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Isolation and Characterization of Fast-Migrating Human Glioma Cells in the Progression of Malignant Gliomas

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Gliomas are the most common primary brain tumors. The most malignant form, the glioblastoma multiforme (GBM; WHO IV), is characterized by an invasive phenotype, which enables the tumor cells to infiltrate into adjacent brain tissue. When investigating GBM migration and invasion properties in vitro, in most cases GBM cell lines were analyzed. Comprehensive investigations focusing on progression-dependent characteristics of migration processes using fresh human glioma samples of different malignancy grades do not exist. Thus, we isolated fast-migrating tumor cells from fresh human glioma samples of different malignancy grades (astrocytomas WHO grade II, grade III, GBM, and GBM recurrences) and characterized them with regard to the transcription of genes involved in the migration and invasion, tumor progression, epithelial-to-mesenchymal transition, and stemness. In addition, we transferred our results to GBM cell lines and glioma stem-like cells and examined the influence of temozolomide on the expression of the above-mentioned genes in relation to migratory potential. Our results indicate that "evolutionary-like" expression alterations occur during glioma progression when comparing slow- and fast-migrating cells of fresh human gliomas. Furthermore, a close relation between migratory and stemness properties seems to be most likely. Variations in gene expression were also identified in GBM cell lines, not only when comparing fast- and slow-migrating cells but also regarding temozolomide-treated and untreated cells. Moreover, these differences coincided with the expression of stem cell markers and their migratory potential. Expression of migration-related genes in fast-migrating glioma cells is not only regulated in a progression-dependent manner, but these cells are also characterized by specific stem cell-like features.

Key words: Migration; Invasion; Epithelial-to-mesenchymal transition (EMT); Stemness; Gliomas; Progression

INTRODUCTION

Gliomas are the most common type of primary brain tumors, and the most malignant form, glioblastoma multiforme (GBM), accounts for about 15% of all brain tumors and more than 50% of all astrocytomas[1]. The current standard of treatment for GBM is surgical resection of the tumor, followed by adjuvant radio- and chemotherapy. However, median survival time for GBM patients is still poor, approximately 12–15 months, despite multimodal therapy[2]. Currently, there are no effective long-term treatments for this disease. One of the primary reasons for poor prognosis is the GBM's invasive phenotype, which enables the tumor cells to infiltrate like guerilla warriors[3] into adjacent normal brain tissue and frequently results in the appearance of recurrence, yielding ineffective treatment strategies[4].

To shed light on the mechanism that drives GBM invasion, some groups started to isolate and characterize the profile of migratory GBM cell populations. Comparing the transcriptional profile of 10 human glioma cell lines, Demuth et al.[5] developed a 22-gene signature capable of classifying glioma cultures based on their migration rate. Both Yang et al.[6] and Loftus et al.[7] performed microRNA (miRNA) profiling in cultured migrating glioma cell lines, identifying several genes involved in the migration potential of human glioma cells (e.g., KHSRP, HCFC1, and Pyk2), and were able to verify their results in different clinical samples. In addition, Nevo et al.[8] used a microarray profiling approach on a human glioma stem cell xenograft model to explore gene expression changes in situ in invading glioma cells compared to the tumor core and were able to show a potential role of, for example, CD44, PARD3, and OLIG2

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in invasion processes. Xue et al.[9] used GSE28167, human glioma U251MG cells cultured on strictly aligned versus randomly orientated electrospun nanofibers, to analyze a gene profile aiming to identify potential genes, miRNAs, and target sites of migratory cells. Moreover, Mariani et al.[10] performed a cDNA array on populations of a glioma cell line exposed, or not, to a motility-inducing substrate of cell-derived extracellular matrix proteins and were able to show an association of cell motility with reduced transcription of proapoptotic and proliferation-associated genes.

Taken together, the common feature of these investigations was to identify specific characteristics of migratory cells of malignant gliomas usually using cultured glioma cell lines and focusing especially on the highly malignant form of gliomas, the glioblastomas. However, the invasiveness does not necessarily correlate with the malignancy grade of gliomas[11], and in contrast to cultured glioma cell lines, an in vivo tumor is a heterogeneous tissue with tumor cells at different stages of differentiation that are potentially characterized by a stage-specific migratory potential[12].

Thus, in our approach, we isolated tumor cells from fresh human glioma samples of different malignancy grades [astrocytomas World Health Organization (WHO) grade II, grade III, glioblastomas, and glioblastoma recurrences], divided them according to their migratory potential, and characterized the transcription of typical genes involved in migration and invasion, tumor progression, and epithelial-to-mesenchymal transition [EMT; in the following, termed "guerilla gene set": integrin subunit α 5 (ITGA5), integrin subunit β 4 (ITGB4), fibronectin 1 (FN1), CXCR7, glial acidic fibrillary protein (GFAP), vimentin (VIM), snail1 (SNAI1), snail2/slug (SNAI2), and TWIST1]. Since a close relation between migration and stemness of glioma cells is well known [e.g., by downregulation of the tumor suppressor "suppressor of cancer cell invasion (SCAI)" and thereby activation of the Wnt-/β-catenin signaling pathway or by overexpression of integrin α3 in glioma-stem like cells[13,14]], we put an additional focus on the stem cell characteristics of isolated fast-migrating glioma cells [in the following, termed "stem cell markers": prominin-1 (PROM1), musashi-1 (MSI1), nestin (NES), sex-determining region Y-box 2 (SOX2), and CXCR4]. In addition, we aimed to transfer our results to different commercial glioma cell lines and glioma stem-like cells generated from those. Finally, we examined the influence of temozolomide on the expression of the previously mentioned genes in relation to the migratory potential of different glioma cell lines.

