGF	4.15	0.000009
Insulin-like growth factor binding protein 2, 36 kDa	IGFBP2	10.79	0	Chondroitin sulfate proteoglycan 2 (versican)	CSPG2	4.15	0
Coagulation factor II (thrombin) receptor	F2R	9.08	0	Insulin-like growth factor-binding protein 3	IGFBP3	4.1	0.000053
Osteoblast-specific factor 2 (fasciclin I-like)	POSTN	8.65	0.000528	KDEL endoplasmic reticulum protein retention receptor 2	KDELRL2	4.07	0
T-lymphokine-activated killer cell cell-originated protein kinase	PBK	7.42	0	Insulin-like growth factor-binding protein 5	IGFBP5	4.06	0
Epidermal growth factor receptor	EGFR	7.04	0.000005	MHC class II, DR α	HLA-DRA	4.04	0
Transforming growth factor- β induced, 68 kDa	TGFB1	6.81	0.000002	Macrophage scavenger receptor 1	MSR1	4.04	0
Tenascin C (hexabrachion)	TNC	6.8	0	CD99 antigen	CD99	4	0
Collagen type IV, $\alpha 1$	COL4A1	6.78	0	MHC class II, DR $\beta 4$	HLA-DRB4	3.99	0
Tissue inhibitor of matrix metalloproteinase 1	TIMP1	6.5	0	MHC class II, DR $\beta 1$	HLA-DRB1	3.98	0
Annexin A1	ANXA1	6.16	0	Chemokine (C-X-C motif) receptor 4	CXCR4	3.98	0.000001
Centromere protein F, 350/400 kDa (mitosin)	CENPF	6.15	0.000005	Caveolin 1, caveolae protein, 22 kDa	CAV1	3.94	0.000002
Sex-determining region Y (SRY)-box 11	SOX11	5.99	0.000057	Protein S (α)	PROS1	3.93	0
Collagen type I, $\alpha 2$	COL1A2	5.85	0.000301	Thymidylate synthetase	TYMS	3.89	0
Fibronectin 1	FN1	5.84	0	Serine (or cysteine) proteinase inhibitor, clade A, member 3	SERPINA3	3.84	0
Vascular cell adhesion molecule 1	VCAM1	5.57	0.000003	Complement component 5 receptor 1 (C5a ligand)	C5R1	3.82	0.000083
Collagen type IV, $\alpha 2$	COL4A2	5.36	0	Regulator of G-protein signaling 1	RGS1	3.8	0.000157
Chromosome 8 open reading frame 4	C8orf4	5.32	0.000196	MHC class II, DR α	HLA-DRA	3.77	0
FLJ20933 fis, clone ADSE01388		5.3	0.001651	Peroxiredoxin 4	PRDX4	3.76	0
Chemokine (C-C motif) ligand 3	CCL3	5.29	0.000347	Chitinase 3-like 1 (cartilage glycoprotein-39)	CHI3L1	3.76	0.000005
Epithelial membrane protein 1	EMP1	5.27	0	ESTs, weakly similar to cytokine receptor-like factor 2		3.75	0
Sex-determining region Y (SRY)-box 4	SOX4	5.27	0	MHC class II, DR $\beta 6$	HLA-DRB6	3.75	0
Collagen type VI, $\alpha 2$	COL6A2	5.2	0.002623	KIAA0101 gene product	KIAA0101	3.74	0
Hypothetical protein FLJ23468	MLF1IP	5.17	0	Integrin, $\beta 2$ [antigen CD18 (p95)]	ITGB2	3.74	0
Matrix G1a protein	MGP	5.04	0.000002	Interleukin-8	IL8	3.73	0.002226
CD163 antigen	CD163	5.03	0.000009	Matrix metalloproteinase-9	MMP9	3.73	0.004374
Stabilin 1	STAB1	4.93	0	Pre-B-cell colony-enhancing factor	PBEF1	3.73	0.000045
Complement component 1, R subcomponent	C1R	4.88	0	Complement component 3	C3	3.72	0
Laminin, $\beta 1$	LAMB1	4.87	0.000052	MHC class II, DQ $\beta 1$	HLA-DQB1	3.7	0
Annexin A2	ANXA2	4.69	0	EGF-containing fibulin-like extracellular matrix protein 1	EFEMP1	3.69	0.000002
CD14 antigen	CD14	4.67	0	MHC class II, DQ $\alpha 1$	HLA-DQA1	3.69	0.000016
CD44 antigen	CD44	4.61	0	Vesicle-associated membrane protein 8 (endobrevin)	VAMP8	3.67	0
Ribosomal protein, large P2	RPLP2	4.58	0	v-maf musculoaponeurotic fibrosarcoma oncogene homologue B	MAFB	3.67	0
Secreted protein acidic and rich in cysteine (osteonectin)	SPARC	4.56	0	Hematopoietic cell-specific Lyn substrate 1	HCLS1	3.65	0
Cysteine and glycine-rich protein 2	CSRP2	4.53	0	Complement component 1, Q subcomponent, β polypeptide	C1QB	3.64	0
Thymosin, β , identified in neuroblastoma cells	TMSL8	4.49	0.000158	Neuromedin B	NMB	3.64	0
Nucleolar protein ANKT	NUSAP1	4.47	0	MHC class II, DM α	HLA-DMA	3.63	0
Epithelial membrane protein 3	EMP3	4.44	0	Glycoprotein (transmembrane) nmb	GPNMB	3.62	0
Sex-determining region Y (SRY)-box 2	SOX2	4.31	0	Zinc finger protein 36, C3H type-like 2	ZFP36L2	3.61	0
MHC class II, DP $\beta 1$	HLA-DPB1	4.3	0	Sec61 γ	SEC61G	3.6	0
Growth differentiation factor 8	GDF8	4.29	0.004637	CD24 antigen (small cell lung carcinoma cluster 4 antigen)	CD24	3.59	0.000115
Nestin	NES	4.28	0	δ -like 3 (Drosophila)	DLL3	3.58	0.00054
Hypothetical protein FLJ20716	FAM70A	4.28	0	High-mobility group box 2	HMGB2	3.57	0
Lung type I cell membrane-associated glycoprotein	PDPN	4.28	0	Inhibitor of DNA binding 3	ID3	3.56	0

(Continued on the following page)

Table 1. Top 100 Primary Glioblastoma-Associated Genes Expressed at Higher Levels Compared with Normal Brain Tissue (Cont'd)

Gene	Symbol	Fold change	P	Gene	Symbol	Fold change	P
Complement component 1, q subcomponent, receptor 1	CIQR1	4.23	0	Transcriptional coactivator with PDZ-binding motif (TAZ)	WWTR1	3.55	0
Apolipoprotein C-1	APOC1	4.22	0	Heme oxygenase (decycling) 1	HMOX1	3.55	0.000044
RNA-binding motif protein 3	RBM4	4.2	0	Fatty acid-binding protein 7, brain	FABP7	3.53	0.000002
Chondroitin sulfate proteoglycan BEHAB/brevican	BCAN	4.2	0	Connective tissue growth factor	CTGF	3.53	0.000014
SMC4 structural maintenance of chromosomes 4-like 1	SMC4L1	4.17	0	Retinitis pigmentosa 2 (X-linked recessive)	RP2	3.51	0

NOTE: Analysis was based on a cutoff of a 2.5-fold increase in relative expression ($P < 0.05$) in primary glioblastoma compared with normal brain tissue.

and endothelial cells (15-17), has been shown. Likewise, mesenchymal stem cells (MSC), which can give rise to various cell types of mesenchymal tissues, such as bone, cartilage, adipose, and muscle (18), could differentiate into astrocytes and neurons (19, 20). A microarray analysis of a single MSC further explored a panel of lineage-related transcripts associated with osteoblast, fibroblast, muscle, adipocyte, epithelial cells, endothelial cells, and neural/glial cells (21-25). Recently, transcription profiling has uncovered an overlapping genetic program in various types of stem cells (26) and therefore supports the concept that somatic stem cells have a broad differentiation repertoire. In the present study, we show that primary glioblastoma tumors and their passaged tumor cell lines express cellular and molecular characteristics of MSC. Further, when treated with adipogenic, osteogenic, or chondrogenic induction medium, primary glioblastoma cell lines are capable of differentiating into mesenchymal lineage cell types, thus implying that a subset of primary glioblastomas may use mesenchymal properties to attain their malignant phenotype.

Results

Primary Glioblastoma Biopsies Express Mesenchymal and NSC-Associated Genes

To identify gain-of-function genes associated with acquisition of malignant features of glioblastoma, we did a large-scale DNA microarray analysis of 101 glial brain tumors (14). Through a series of comparative analyses relative to lower-grade astrocytomas, we have identified a list of primary glioblastoma-associated genes that are distinct from being identified in progression from a lower grade (secondary glioblastoma). A series of genes that are highly expressed in mesenchymal tissues but not in neural or glial cells were identified, including bone, cartilage, tendon, ligament, fat, and muscle (e.g., *YKL-40*, *lysyl oxidase*, *PDPN*, *collagen type V* and *VI*, *biglycan*, *mesenchyme homeobox 2*, *fatty acid-binding protein*, *CA3*, and *tangelin*; ref. 14). To access and capture genes that are potentially associated with cellular properties and tumorigenesis of primary glioblastoma, a comparative analysis of primary glioblastoma ($n = 46$) relative to normal brain tissue

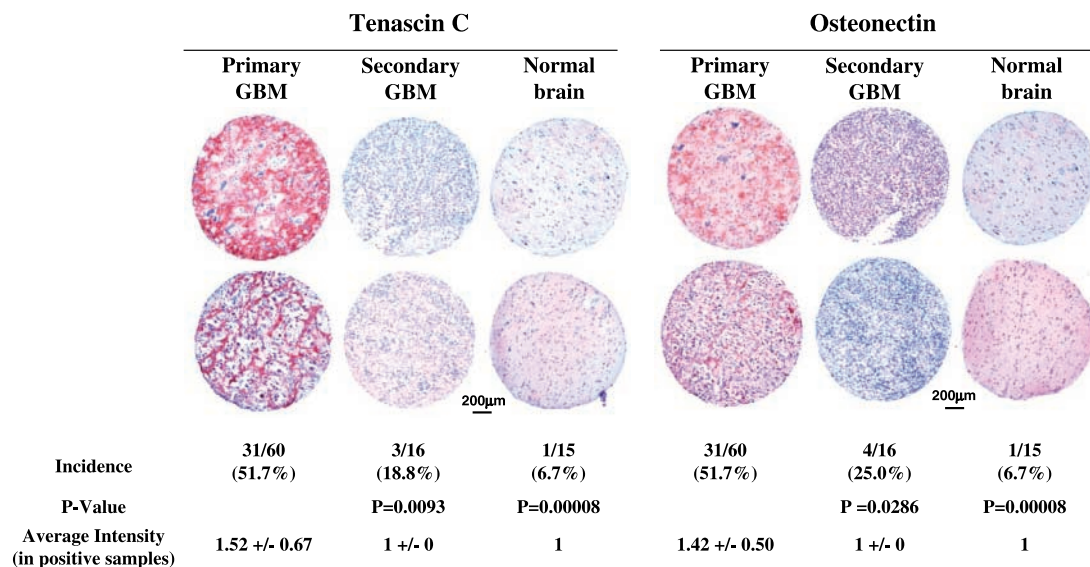
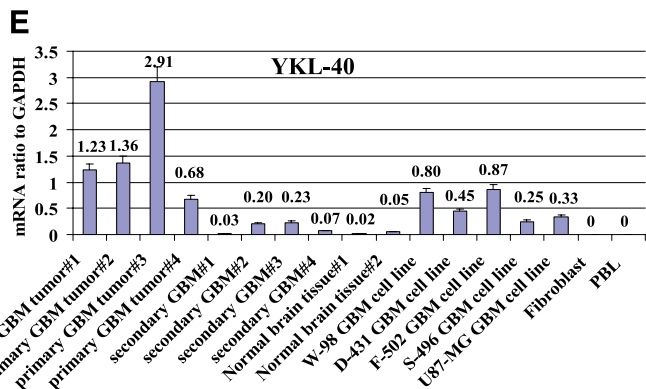
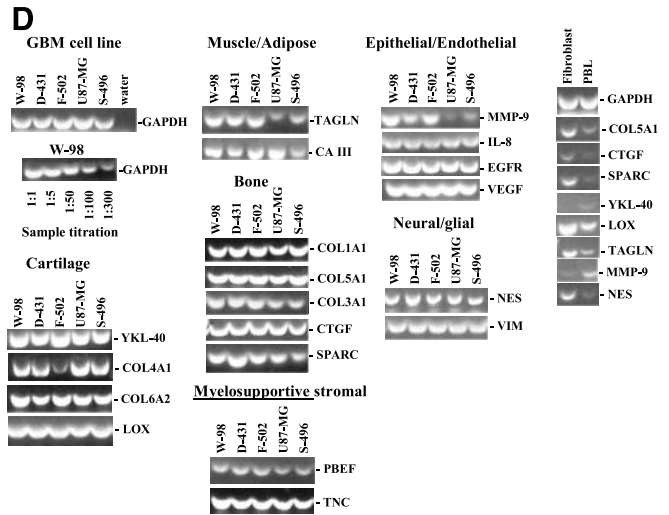
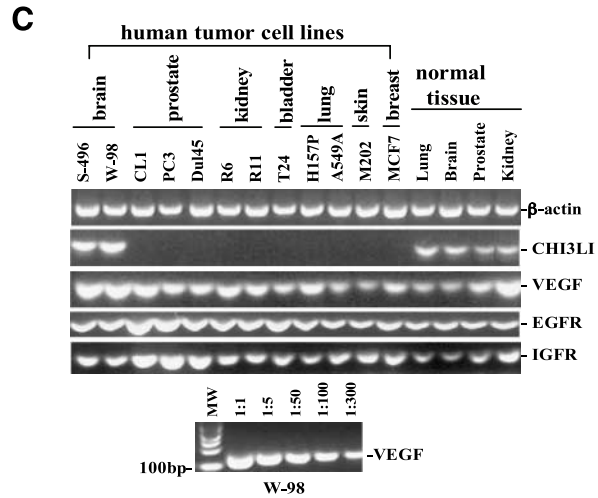
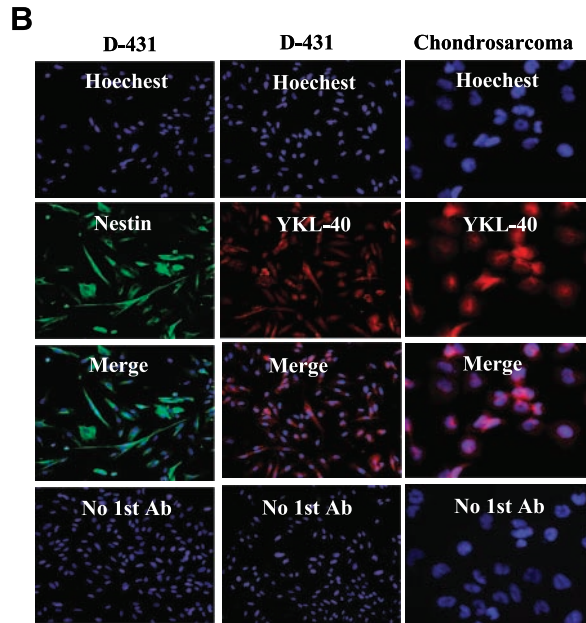
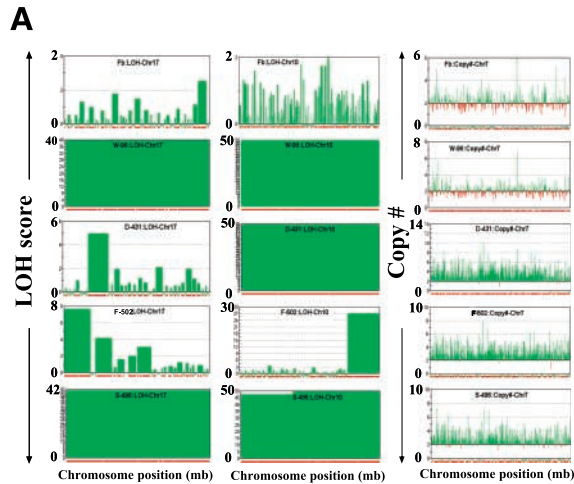