MATERIALS AND METHODS

Tumor Specimens

In total, 19 glioma samples of different tumor entities from 17 patients (1 patient with a primary and a recurrent tumor after 4 months, one tumor subdivided according to intraoperative visualized tumor heterogeneity into two samples) were surgically dissected tissues from the Department of Neurosurgery (Kiel, Germany), which were obtained in accordance with the Helsinki Declaration of 1975 with approval of the ethics committee of the University of Kiel, Germany, after written, informed consent was obtained from the donors (file reference: D 408/14). Tumors were classified according to the WHO criteria. The diagnosis was established by a pathologist. One tumor sample corresponded to an astrocytoma WHO II (Astro II), 4 to astrocytomas WHO III (Astro III), and 14 to glioblastomas WHO IV (GBM; 10 primaries, 4 recurrences). See Table 1 for a detailed sample and the patients' information.

CD11b and CD3 MACS Depletion Procedure

Freshly obtained human glioma samples were used for MACS® technology to deplete CD11b- and CD3-expressing cells (macrophages, microglia, T lymphocytes, and NK cells) as described previously[15]. In detail, single-cell suspensions of 400 mg of tumor tissues were generated using the Neural Dissociation Kit (T) (Miltenyi Biotech GmbH, Gladbach, Germany). Cells were immediately labeled with both CD11b and CD3 MicroBeads (Miltenyi Biotech GmbH) and separated using MACS LS columns according to the manufacturer's instructions. To verify the purification of the native residual glioma cell fractions (and as a positive control for the immune cell fractions), these were analyzed by reverse transcription and quantitative real-time polymerase chain reaction (qRT-PCR) concerning CD3 and ionized calcium-binding adapter molecule 1 (Iba-1) gene expression (see below), and stained by immunocytochemistry to exclude or detect CD3/CD11b (immune cells) and GFAP (glioma cells) cells (see below). In addition, transcription of the "guerilla gene set" and the "stem cell markers" in the native residual glioma cell fractions was determined by qRT-PCR (see below).

Immunocytochemistry

The identity of freshly isolated glioma cells ($CD11b^-$ and $CD3^-$, $GFAP^+$) was confirmed by immunofluorescence using a mouse anti-human CD11b antibody (sc-1186; 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), a rabbit anti-human CD3 antibody (PA5-29387; 1:200; Thermo Fisher Scientific, Rockford, IL, USA), and a mouse or rabbit anti-human GFAP antibody (MAB 3402; 1:500; Merck Millipore, Darmstadt, Germany; or sc-9065; 1:100; Santa Cruz Biotechnology). In detail, isolated glioma cells were fixed with methanol–acetone (1:1; ice cold) for 10 min, and nonspecific binding was blocked with 0.1% (w/v) bovine serum albumin/0.2% (w/v) glycine in PBS for 60 min. Fixed cells were incubated with anti-CD3 (rabbit) and anti-GFAP (mouse) or with anti-CD11b (mouse) and anti-GFAP (rabbit) primary antibodies overnight at 4°C.

Table 1. Clinical Data on Glioma Samples

					Previous Clinical Background			
ID	Pat.	Sex	Age (at Surgery)	Diagnosis	Diagnosis	Elapsed Time (After First Surgery)	Therapy	Additional Information
Gu25	1	F	33	GBM oligo, WHO IV primary				
Gu37	2	M	77	GBM, WHO IV primary				
Gu48	3	F	79	GBM, WHO IV recurrent	GBM, WHO IV primary	19 months	STUPP	Recurrence under chemotherapy
Gu52	4	M	41	GBM, WHO IV secondary	Astrocytoma, WHO III recurrent	14 months		
Gu55	5	M	50	Astrocytoma, WHO II recurrent	Astrocytoma, WHO II Primary	7 months		
Gu58	6	F	65	GBM, WHO IV primary				
Gu59	7	M	34	GBM, WHO IV primary				
Gu60 low	8	M	25	Astrocytoma, WHO III primary				
Gu60 high	8	M	25	Astrocytoma, WHO III primary				
Gu63	9	M	80	GBM, WHO IV primary				
Gu64	10	M	51	GBM, WHO IV recurrent	GBM, WHO IV primary	14 months	STUPP	Recurrence under chemotherapy
Gu65	11	M	47	GBM, WHO IV secondary	Astrocytoma, WHO III primary	11 years	STUPP	
Gu67	12	F	78	GBM, WHO IV primary				
Gu69	13	M	68	Astrocytoma, WHO III primary				
Gu71	14	F	67	GBM, WHO IV primary				
Gu72	7	M	34	GBM, WHO IV recurrent	GBM, WHO IV primary	4 months	STUPP	Recurrence under chemotherapy
Gu91	15	F	44	Astrocytoma, WHO III recurrent	Astrocytoma, WHO III primary	4 months		
Gu93	16	F	55	GBM, WHO IV primary				
Gu97	17	F	74	GBM, WHO IV recurrent	GBM, WHO IV, primary	8 months	STUPP	Recurrence under chemotherapy
Mean			55.7					

Listed is information on gender, age at surgery, and diagnosis of the investigated glioma samples, with previous clinical background data concerning diagnosis of previous surgically resected tumors, elapsed time, therapy schemes, and additional data as far as available.