FIGURE 1. Primary glioblastoma tumors express mesenchymal phenotype. Representative immunohistochemical staining of TNC and osteonectin in clinical glioblastoma (GBM) subgroups and normal brain. Subsets of the images from a tissue array. Each core is 0.06 mm across. Strong positivity of TNC and osteonectin staining was detected in two primary glioblastoma. Negative/weak staining was detected in two secondary glioblastoma and two normal brain cores. The incidence of the expressions of two markers was significantly higher in primary glioblastoma compared with secondary glioblastoma and normal brain tissue, and the average intensity of staining in positive samples was higher in primary glioblastoma when compared with secondary glioblastoma and normal brain cores. Staining intensity was scored on a scale of 0 to 2 as described in Materials and Methods. Magnification, $\times 400$.

($n = 10$) was done. Probe set signals on the U133A array that were ≥ 2.5 -fold in primary glioblastoma versus the normal brain tissue with a pairwise t test with a $P < 0.05$ were selected and the top 100 genes were presented in Table 1 (see Supplementary Table S1 for more detailed description). As anticipated, genes that were identified previously as primary glioblastoma progression-associated genes reappeared in this gene list, which reflected the status of inflammation, coagulation, immune/

complement responses, angiogenesis, and ECM remodeling (14), whereas a new series of genes that are associated with MSC, MSC differentiation, skeletal/cartilage development, morphogenesis, organogenesis, and embryonic neuroepithelial stem cells (dChip gene information) were identified (Table 1; refs. 21-25). Particularly, several mesenchyme-specific genes, normally associated with osteoblasts and osteoblastic differentiation, are highly expressed, including *POSTN* (27), *TNC*



(28, 29), *SPARC/osteonectin* (30), *collagen type I/III* (31), *CTGF* (32), *ANXA2* (33), *IGFBP3/IGFBP5* (34, 35), *osteostatin* (*GPNMB*; ref. 36), *CSPG2* (28), and *TAZ* (*WWTR1*; ref. 37). The coexpression of TNC, SPARC, collagens, osteostatin, and ANX2 suggests the presence of a molecular program of epithelial-mesenchymal transition (EMT), which is frequently seen in malignant tumor with a highly invasive phenotype (38-40). TAZ was recently identified as a transcriptional modulator of MSC differentiation that promotes the development of the osteoblastic component and represses peroxisome proliferator-activated receptor- γ -dependent adipogenesis (37). Moreover, myostatin (GDF8), IGFBP3/IGFBP5, and ID3 also have been suggested to play a role in the suppression of myogenesis and adipogenesis (41-45). Simultaneously, several NSC-associated genes were overexpressed in primary glioblastoma tumors, including *NES*, *SOX2*, *SOX4*, *SOX11*, *CXCR*, and *DLL3*. The overexpression of *NES* transcript reflected the characteristic of neuroepithelial tumor cells. *SOX2* is expressed in embryonic neural epithelial stem cells (46) and is essential to maintain pluripotent, self-renewal, and undifferentiated phenotypes of embryonic stem cells (47). *SOX4* and *SOX11* play a role in central nervous system development (48), and *SOX4* gene recently was characterized as a component of the parathyroid hormone signal transduction pathway in osteoblastic cells and has an important function in bone formation (49). The chemokine receptor *CXCR4* has been regarded as a MSC and NSC receptor that mediates MSC-specific migration to bone marrow and NSC migration to injury sites in the central nervous system (50, 51). *CXCR4*-stromal cell-derived factor-1 pathway was suggested to play an important role in promoting glioma survival, proliferation, and migration (52, 53). *DLL3* has been shown to function as an inhibitor of Notch signaling that inhibited glial differentiation of mouse neural progenitors (54). The distinctive gene expression profile in a set of glioblastoma and normal brain tissue samples was verified previously by a real-time quantitative reverse transcription-PCR (RT-PCR) analysis (14). This transcription profile of primary glioblastoma is therefore indicative of NSC- and MSC-like properties in glioblastoma tumors.

It is possible that the mesenchymal gene expression detected was reflecting a mixture of normal stromal cells within the tumor. To assess this possibility, a tissue microarray consisting

of tumor cores and matching normal brain counterparts from 60 primary glioblastomas and 16 secondary glioblastomas was constructed to allow visualization of cancer cells distinct from the supporting normal tissue. The detection of YKL-40/cartilage glycoprotein-39 (cartilage/chondrocyte-associated gene) overexpressed in clinically defined primary glioblastoma cells but not in secondary glioblastoma cells was published previously (14). Here, we have further tested two other MSC-associated markers, TNC (myelosupportive stromal-associated gene) and SPARC/osteonectin (skeletal development-associated gene), which were identified previously in glioblastoma tumors. Our results showed that both markers were overexpressed in the primary glioblastoma samples, and the incidence as well as expression level were significantly higher in primary glioblastoma than in secondary glioblastoma and normal brain (Fig. 1). Although TNC and osteonectin are expressed in glioblastoma tumors, our analysis pointed out that TNC and osteonectin are significantly overexpressed in primary glioblastoma (mesenchymal phenotype) compared with secondary glioblastoma (proliferation/cell cycle phenotype). The mesenchymal gene expression was detected broadly throughout the population of malignant cells and ECM showing cellular atypia.

Primary Glioblastoma Biopsy-Derived Tumor Lines Express Molecular Signatures of MSC

To examine whether MSC and mesenchymal lineage cell type-associated transcripts detected in primary glioblastoma biopsies are expressed in glioblastoma-derived cells, four permanent glioblastoma biopsy-derived glioblastoma cell lines (W-98, D-431, F-502, and S-496) were selected for molecular characterization. To rule out the possibility that glioblastoma cell lines are stromal cells derived from angiogenic tumors, we determined the genomic and molecular identities of cell lines. All four cell lines carry chromosomal abnormalities commonly detected in glioblastomas using a high-density single nucleotide polymorphism array analysis (Fig. 2A). Notably, D-431 and F-502 exhibit loss of heterozygosity (LOH) in chromosome 10 but not in chromosome 17, whereas W-98 and S-496 exhibit LOH in both chromosomes. Additionally, amplification of chromosome 7 was detected in D-431, F-502, and, lesser degree, S-496. Frequent LOH for chromosome 10 and gain of

FIGURE 2. Glioblastoma biopsy-derived glioblastoma cell lines express genomic, cellular, and molecular properties indicative of mesenchymal phenotype. **A.** Genomic abnormalities that are associated with mesenchymal phenotype of glioblastoma were detected in glioblastoma cell lines. Whole genomic DNA of glioblastoma cell lines (W-98, D-431, F-502, and S-496) and the fibroblast cell line were evaluated for allelic imbalances and chromosomal copy number abnormalities by using a high-density single nucleotide polymorphism array analysis (Affymetrix XbaI 131 Array). X axis, length of chromosomes 17, 10, and 7; Y axis, score of the evidence of LOH on chromosome 10 or 17 or gain of gene copy number on chromosome 7. D-431 and F-502 exhibit LOH on chromosome 10 accompanied by gain of genes copy number on chromosome 7, whereas W-98 and S-496 exhibit LOH on chromosomes 10 and 17 and lesser degree of gain of chromosome 7. Fibroblast cell line had no evidence of genomic abnormalities. **B.** Coexpression of neural and mesenchymal-associated gene in glioblastoma cell lines. Expression of NES and YKL-40 was determined using immunocytochemical analysis. Rat chondrosarcoma cell lines served as positive control cells for YKL-40. Representative of four glioblastoma cell lines. Magnification, $\times 100$. **C.** Specific expression of YKL-40 in glioblastoma cell lines. Semiquantitative RT-PCR analysis was done to determine the expression of YKL-40, EGFR, insulin-like growth factor-I receptor (*IGFR*), and vascular endothelial growth factor (*VEGF*) in various types of human cancer cell lines. Overexpression of EGFR, vascular endothelial growth factor, and insulin-like growth factor-I receptor was detected in all tested human cancer cell lines, whereas only glioblastoma cell lines uniquely express YKL-40 in the same culture condition (10% serum), indicative of mesenchymal origin. **D.** Glioblastoma cell lines express multiple MSC-associated genes. Semiquantitative RT-PCR analysis was done to verify that MSC-associated genes identified in primary glioblastoma tumors (Table 1) are expressed in glioblastoma cell lines. Transcripts of multiple cell lineages in glioblastoma cell lines (W-98, D-431, F-502, U87-MG, and S-496), normal newborn foreskin fibroblasts, and peripheral blood lymphocytes (*PBL*) were analyzed. PCR products were grouped based on the tissue lineage. All determinations were done in duplicates. The full name of each labeled gene symbol is described in Table 1. **E.** Differential expression of mesenchymal lineage-associated gene in glioblastoma subtypes. Real-time quantitative RT-PCR analysis of YKL-40 in glioblastoma tumor subtypes, normal brain tissues, glioblastoma cell lines, normal fibroblast, and freshly peripheral blood lymphocytes was done. The quantitation of specific transcript in samples was calculated and expressed by the YKL-40 mRNA ratio to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

chromosome 7 have been linked to mesenchymal glioblastoma subtype (55, 56). To further verify the glioblastoma origin of tumor lines, immunocytochemical staining of YKL-40 and NES was done. Homogeneous coexpression of NES and YKL-40 in glioblastoma cells was shown in all glioblastoma cell lines (Fig. 2B). Transcripts of YKL-40 and NES were also detected in glioblastoma cell lines using semiquantitative RT-PCR amplification (Fig. 2C and D). Notably, glioblastoma cell lines overexpress EGFR, vascular endothelial growth factor, and insulin-like growth factor-I receptor as other types of tested human cancer cell lines, yet YKL-40 was only detected in glioblastoma cell lines among the tested cancer cell lines, suggesting the mesenchymal origin of glioblastoma (Fig. 2C). To test whether mesenchymal tissue- and MSC-associated genes identified in glioblastoma tumors (Table 1) are indeed expressed in glioblastoma cell lines, glioblastoma cell lines were subjected to RT-PCR analysis. The results indicated that glioblastoma cell lines simultaneously express a host of transcripts typically expressed in mature mesenchymal lineage cell types (Fig. 2D), including bone (collagen type I, III, and V, CTGF, and SPARC), cartilage/ligament/tendon (CHI3L1/YKL-40, collagen type IV and VI, and lysyl oxidase), muscle/adipose (transgelin and CA3), myelosupportive stromal tissue (PBEF1 and TNC), and endothelial/epithelial cells (IL8, MMP9, EGFR, and VEGF). Concurrently, transcripts of undifferentiated NSC, NES, and vimentin, were also detected in all glioblastoma cell lines. A weak signal of glial fibrillary acidic protein can only be detected in F-509 using higher number of PCR cycles (40 cycles; data not shown). Three of the four glioblastoma cell lines, derived from clinically defined primary glioblastomas, had similar transcription profiles. Notably, S-496, derived from a clinically defined secondary glioblastoma, had lower expression levels of several mesenchymal tissue-associated genes. U87-MG, a commonly studied glioblastoma cell line, also exhibited mesenchymal expression characteristics similar to those of the primary glioblastoma samples, whereas several of the genes tested for expression were barely detectable in fibroblast cells or peripheral blood lymphocytes. To verify that glioblastoma subtypes express a differential level of MSC-associated transcript, a real-time quantitative RT-PCR analysis of YKL-40 expression was done. A higher expression level of YKL-40 transcript was detected in both primary glioblastoma tumors and cell lines (W-98, D-431, and F-502) compared with secondary glioblastoma tumors, secondary glioblastoma cell line (S-495), and normal brain tissue. YKL-40 transcript was not detectable in both fibroblasts and peripheral blood lymphocytes.

Glioblastoma Cell Lines Expressed Surface Antigen Profile Similar to That of MSC

To test whether the glioblastoma cells express surface markers associated with MSC, we determined the surface expression of CD29 (integrin β_1 /fibronectin receptor), CD44 (stromal hyaluronic acid and osteopontin receptors), CD90 (Thy-1 cell surface antigen), and CD105 [endoglin; transforming growth factor- β 3 (TGF- β 3) receptor], four important surface markers that have been used partly for defining MSC (18, 25, 57), in glioblastoma cell lines. Using a four-color fluorescence-activated cell sorting analysis, the majority of the cell population in culture coexpressed all four antigens in all

three primary glioblastoma cell lines [W-98 (61.7%), D-431 (67.3%), and F-502 (86.5%)], whereas negligible CD105⁺ cells were detected in the secondary glioblastoma cell line (S-496; Fig. 3A). A lesser percentage of the long passaged U87-MG cells expressed CD90 (7.4%) and CD105 (24.8%), whereas purified peripheral blood lymphocytes had few positive cells for any of four markers. All glioblastoma cell lines are negative for CD14, CD34, and CD31 (data not shown). Other MSC-associated markers, including CD73, CD109, and CD140B mRNA, were also detected in all four glioblastoma cell lines using RT-PCR analysis (data not shown).

To determine whether the differential expression of CD105 detected between primary and secondary glioblastoma cell lines was also observed *in vivo*, tissue microarrays were stained with anti-CD105 antibody. The result indicated that CD105 was strongly expressed in a large fraction of malignant-appearing tumor cells in primary glioblastoma biopsies (19 of 45), whereas both secondary glioblastoma (0 of 16; $P = 0.000867$) and normal brain tissue (0 of 18; $P = 0.0004856$) were stained negatively for CD105. CD105 was strongly expressed by endothelial cells as expected.

Differentiation Plasticity of Glioblastoma Cells

As the both primary glioblastoma tumors and their derived cell cultures retained the molecular expression pattern suggestive of a MSC-like characteristics, we sought to functionally test whether glioblastoma cells possess stem-like plasticity. To prevent cell selection, the cell differentiation was tested in both cloned and noncloned cell cultures. Noncloned glioblastoma cells were treated with adipogenic, osteogenic, and chondrogenic induction medium and the cells were analyzed for the induction into the various cell types. Under the adipogenic treatment, three tested primary glioblastoma cell lines (W-98, D-431, and F-502) formed oil red-O-positive cells with cytoplasmic accumulation of lipid vacuoles and eccentric deviation of nucleus within cells (Fig. 4A). In contrast, the treated secondary glioblastoma cell line (S-496) only showed weak staining. The treated human foreskin fibroblasts and all nontreated glioblastoma cells were negative for oil red-O staining. Osteogenic differentiation of glioblastoma cell lines was evident with the detection of alkaline phosphatase-positive cells (Fig. 4B), which were observed in all three induced primary glioblastoma cell lines. F-502, which showed the strongest activity of alkaline phosphatase, also formed strong calcium deposits (Fig. 4B), which were stained with silver by von Kossa method, whereas only few deposits can be detected in the cultures of W-98 and D-431 (data not shown). In contrast, only a few alkaline phosphatase-positive cells were identified in S-496 cultures and fibroblast cultures. Chondrogenic differentiation of glioblastoma cells was induced in serum-free medium supplemented with TGF- β 3. Collagen type II-positive cells were detected in all three induced primary glioblastoma cell lines (Fig. 4C) by immunohistochemical staining. Unexpectedly, collagen type II-positive cells were also seen in the fibroblast culture. None of the nontreated cells was positive for collagen type II. The treated secondary glioblastoma cell line that lack of CD105 marker did not survive through the culture of chondrogenic treatment in the serum-free medium, suggesting that CD105 is indeed a