The primary antibodies were omitted for negative controls. Next, cells were incubated with a mixture of Alexa Fluor 488- or 555-coupled secondary antibodies (donkey anti-mouse or anti-rabbit IgG; 1:1,000; Invitrogen, Carlsbad, CA, USA) for 1 h at 37°C in darkness. Nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI; 1:30,000; 30 min at room temperature; Sigma-Aldrich, St. Louis, MO, USA). After embedding in Immu-Mount (Thermo Fisher Scientific), digital photography was performed using a Zeiss fluorescence microscope and a Zeiss camera (Zeiss, Jena, Germany).

Reverse Transcription and Real-Time PCR (qRT-PCR)

RNA was isolated with the TRIzol® reagent (Invitrogen) or with the ARCTURUS® PicoPure® RNA Isolation Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. DNase digestion, cDNA synthesis, and qRT-PCR were performed as described

previously[15] using TaqMan primer probes (Applied Biosystems): glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1), CXCR4 (Hs00237052_m1), CXCR7 (Hs00664172_s1), FN1 (Hs00277509_m1), GFAP (Hs00157674_m1), Iba-1 (Hs00610419_g1), SNAI1 (Hs00195591_m1), SNAI2 (Hs00950344_m1), TWIST1 (Hs01675818_s1), VIM (Hs00185584_m1), CD133 (PROM1) (Hs00195682_m1), NES (Hs00707120_s1), SOX2 (Hs00602736_s1), MSI1 (Hs00159291_m1), ITGA5 (Hs00233743_m1), ITGB4 (Hs00236216_m1), and CD3 (Hs01062241_m1). Fluorescence data were measured and converted into cycle threshold (Ct) data, and ΔCt values of each sample were calculated as $Ct_{\text{gene of interest}} - Ct_{GAPDH}$. Relative gene expression was calculated with $y = 2^{(\text{normalized CT nonmigrated} - \text{normalized CT migrated})} = n$-fold expression compared to slow-migrating cells/control. A ΔCt value of 3.33 corresponds to a one-magnitude-lower gene expression compared to GAPDH.

Pore Migration Assay to Isolate Fast-Migrating Glioma Cells

Isolated (and immune cell depleted) native glioma cells (7.0×10^4 up to 2.5×10^5) were seeded in a six-well plate (Thermo Fisher Scientific) onto a cell culture insert with a membranous bottom (pore size: 8 μm; Merck Millipore). While the growth medium [Dulbecco's modified Eagle's medium (DMEM); Invitrogen] in the upper chamber above the membrane contained only 2% of fetal bovine serum (FBS; Invitrogen), the medium below the membrane contained 10% FBS, favoring migration of fast-migrating cells into the lower cell chamber. The migration process was stopped after 24 h, and migrated cells were collected from the lower chamber and the bottom side of the insert by a cell scraper (fast-migrating fraction). In addition, nonmigrated cells were collected from the upper chamber as well (slow-migrating fraction). Cells were harvested by centrifugation at 1,400 rpm for 5 min. RNA was isolated using the ARCTURUS® PicoPure® RNA Isolation Kit (Thermo Fisher Scientific). To analyze the gene expression profiles of the "guerilla gene set" and the "stem cell markers," qRT-PCR was performed for both fractions as described above.

Cultivation of Glioma Cell Lines

The human glioblastoma cell lines A172 (ECACC 880624218), T98G (ECACC 92090213), and U251MG (ECACC 89081403; formerly known as U373MG) were obtained from the European Collection of Cell Cultures (ECACC; Salisbury, UK) and cultured in DMEM plus 10% FBS (Invitrogen) as described previously[16]. The different cell lines were checked for purity by immunostaining with cell type-specific markers and for the absence of *Mycoplasma* contamination by staining with bisbenzimide.

To determine gene expression profiles of the "guerilla gene set" and the "stem cell markers" in native glioma cell lines, RNA was isolated with the TRIzol® reagent, and qRT-PCR was performed as described above. In addition, to isolate fast-migrating cells of A172, T98G, and U251MG glioma cell lines, cells were allowed to migrate through a membrane with 8-μm pore size along a serum gradient, and fast- and slow-migrating cells were collected. RNA was isolated, and qRT-PCR was performed with regard to transcription of the "guerilla gene set" and the "stem cell markers" as described above.

Glioma Stem-Like Spheroids

Glioma stem-like cells were generated from the human glioblastoma cell lines (A172, T98G, and U251MG) by sequential cultivation in neurosphere medium[17] plus 20 ng/ml basic fibroblast growth factor (bFGF; ImmunoTools, Friesoythe, Germany) and 20 ng/ml epidermal growth factor (EGF; PeproTech, Rocky Hill, NJ, USA)[16,18–20]. Developing glioma spheroids were kept for 6 weeks with a dissociation procedure by trypsinization every other week. To verify whether stem-like cells have been successfully generated, one fraction was differentiated in stem cell medium containing 10% FBS without additional growth factors for 9 days, RNA was isolated using the ARCTURUS® PicoPure® RNA Isolation Kit, and transcription of SOX2, PROM1, MSI1, NES, and CXCR4 was determined in both glioma stem-like spheroids and differentiated fractions by qRT-PCR. In addition, gene expression profile of the "guerilla gene set" was analyzed in glioma stem-like cells by qRT-PCR as described above.

Temozolomide-Stimulated Glioma Cell Lines

Native T98G, U251MG, and A172 cells (5.0×10^5) were stimulated with 500 μM temozolomide [Sigma-Aldrich; dissolved in dimethyl sulfoxide (DMSO)] in DMEM supplemented with 10% FBS for 10 days. Controls were solely stimulated with an equal volume [0.2% (v/v)] of DMSO. Medium was changed every third day. To isolate fast-migrating cells, a pore migration assay was performed with temozolomide-stimulated native T98G, U251MG, and A172 cells, and fast- and slow-migrating cells were collected. RNA was isolated, and qRT-PCR concerning transcription of the "guerilla gene set" and "stem cell markers" was performed as described above ($n=5$). In addition, after 10 days of stimulation with temozolomide, 1.5 to 2.0×10^4 A172, U251MG, or T98G cells were seeded in a culture dish with a grid (8 cm^2; Thermo Fisher Scientific), and stimuli (DMSO or temozolomide) were added to the medium. When the cells had formed

a monolayer, they were carefully scratched with a 20-µl pipet tip. Immediately afterward, dead and detached cells were aspirated, and new medium was added to the dishes supplied with the appropriate stimuli. For the wound healing process, the scratch was viewed under a transmitted-light microscope (Zeiss) over 8 h at exactly the same position, and for visualization, several pictures were taken after certain time intervals. Scratch areas were measured using the ImageJ software, and differences between 8 and 0 h were calculated as $x = (\text{free area}^{0h} - \text{free area}^{8h})/\text{free area}^{0h}$ (yielding the settled area in percentage; $n = 6$).

Statistical Analysis

For statistical analysis, a two-tailed Student's *t*-test with matched samples was used. Significance levels were $p < 0.05$ and $p < 0.01$.

RESULTS

Migration-Associated Gene Expression in Human Glioma Samples With Respect to Glioma Progression

Prior to isolation and characterization of fast-migrating glioma cells from freshly obtained surgical human glioma specimens of different malignant grades, we depleted interfering immune cells by MACS separation technology. After dissociation and digestion in order to generate a homogenous single-cell suspension, specific CD3 and CD11b magnetic bead-labeled antibodies were used for depletion of macrophages, microglia, T lymphocytes, and NK cells. Afterward, purity of the remaining tumor cell fraction was proven both by qRT-PCR and immunocytochemistry (Fig. 1A). As exemplarily shown for preparations Gu58 (Fig. 1A, left) and Gu63 (Fig. 1A, right), immunocytochemistry of immune cell-depleted tumor fractions did not yield any positive staining signals for CD3 (identifying T and NK cells) or CD11b (identifying microglia/macrophages) in either human glioblastoma, whereas most isolated cells were $GFAP^+$ (glial cell-specific marker). According to tumor heterogeneity and reflecting the fact that GFAP staining intensity is known to be variable in different glial tumor cells and tissues[21], the amounts of $GFAP^+$ cells differed between individual preparations (Fig. 1A, top). Additionally, in comparison to the MACS-depleted immune cell fraction, which served as a positive control, the residual tumor cell preparations nearly lacked mRNA expression of CD3 or Iba-1 (last named to identify microglia/macrophages), implicating that the depletion of immune cells had been successful (Fig. 1A, bottom).

To characterize the expression levels of the "guerilla gene set" and the "stem cell markers" in the whole residual tumor, cell preparations including slow- and fast-migrating tumor cells as well as tumor cells with stem or dormant cell properties, we performed qRT-PCR on these genes in the next step. Results are shown in Figure 1B, in which single ΔCt values of astrocytomas, glioblastomas, and recurrences are demonstrated. It should be kept in mind that a ΔCt value of 3.33 corresponds to a one-magnitude-lower gene expression.

Overall, all investigated genes were found to be expressed in considerable amounts in all glioma preparations. ITGA5, ITGB4, FN1, CXCR7, GFAP, and VIM were expressed at comparably high levels, while transcription factors SNAI1, SNAI2, and TWIST1 were found at moderate levels and "stem cell markers" at moderate to high levels. Interestingly, in comparison to astrocytomas and glioblastomas, genes of the "guerilla gene set" were found at higher expression levels, especially in two recurrent samples. However, referring to tumor heterogeneity and in accordance with our own published results, where we previously showed a broad range of expression for the individual genes[22–24], expression of investigated genes did not show clear differences between different tumor entities or progression stages.