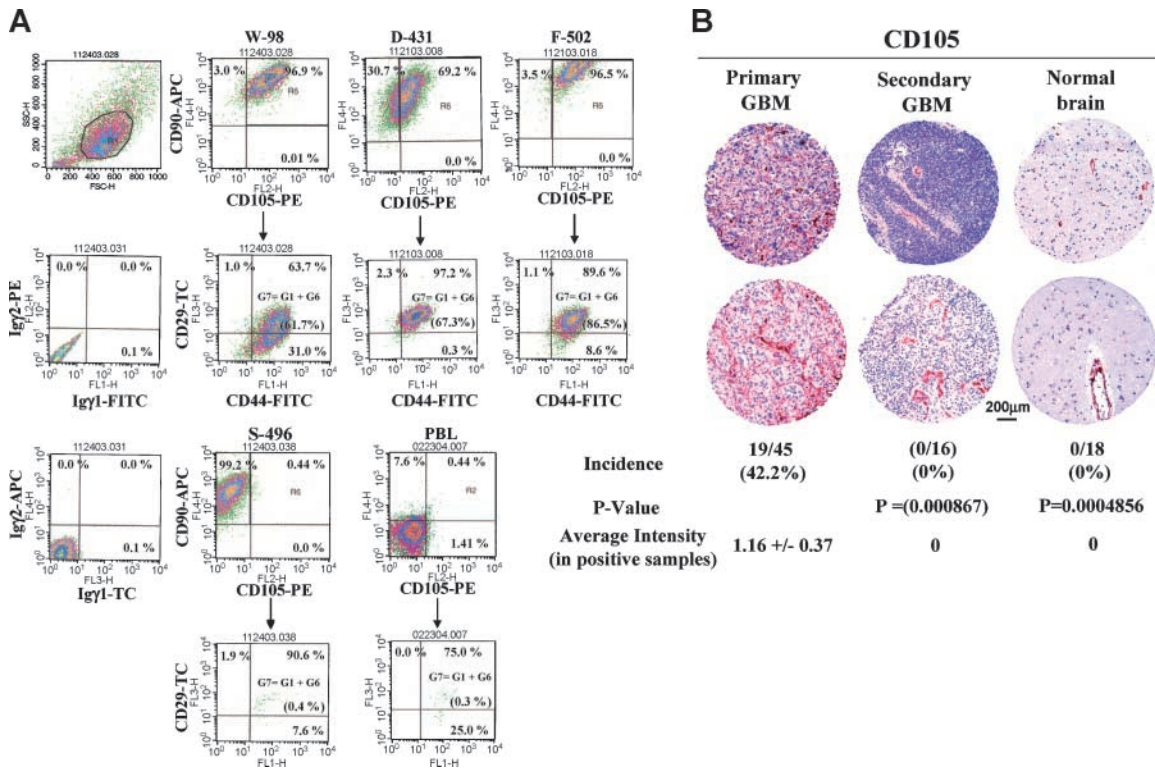


FIGURE 3. Expression of MSC-associated surface markers in glioblastoma cells. **A.** Coexpression of CD90, CD105, CD29, and CD44 in glioblastoma cell lines analyzed using four-color fluorescence-activated cell sorting analysis. The percentage of cells coexpressing four surface markers in each cell line was calculated by percentage of cells coexpressing CD90 and CD105 multiplied by percentage of cells coexpressing CD29 and CD44 in the cell population that are double positive for CD90 and CD105. **B.** Specific expression of CD105 in primary glioblastoma tumors. Representative immunohistochemical staining of CD105 in clinical glioblastoma subgroups and normal brain. A subset of the images from a tissue array. Strong positivity of CD105 antibody staining was detected in two primary glioblastomas. Negative staining was detected in two secondary glioblastomas and two normal brain cores. Endothelial cells were stained positively for CD105. The incidence of the expressions of CD105 in primary glioblastoma was 19 of 45, whereas no positive staining was detected in secondary glioblastoma (0 of 16) or normal brain tissue (0 of 18). Magnification, $\times 400$.

functional marker for MSC and that chondrogenesis is driven by TGF- β -CD105 signaling. The mesenchymal differentiation was further shown in the cloned and expanded glioblastoma cell lines as well as in bone marrow-derived stromal cells (data not shown). The transcription level of lineage-specific genes in glioblastoma cells was determined using a real-time quantitative RT-PCR assay. Although the lineage-specific protein cannot be detected in noninduced cells (Fig. 4D), low levels of baseline mRNA expression were detected using RT-PCR amplification. As such, the highest amount of lineage-specific mRNA level postinduction did not always correspond with the highest fold increase when compared with baseline level. When compared with nontreated cultures, markedly increased lineage-specific mRNA levels of adipocyte (fatty acid-binding protein 4), bone (alkaline phosphatase), and cartilage (collagen type II) were detected in glioblastoma cell cultures from primary tumors, which were treated with the corresponding agent. The transcripts of alkaline phosphatase were strongly induced (42-fold) in S-496 (secondary glioblastoma cell line). Cells with positive immunostaining of collagen type II were detected in a fibroblast cell culture treated with a chondrogenic medium. The quantitative RT-PCR, however, displayed lower induction of collagen type II mRNA expression when compared with glioblastoma cell lines.

Conversely, when these glioblastoma cell lines were cultured in the serum-free medium supplemented with EGF and fibroblast growth factor (NSC culture condition), they formed glioblastoma spheres (neurosphere-like) and could be passaged in sphere cultures that express CD133, NES, and SOX2.⁷ Moreover, these NSC-like glioblastoma spheres can be induced for full or partial differentiation into neuronal, oligodendrocytic, and astrocytic differentiation as described by others (4-7), suggesting that glioblastoma cells express stem-like properties and plasticity.

Discussion

Previously, we have used large-scale gene expression analysis to characterize primary and secondary glioblastomas. By comparing with low-grade astrocytoma, we have identified a series of glioblastoma-associated genes that could distinguish primary glioblastomas from the secondary glioblastomas (14). The comparison with lower-grade astrocytomas was done to attempt selecting for genes, which are specific to the end malignant transformation into the highly invasive glioblastomas. Secondary glioblastoma-associated genes primarily

⁷ Unpublished data.

include mitotic cell cycle components, suggesting loss of function in prominent cell cycle regulators, whereas primary glioblastoma-associated genes highlight genes expressed in mesenchyme-derived tissues and stromal response, suggesting

the augmentation of extracellular signaling operating the malignancy. To identify genes potentially link to cellular properties and tumorigenesis of primary glioblastoma, in this study, we compared primary glioblastoma with normal brain