This impression completely changed after isolation of fast-migrating tumor cells using a pore migration assay and comparing the expression results of the "guerilla gene set" and "stem cell markers" between slow- and fast-migrating tumor cells of individual glioma samples, respectively (Fig. 2). Here the relative gene expression of fast-migrating cells was normalized to slow-migrating cells.

To begin with, extensive differences in gene expression were observed between fast- and slow-migrating cells comprising all tumor entities and progression stages. Interestingly, the patterns of up- or downregulation varied between the groups of different tumor entities. While the investigated genes were often expressed at lower levels in fast-migrating cells of astrocytomas of grades II and III (Astro, irrespective of primary or recurrent astrocytomas), they were upregulated in those of firstly diagnosed glioblastomas [GBM(p) indicating primary, GBM(s) indicating secondary glioblastomas]. Fast-migrating cells of glioblastoma recurrences showed a predominant downregulation pattern [GBM(r)]. (Fig. 2). Thus "evolutionary-like" expression changes of the "guerilla gene set" and the "stem cell markers" seem to occur during glioma progression when comparing slow- and fast-migrating cells. Since these results were applicable for the expression of the "guerilla gene set" and the "stem cell markers," a close relation between migratory and stemness properties of glioma cells seems to be very likely.

When looking more closely to differences between slow- and fast-migrating cells of GBMs, the highest expression differences were detectable for ITGA5, CXCR7, VIM, SNAI1, and TWIST1 (Fig. 2). Upregulation of these genes varied in a broad range, regarding both different GBM samples and the investigated genes. In

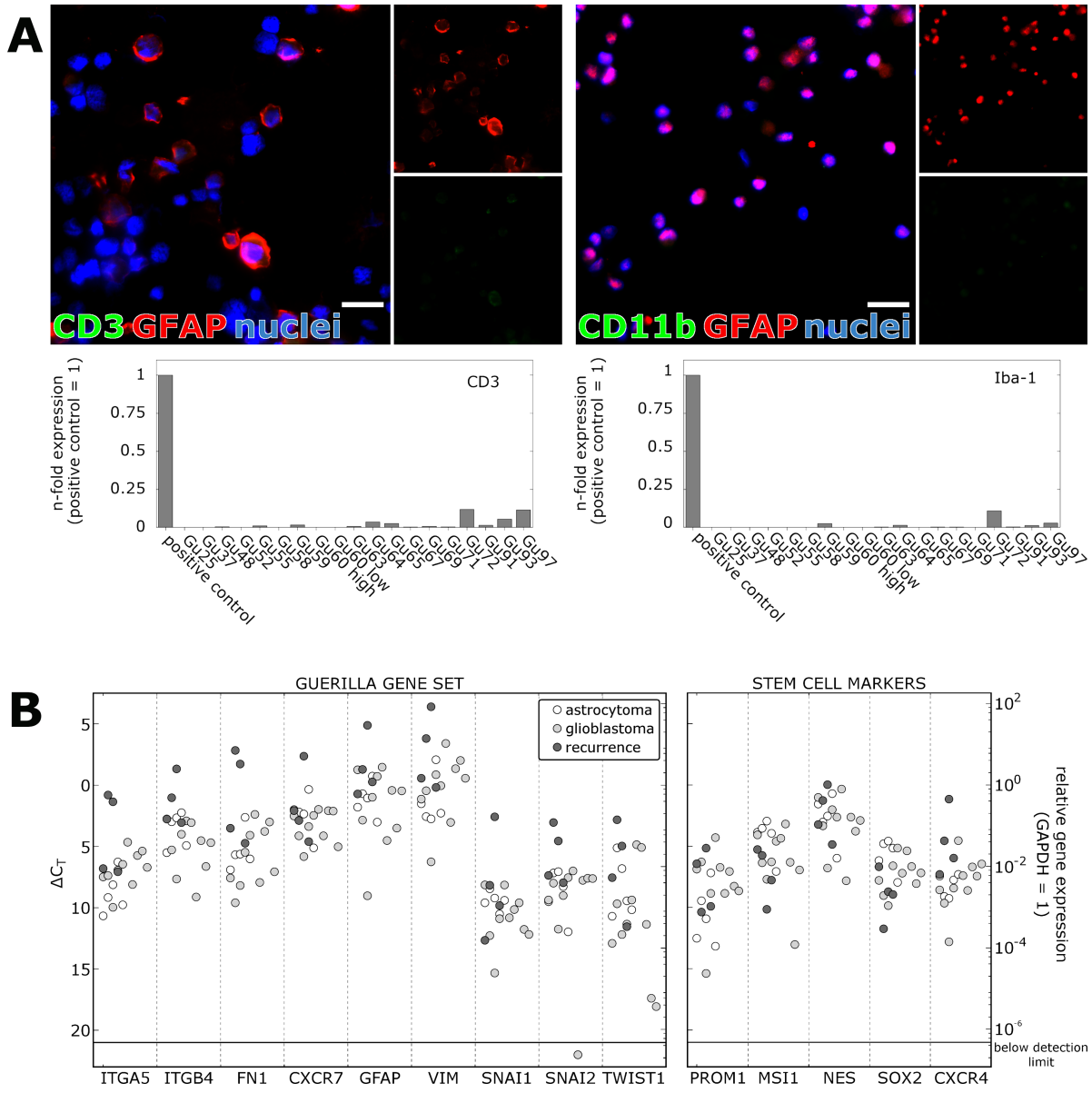
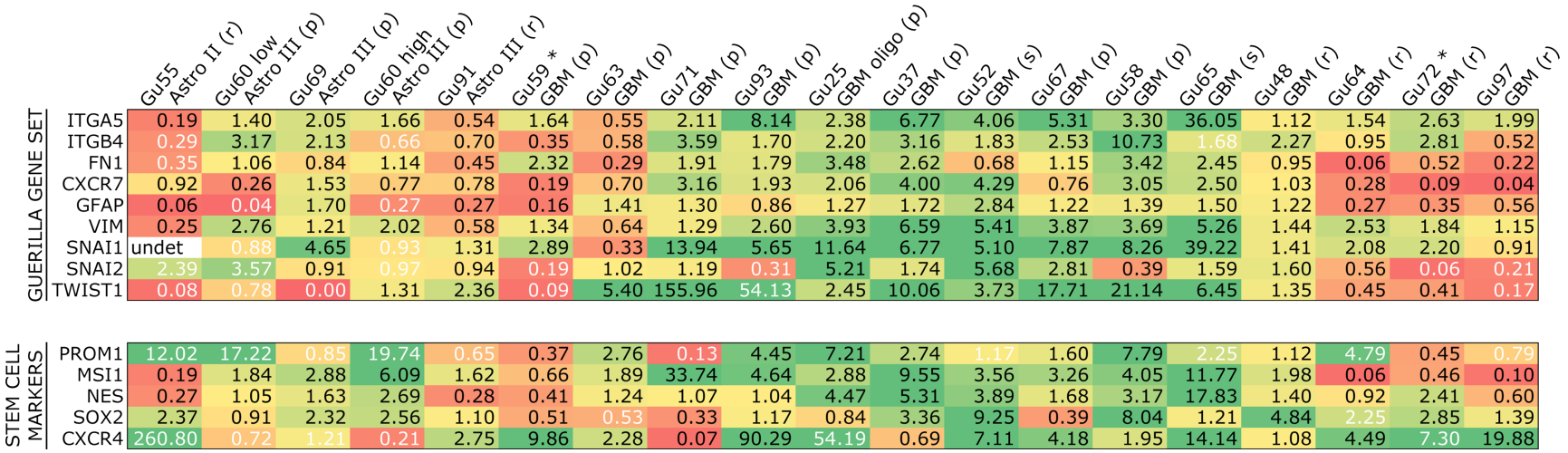


Figure 1. (A) Successful depletion of immune cells from dissociated human glioma cell preparations and (B) transcription of the "guerilla gene set" and "stem cell markers" in these cell preparations. (A) After dissociation of solid human gliomas single-cell suspensions were subjected to magnetic cell separation to deplete potentially interfering $CD3^+$ and $CD11b^+$ immune cells. Top: Successful depletion of $CD3^+$ and $CD11b^+$ cells was proven by immunocytochemistry, which did not yield any staining signals for CD3- or CD11b-specific antibodies (secondary antibodies are labeled green). The majority of cells of the single-cell suspensions showed positive staining for the glial-specific marker GFAP (red); nuclei were stained blue with DAPI. Shown examples from preparations Gu58 (left) and Gu63 (right) are representatives. Scale bars: 20 µm. Bottom: Residual cells were additionally analyzed by qRT-PCR regarding their transcription of CD3 (NK and T-cell marker) and Iba-1 (microglia/macrophage marker) in comparison to the depleted immune cell fraction (positive control = 1). CD3 and Iba-1 expression was undetectable or nearly lacking for all preparations used for further experimental characterization proving the successful depletion of interfering immune cells. (B) By qRT-PCR, we investigated the basic transcription of the "guerilla gene set" (ITGA5, ITGB4, FN1, CXCR7, GFAP, VIM, SNAI1, SNAI2, and TWIST1) and the "stem cell markers" (PROM1, MSI1, NES, SOX2, and CXCR4) prior to further functional characterization. We observed moderate to high levels of ITGA5, ITGB4, FN1, CXCR7, GFAP, and VIM and somewhat lower levels of the transcription factors SNAI1, SNAI2, and TWIST1. Stem cell markers were expressed at moderate to high levels. In general, patient individual transcription data showed a broad variation range (at least 10-fold variation between lowest and highest expression value), in which a clear discrimination between tumor entities or progression stages was not observed. ΔCt values of astrocytomas are shown in white circles (each circle marking an individual tumor cell preparation), of primary and secondary glioblastomas in light gray, and of recurrences in dark gray. ΔCt values indicate the relative gene expression in comparison to the housekeeping gene GAPDH in a logarithmic plotting (left axis), which means a 3.33 lower ΔCt value denotes a 10-fold lower gene expression (depicted in a logarithmic scale on the right axis).