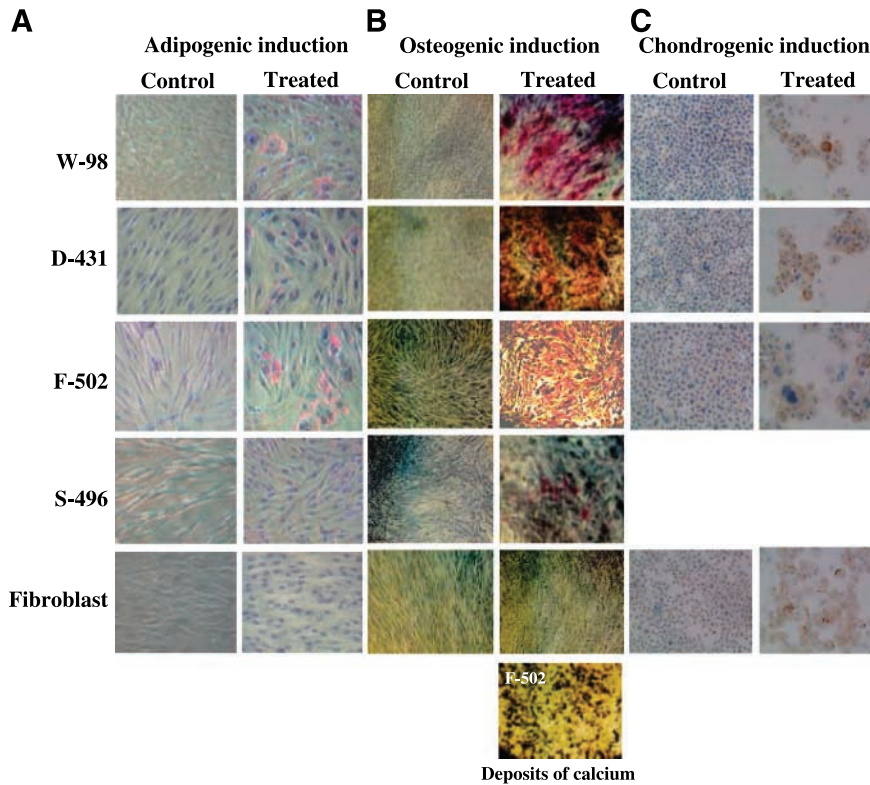
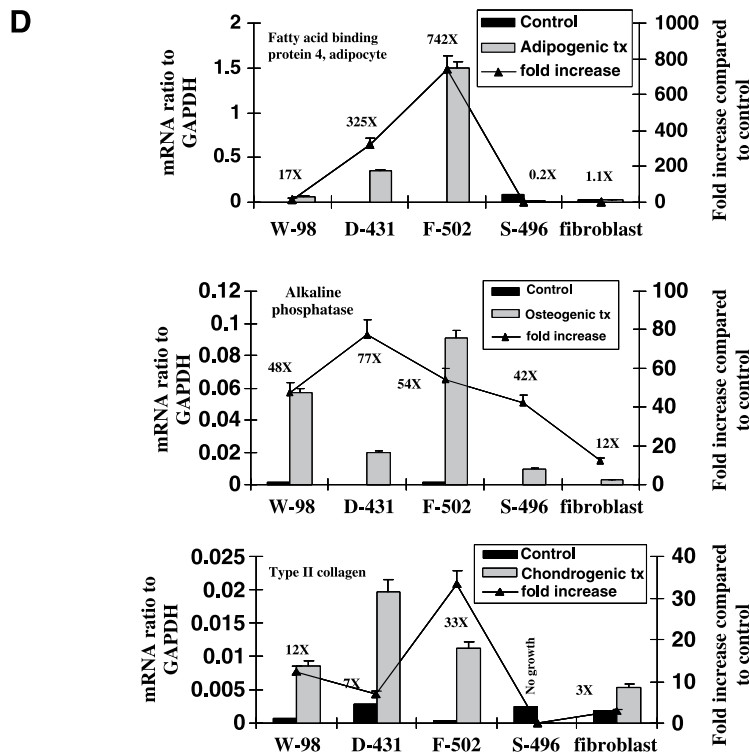


FIGURE 4. Mesenchymal differentiation plasticity of primary glioblastoma cells. **A.** Adipogenic differentiation. In comparison with nontreated controls, adipogenic differentiation in primary glioblastoma cell cultures was evident by the accumulation of lipid vesicles (W-98, D-431, and F-502) that stain with oil red-O. Such changes were not observed in cells derived from fibroblasts and lesser degree in secondary glioblastoma cells (S-496). Magnification, $\times 400$. **B.** Osteogenic induction. Osteogenic differentiation in primary glioblastoma cell cultures (W-98, D-431, and F-502) was indicated by the various degree of positive stain for alkaline phosphatase. Only a few positive cells were identified in secondary glioblastoma cell line (S-496) and cultured fibroblasts. No staining was detected in nontreated cells. F-502 cells stained by von Kossa technique show the presence of calcium. Magnification, $\times 400$. **C.** Chondrogenic induction. Primary glioblastoma cells treated with chondrogenic medium show positive immunostaining for the collagen type II (F-502, D-431, and W-98). Lesser degree of staining was indicated in fibroblast cultures and negative staining for nontreated cells. Magnification, $\times 400$. **D.** Induction of lineage-specific mRNA expression in glioblastoma cells treated with differentiation agents. A real-time quantitative RT-PCR analysis of lineage-specific mRNA expression was done in two sets of nontreated, adipogenic, osteogenic, and chondrogenic medium-treated cell cultures. Total RNA was analyzed for the lineage-specific mRNA expression that is restricted to the adipocytic (fatty acid-binding protein), osteoblastic (alkaline phosphatase), or chondrocytic (collagen type II) lineage. The quantitation of specific transcripts in cells was calculated and expressed by the lineage-specific mRNA ratio to glyceraldehyde-3-phosphate dehydrogenase. The fold increase was calculated by comparison of mRNA levels in treated and nontreated samples. Representative of two sets of cultures.



tissue. We identified a series of fundamental MSC-associated genes overexpressed in glioblastoma biopsies, raising the question whether primary glioblastoma fundamentally possess mesenchymal properties or acquire these properties under environmental guidance during the tumor progression.

Evidently, that *YKL-40*, *TNC*, *osteonectin*, and *CD105*, four genes that are ontogenically associated with mesenchymal tissues but not to neural lineage, were overexpressed in primary glioblastoma tumor cells suggest that primary glioblastoma possess mesenchymal properties. The finding of glioblastoma cell lines expressing cellular and molecular signatures of MSC and were able to be induced and differentiated into mesenchymal lineage cell types further supports the concept that a subset of primary glioblastoma possesses MSC-like properties. Many bone/cartilage-associated factors have been implicated in angiogenesis/neovascularization, cell proliferation, antiapoptosis, EMT, and cell migration; therefore, these mesenchymal-associated factors may participate in promoting tumorigenesis and malignant progression. If primary glioblastomas truly possess MSC-like properties and constitutively express bone/cartilage-associated factors, it may explain in part for its developmental aggressiveness without an identifiable less malignant precursor lesion in contrast to that secondary glioblastoma. Indeed, our previous study, which includes 63 glioblastoma tumors (13), showed that glioblastoma subtype (group HC2B) defined by overexpression of ECM/mesenchymal components is associated with shorter survival and is enriched in primary glioblastoma (14). Further, 52% (24 of 46) of primary glioblastoma overexpress mesenchymal-associated genes, whereas 85% of recurrent primary glioblastoma that had previous treatment express mesenchymal phenotype (14), suggesting that mesenchymal properties confer the malignant and antiapoptotic phenotype. Loss of chromosome 10 and chromosome 7/7p amplification are the most frequently observed in primary glioblastomas that are associated with poor prognosis (56). A gain of chromosome 7 has been suggested to confer radiation resistance (58). Many mesenchymal-associated genes on chromosome 7, which are up-regulated in primary glioblastoma, were reported to be associated with Akt phosphorylation, antiapoptosis, hypoxia, angiogenesis, and EMT (ref. 14; Table 1). Therefore, the overexpression of mesenchymal properties in glioblastoma cells is likely to confer the resistance to treatment and tumor recurrence. Similar observations have been reported by Phillips et al. (55), which indicate that glioblastoma characterized by resemblance to tissue of mesenchymal origin is associated with gain of chromosome 7 accompanied by a loss of chromosome 10, robust angiogenesis, up-regulation of Akt activation, and overexpression of *YKL-40*, *CD44*, signal transducer and activator of transcription 3, and vimentin.