		Gu55 Astro II (r)	Gu60 low Astro III (p)	Gu69 Astro III (p)	Gu60 high Astro III (p)	Gu91 Astro III (r)	Gu59 * GBM (p)	Gu63 GBM (p)	Gu71 GBM (p)	Gu93 GBM (p)	Gu25 GBM oligo (p)	Gu37 GBM (p)	Gu52 GBM (s)	Gu67 GBM (p)	Gu58 GBM (p)	Gu65 GBM (s)	Gu48 GBM (r)	Gu64 GBM (r)	Gu72 * GBM (r)	Gu97 GBM (r)
GUERILLA GENE SET	ITGA5	0.19	1.40	2.05	1.66	0.54	1.64	0.55	2.11	8.14	2.38	6.77	4.06	5.31	3.30	36.05	1.12	1.54	2.63	1.99
	ITGB4	0.29	3.17	2.13	0.66	0.70	0.35	0.58	3.59	1.70	2.20	3.16	1.83	2.53	10.73	1.68	2.27	0.95	2.81	0.52
	FN1	0.35	1.06	0.84	1.14	0.45	2.32	0.29	1.91	1.79	3.48	2.62	0.68	1.15	3.42	2.45	0.95	0.06	0.52	0.22
	CXCR7	0.92	0.26	1.53	0.77	0.78	0.19	0.70	3.16	1.93	2.06	4.00	4.29	0.76	3.05	2.50	1.03	0.28	0.09	0.04
	GFAP	0.06	0.04	1.70	0.27	0.27	0.16	1.41	1.30	0.86	1.27	1.72	2.84	1.22	1.39	1.50	1.22	0.27	0.35	0.56
	VIM	0.25	2.76	1.21	2.02	0.58	1.34	0.64	1.29	2.60	3.93	6.59	5.41	3.87	3.69	5.26	1.44	2.53	1.84	1.15
	SNAI1	undet	0.88	4.65	0.93	1.31	2.89	0.33	13.94	5.65	11.64	6.77	5.10	7.87	8.26	39.22	1.41	2.08	2.20	0.91
	SNAI2	2.39	3.57	0.91	0.97	0.94	0.19	1.02	1.19	0.31	5.21	1.74	5.68	2.81	0.39	1.59	1.60	0.56	0.06	0.21
	TWIST1	0.08	0.78	0.00	1.31	2.36	0.09	5.40	155.96	54.13	2.45	10.06	3.73	17.71	21.14	6.45	1.35	0.45	0.41	0.17
STEM CELL MARKERS	PROM1	12.02	17.22	0.85	19.74	0.65	0.37	2.76	0.13	4.45	7.21	2.74	1.17	1.60	7.79	2.25	1.12	4.79	0.45	0.79
	MSI1	0.19	1.84	2.88	6.09	1.62	0.66	1.89	33.74	4.64	2.88	9.55	3.56	3.26	4.05	11.77	1.98	0.06	0.46	0.10
	NES	0.27	1.05	1.63	2.69	0.28	0.41	1.24	1.07	1.04	4.47	5.31	3.89	1.68	3.17	17.83	1.40	0.92	2.41	0.60
	SOX2	2.37	0.91	2.32	2.56	1.10	0.51	0.53	0.33	1.17	0.84	3.36	9.25	0.39	8.04	1.21	4.84	2.25	2.85	1.39
	CXCR4	260.80	0.72	1.21	0.21	2.75	9.86	2.28	0.07	90.29	54.19	0.69	7.11	4.18	1.95	14.14	1.08	4.49	7.30	19.88

Figure 2. Relative gene expression differences between fast-migrating and slow-migrating human glioma cell preparations. Nineteen single-cell preparations of 18 individual tumors from 17 patients (ASTRO, astrocytoma; GBM, glioblastoma; p, primary; s, secondary; r, recurrent) were subjected to the pore migration assay. Upper and lower cell fractions were analyzed regarding their transcription of the "guerilla gene set" (top) and the "stem cell markers" (bottom) by qRT-PCR. One astrocytoma (Gu60) with distinct different regions of malignancy was subdivided in two samples. Gu59 and Gu72 were obtained as primary (Gu59) and recurrent (Gu72) glioblastomas from the same patient. Relative gene expression between fast-migrating and slow-migrating glioma cells is depicted in a color-coded way, showing higher expression of the gene of interest in fast-migrating glioma cells in increasing green shadings ($y > 1$), and lower expression in comparison to slow-migrating cells in increasing red shadings ($y < 1$). When expression was about equal in both fractions, the background color is yellow ($y \sim 1$). If the Ct value of one fraction was below the detection limit, relative expression was calculated using the detection limit (40 cycles), and data are displayed in white numbers. In case of both fractions being undetectable, this was marked "undet." In general, we could observe that gene expression of the "guerilla gene set" was decreased in fast-migrating cells of astrocytomas of grades II and III, but mostly increased in primary and secondary glioblastomas. In recurrences however, we again observed lower expressions of the "guerilla gene set" in fast-migrating cells. This remarkably hints to an "evolutionary-like" development especially of the fast-migrating tumor cells during glioma progression. Regarding the expression of "stem cell markers," we observed specific regulation patterns between fast- and slow-migrating glioma cells that were concerning their tendency comparable to those of the "guerilla gene set"; however, the regulation patterns were not as uniform as observed for the "guerilla gene set."

contrast, especially FN1, CXCR7, GFAP, TWIST1, and SNAI2 were uniformly downregulated in fast-migrating cells of GBM recurrences. Interestingly, this could also be observed for GFAP in astrocytomas of grades II and III. In addition, MSI1 was clearly upregulated in fast-migrating cells of primary and secondary GMB, but strongly downregulated in GBM recurrences. However, for "stem cell markers," the regulation pattern was not as uniform as for the "guerilla gene set" when looking at different tumor entities.