Using tissue microarrays, we have verified four MSC-associated genes that are overexpressed in primary glioblastoma cells [i.e., *YKL-40* (14), *TNC*, *osteonectin*, and *CD105*], indicative of mesenchymal origin in a subset of primary glioblastoma. Glioblastoma cells express four MSC-associated surface markers that can be induced to differentiate into mesenchymal lineage-like cell types defined by morphologic, histologic, immunologic, and molecular characteristics that have been well documented in the literature. Although up-

regulation of tissue-specific genes postdifferentiation treatment supported the induction of mesenchymal lineage cell type differentiation, we, however, have observed differential magnitude/degree of differentiation induction among the cells in cultures and between the cell lines, suggesting that mesenchymal glioblastoma cells are not normal MSC. Gene mutation may prevent poorly differentiated glioblastoma cells from terminal differentiation (47) even under the differentiation treatment. *YKL-40* is a growth factor for connective tissue cells and a potent migration factor for endothelial cells (59). Recently, overexpression of *YKL-40* was reported to be associated with poorer response to radiation and shorter overall survival in glioblastomas (55, 60). *TNC* is a secreted ECM protein highly up-regulated in many types of cancers and has been shown to play a role in promoting angiogenesis and invasion of human glioma cells (61-63). *Osteonectin* is a secreted glycoprotein that binds to ECM and promotes tissue remodeling, angiogenesis, and embryonic development (64). A study showed that *osteonectin* mediates cellular survival of gliomas through Akt activation (65). *CD105*, the TGF- β 3 receptor, plays a role in TGF- β signaling in the control of chondrogenic and osteogenic differentiation (66). TGF- β -*CD105* signaling pathway, therefore, may contribute to EMT phenotype as well as the *in vivo* mesodermal differentiation in primary glioblastoma (12). This hypothesis is further supported by the detection of overexpression of *TGFBI* gene in primary glioblastoma tumors, one of the most highly represented genes in MSC (25). TGF- β signaling has been shown to stimulate tumor cell motility, invasiveness, neovascularization, and EMT. Synergistically, *CD44* (4-fold increase in glioblastoma tumors compared with normal brain tissue) is a cell surface receptor for hyaluronate and osteopontin, and their interactions can synergize tumor cell growth, migration, and cellular chemotaxis (67). Integrin β_1 (*CD29*) is a predominant adhesion receptor used by stromal precursor cells for adhesion, proliferation, and development into osteoblast-like cells (68). The expression of *Thy-1* (*CD90*) glycoprotein implicated a myofibroblastic/EMT phenotype (69). Therefore, the link between mesenchymal glioblastoma and poor prognosis is likely triggered by the activation of inflammatory/stromal/angiogenesis program driven by MSC-associated markers through autocrine and paracrine fashion in tumor *in situ*.

The identification of genes that are associated with embryonic neural epithelial stem cells (e.g., *SOX* genes and *NES*) revealed the neural origin of primary glioblastoma as well as its stem-like status. *SOX* transcripts were also detected in glioblastoma cell lines (data not shown). *NES* and *SOX* proteins are often regarded as markers for neural primordium and have been shown to have regulatory link (70). EMT is the hallmark of embryo development and an essential process that governs morphogenesis (71-73). Therefore, mesenchymal properties and highly invasive phenotype of primary glioblastoma may simply reflect the reactivation of the molecular properties of radial glial cells, which migrate out of the subventricular zone during embryogenesis. Alternatively, gene mutation, for instance, overexpression of *EGFR* gene or *PTEN* deletion, may initiate a distinct program of gene expression and turn on mesenchymal master genes. It has been shown that EGF

but not platelet-derived growth factor can stimulate the differentiation of MSC into bone-forming cells (74). TGF- β , Ras, and phosphatidylinositol 3-kinase/Akt signaling, components associated with EGFR and PTEN pathway, coordinated activation EMT (70, 75-79), which has been implicated in tumor recurrence and self-renewal (40).

In summary, we have shown that primary glioblastoma tumors and cell lines express the molecular signatures of MSC and NSC. These molecular properties may therefore confer their capability of differentiation into both neural and mesenchymal lineage cell types. It is likely that these properties will greatly affect the biological/pathologic behavior of the glioblastoma cells *in vivo* not only in the developmental stage but also during the malignant progression. This finding may affect the design of effective therapies currently not considered for primary glioblastomas that are more commonly used for mesenchymal origin tumors. Thus, MSC-like properties may play a role in the tumorigenesis, invasive progression, and tumor recurrence.

Materials and Methods

Tumor Samples

Tumor samples presented here are derived from a published microarray study (14). The patient tumors and normal samples were collected either from autopsies of glioblastoma patients within 24 hours of death or from patients who underwent surgery at University of California at Los Angeles Medical Center. All samples were collected under protocols approved by the University of California at Los Angeles Institutional Review Board. All histopathologic typing and tumor grading were done by one neuropathologist (P.S.M.) according to the WHO criteria. The subgrouping of glioblastomas was based on clinical presentation. Secondary glioblastomas were called if there was previous pathologic evidence of lower-grade glioblastoma. All tumors without prior evidence of progression from a lower-grade tumor were clinically classified as primary glioblastomas.

Microarray Procedures and Data Analysis

Total RNA was isolated from tumor samples using a Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). Complementary DNA was generated and converted to cRNA probes using standard Affymetrix protocols and hybridized to U133A oligonucleotide microarray (GeneChip Human Genome U133A, Affymetrix, Santa Clara, CA), which represents ~14,500 human transcripts. The chips were scanned using the GeneArray scanner (Affymetrix). The CEL files generated by the Affymetrix Microarray Suite version 5.0 were converted into DCP files using the DNA-Chip Analyzer (dChip 1.3; <http://biosun1.harvard.edu/complab/dchip/>). The DCP files were globally normalized, and gene expression values were generated using the dChip implementation of perfect-match minus mismatch model-based expression index. The group comparisons were done in dChip. In addition, to avoid inclusion of low-level and unreliable signals, the higher signal needed to exceed 100 and be called present by Microarray Suite version 5.0 in >20% of the samples. Gene annotation and tissue distribution was obtained from published literature (PubMed), the GeneReport of the Source database ([\[source.stanford.edu\]\(http://source.stanford.edu\)\), human genome expression profile \(\[http://telethon.bio.unipd.it/bioinfo/HGXP_170/index.html#\]\(http://telethon.bio.unipd.it/bioinfo/HGXP_170/index.html#\)\), and University of California at Los Angeles normal tissue transcription database \(<http://www.dev.gmod.org/>\).](http://</p>
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Cell Lines

Four permanent glioblastoma cell lines, W-98, D-431, F-502, and S-496, were established from clinical specimens. W-98, D-431, and F-502 were derived from patients with primary glioblastoma and the S-496 cell line was derived from a patient with secondary glioblastoma. No evidence of histopathology showed gliosarcoma biphasic pattern in tumors. Cell lines were maintained in DMEM/Ham's F-12 (Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics (100 units/mL penicillin and 100 μ g/mL streptomycin). Glioblastoma cell lines were further cloned at 0.5 cell per well in 96-well plate and the single-cell colonies were further expanded to permanent cell lines. The glioblastoma cell line U87-MG and a newborn foreskin fibroblast cell line, Hs. 27, were purchased from American Type Culture Collection (Manassas, VA). Other types of human tumor cell lines, rat chondrosarcoma cell line, and human bone marrow stromal cells as indicated in the text were obtained from multiple laboratories at University of California at Los Angeles and were maintained in the same culture condition.

LOH Analysis

Genomic DNA (500 ng) from each cell line was amplified, labeled, and hybridized under manufacturer's recommended conditions using the GeneChip Human Mapping 10K Array *Xba*I 131 (Affymetrix). Raw allele scores were processed using Affymetrix GeneChip Chromosome Copy Number Tool 1.1 to estimate genome-wide chromosomal gains and losses.

Immunohistochemistry and Immunocytochemical Analysis

A high-density glioblastoma tissue array was constructed consisting of three representative 0.6-mm cores from formalin-fixed, paraffin-embedded tissue blocks from each of 60 primary glioblastomas, 16 secondary glioblastomas, and 15 corresponding normal brain tissues. Microscope slides were treated with 0.01 mol/L sodium citrate buffer (pH 6.0). A 1-hour blocking step was done using 2% normal horse serum followed by the application of primary antibody to TNC (1:200; Chemicon, Temecula, CA), osteonectin (1:1,000; Chemicon), CD105 (1:400; Invitrogen), or control antibody overnight at 4°C. Subsequent immunodetection was done using Vectastain ABC Standard kit (Vector Laboratories, Burlingame, CA) and Vector NovaRED (Vector Laboratories). Staining intensity in tissue array was scored by a neuropathologist (K.Y.) based on a scale of 0 to 2, in which negatively stained specimens were graded 0, weakly positive samples were graded 1, and strongly positive spots were graded 2. The significance of differences in the incidence of marker expression in primary and secondary glioblastomas or normal brain tissue was calculated using one-tailed, two-proportion Z-test. Immunocytochemical staining was done on cell culture, which was seeded on an eight-chamber culture slide (Nunc, Rochester, NY) overnight. Cells were fixed

for 30 minutes with 4% formaldehyde solution and permeabilized with 0.3% Triton X-100/PBS for 10 minutes followed by incubation with 5% goat serum for 20 minutes. After washing with PBS, cells were incubated for 2 hour with anti-YKL-40, anti-NES, control antibody, or no first antibody. After washing, cells were incubated with rhodamine red or Alexa Fluor 488–conjugated goat anti-mouse IgG (1:200). The cells were counterstained with Hoechst 33342 to identify all nuclei.

Fluorescence-Activated Cell Sorting Analysis

Fluorescence-activated cell sorting analyses were done to analyze MSC antigen markers in glioblastoma cell lines. Glioblastoma cells (5×10^5) were stained with antibody cocktails for 30 minutes at 4°C and the free antibody was removed by two washes. The analyses were done on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and $\geq 10,000$ events were collected in each analysis with forward scatter and side scatter as well as four fluorescent measurements: FL1 (FITC), FL2 (PE), FL3 (PE-Cy5), and FL4 (APC). Data were analyzed by using CellQuest 3.1 (De Novo Software, Thornhill, Ontario, Canada). The following antibodies were employed: CD44-FITC, CD105-PE, CD90-APC, CD29-TC, CD34-FITC, CD14-FITC, CD31, and isotype control antibodies (Becton Dickinson; Caltag Laboratories, Burlingame, CA; and R&D Systems, Inc., Minneapolis, MN). For four-color analyses, cells were gated for double positive for CD90-APC and CD105-PE and then analyzed for their coexpression of CD44-FITC and CD29-TC.

In vitro Induction of Multiple Cell Lineage Differentiation

The mesenchymal lineage differentiation was assessed in cloned and expanded glioblastoma cell lines. Adipogenic differentiation was assessed by incubating cells with DMEM-LG plus 10% fetal bovine serum supplemented with 0.5 $\mu\text{mol/L}$ hydrocortisone, 0.5 $\mu\text{mol/L}$ isobutyl methylxanthine, and 60 $\mu\text{mol/L}$ indomethacin (Sigma-Aldrich, St. Louis, MO) for 2 weeks. Osteogenic inductions were carried out by incubating cells with DMEM-LG supplemented with 10% fetal bovine serum, 10^{-8} mol/L dexamethasone, 0.2 mmol/L ascorbic acid, and 10 mmol/L β -glycerophosphate (Sigma-Aldrich) for 3 weeks. Chondrogenic inductions were carried out in suspension culture of micromass pellets or adherent cultures with high cell density. Cells were incubated with serum-free DMEM-LG supplemented with 10 ng/mL TGF- β 3 (R&D Systems), 100 nmol/L dexamethasone, 50 $\mu\text{g/mL}$ ascorbic acid, 100 $\mu\text{g/mL}$ sodium pyruvate, 40 $\mu\text{g/mL}$ proline (Sigma-Aldrich), and ITS Premix (BD Biosciences, San Jose, CA) for 3 weeks. The medium was changed every 3 days. Cells treated postadipogenic induction was stained with oil red-O solution (Sigma-Aldrich) to reveal adipogenic differentiation. To assess osteogenic differentiation and mineralization, cells were stained with alkaline phosphatase staining (Sigma-Aldrich) and von Kossa staining (Newcomer Supply, Middleton, WI). Immunohistochemical staining for collagen type II (Lab Vision Corp., Fremont, CA) was used to assess the chondrogenic differentiation.

Semiquantitative and Real-time Quantitative RT-PCR

Semiquantitative and real-time quantitative RT-PCR was done to verify transcripts of multiple cell lineages in tested samples.

The specific primers were designed from published sequence of mRNA (3′-untranslated region; National Center for Biotechnology Information). The Primer 3 Input (primer3_www.cgi v 0.2) was used to selected primers, and nonredundant specific primer sequences were verified using BLAST Search Genome (<http://genome.ucsc.edu>) and National Center for Biotechnology Information BLAST (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). The primer sequences and expected size of amplified PCR products are listed in Supplementary Table S2. All RNA samples were digested with DNase I (Ambion, Inc., Austin, TX) that free off RNase before reverse transcription. Total RNA (2 μg) was used as a template for RT-PCR. cDNA synthesis was done for 1 cycle at 50°C for 30 minutes and 94°C for 2 minutes, and PCR amplification was carried out for 30 cycles [50°C for 2 minutes and 95°C for 10 minutes (94°C for 15 seconds, 58–61°C for 1 minute, and 72°C for 1 minute) \times 30 cycles]. After amplification, each reaction product (5 μL) was analyzed by 2% agarose gel electrophoresis, and the bands were then visualized by SYBR Green staining. The specificity of the selected PCR products was confirmed by sequencing. Real-time PCR was done to amplify tissue-specific transcripts and was carried out with MJ Opticon PCR Analyzer (MJ Research, Inc., Waltham, MA) using SYBR Green PCR Core Reagents (Applied Biosystems, Foster City, CA). The reactions were cycled 30 times [50°C for 2 minutes and 95°C for 10 minutes (94°C for 15 seconds, 60°C for 1 minute, and 72°C for 1 minute) \times 30 cycles] and the fluorescence was measured at the end of each cycle to construct amplification curves. A melting curve was done to verify the specificity of PCR products. Quantitation of transcripts was calculated based on a titrated standard curve co-run in the same experiment and calibrated with the expression level of housekeeping gene. All determinations were done in duplicate. The following tissue-specific oligonucleotides were used: fatty acid-binding protein 4 (210 bp) sense 5′-AAGTCAAGAGCACCATAACCTTAGA-3′ and antisense 5′-TGGCTTATGCTCTCTCATAAACTCT-3′, alkaline phosphatase (236 bp) sense 5-GAGTACAGACTGCAGACATTCTCAA-3′ and antisense 5′-GTCCTGAGGAGAAAGACCTCAAC-3′, and collagen type II, α 1 (224 bp) sense 5′-CTTTCCAATCTCAGTCACTCTAGGA-3′ and antisense 5′-ATTTGACTCACACCAGTTAGTTTCC.

Accession Numbers

The expression microarray and single nucleotide polymorphism array data have been submitted to the Gene Expression Omnibus database at <http://www.ncbi.nlm.nih.gov/projects/geo/>. The accession number for expression microarray is GSE5107 and the accession number for single nucleotide polymorphism expression microarray is GSE5082.

Acknowledgments

We thank Harley Kornblum and Daniel H. Geschwind for useful comments and the patients who participated in this study.

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