Thus, when isolating fast-migrating cells of fresh surgically resected glioma samples of different malignancy grades, the progression-dependent expression and regulation pattern of the "guerilla gene set" and the "stem cell markers" hint to an "evolutionary-like" process and a close relation between migratory and stemness properties of glioma cells.

Transfer of Migration-Associated Expression Patterns to Human Glioblastoma Cell Lines

To evaluate whether expression patterns of the "guerilla gene set" and the "stem cell markers" observed in fast-migrating cells from fresh glioblastoma samples may also be transferable to fast-migrating cells of commercial human glioblastoma cell lines, we initially measured basal mRNA transcription of investigated genes in T98G, U251MG, and A172 cell lines. As depicted in Figure 3A, nearly all investigated genes were expressed in the different GBM cell lines, with few exceptions. However, broad expression differences existed between both different cell lines and individual genes, and some markers were even expressed at very low levels or were undetectable [e.g., GFAP (T98G, A172)] stem cell markers (especially in T98G).

Next, we isolated fast- and slow-migrating cells from glioblastoma cell lines using the pore migration assay and checked for relative expression differences between fast- and slow-migrating fractions concerning the "guerilla gene set" (Fig. 3B). In accordance with the previous results obtained from fresh surgical GBM samples, all genes of the "guerilla gene set" were upregulated in fast-migrating T98G, U251MG, and A172 cells. Also, a broad expression variety existed ranging, for example, for SNAI2, from 1.30-fold (A172) to 58.31-fold (T98G) upregulation in fast-migrating cell (Fig. 3B, top). In contrast, since "stem cell markers" were expressed at low levels in GBM cell lines (Fig. 3A), the detection of PROM1, MSI1, NES, SOX2, and CXCR4 was very difficult in fast-migrating cells of T98G, U251MG, and A172 cells, yielding hardly reliable results as at least one Ct value (either of native or fast-migrating cells) was not detectable by qRT-PCR (indicated by white numbers) (Fig. 3B, bottom). Although we cannot draw the conclusion that migratory and stemness properties of GBM cells are closely linked with this approach, we were able to confirm the overexpression of the "guerilla gene set" in fast-migrating cells of GBM cells lines.

To overcome this technical problem, we prepared stem-like cells of GBM cell lines and examined whether these "stem cell markers" overexpressing cells in turn were also characterized by an upregulation of the "guerilla gene set." Results are depicted in Figure 3C (comparison of native and stem-like T98G, U251MG, and A172 cells). As expected, all stem-like GBM cells formed spheroids and showed a clear overexpression of PROM1, MSI1, NES, SOX2, and CXCR4 in comparison to the cell lines they were derived from (Fig. 3C, top). When culturing these spheroids under conditions that promote cell differentiation, the expression of the investigated stem cell markers diminished dramatically (depending on individual gene expression differences of ~10- to ~1,000-fold with clearly lower expression in differentiated cells; data not shown), confirming the existence of stemness dynamics in the prepared stem-like cultures.

As shown in Figure 3C (bottom), all genes of the "guerilla gene set" were upregulated in stem-like cells of GBM cell lines, clearly confirming the close connection between migratory and stemness properties of malignant gliomas. Interestingly, FN1 and CXCR7 were the candidates that showed the highest upregulation in stem-like cells, whereas especially TWIST1 but also SNAI2 were only upregulated to lesser extent.

Taken together, isolated fast-migrating and also stem-like cells of GBM cell lines showed migration-associated gene expression profiles similar to those of fresh surgically dissected primary/secondary but not recurrent glioblastoma samples.

Influence of Temozolomide on Migration Properties

To analyze whether the chemotherapeutic agent temozolomide influences migratory properties in different GBM cell lines, we first stimulated T98G, U251MG, and A172 cells for 10 days with a sublethal dose of temozolomide (DMSO serving as a control), and then performed a pore migration assay and measured gene expression of selected genes of the "guerilla gene set" (Fig. 4A). Interestingly, the three GBM cell lines acted individually: while in T98G fast-migrating cell transcription of ITGA5, CXCR7, VIM, and SNAI2 was not regulated upon temozolomide treatment, ITGA5, CXCR7, and SNAI2 expression was significantly downregulated in fast-migrating temozolomide-treated U251MG cells (ITGA5: 0.87 ± 0.07; CXCR7: 0.52 ± 0.33; SNAI2: 0.55 ± 0.15) (Fig. 4A). In contrast, ITGA5, CXCR7, and VIM were significantly upregulated in fast-migrating temozolomide-treated A172 cells (ITGA5: 2.79 ± 1.94; CXCR7: 2.56 ± 1.57; VIM: 2.24 ± 0.52) (Fig. 4A).

To prove whether these different expression profiles result in an altered migratory potential of T98G,

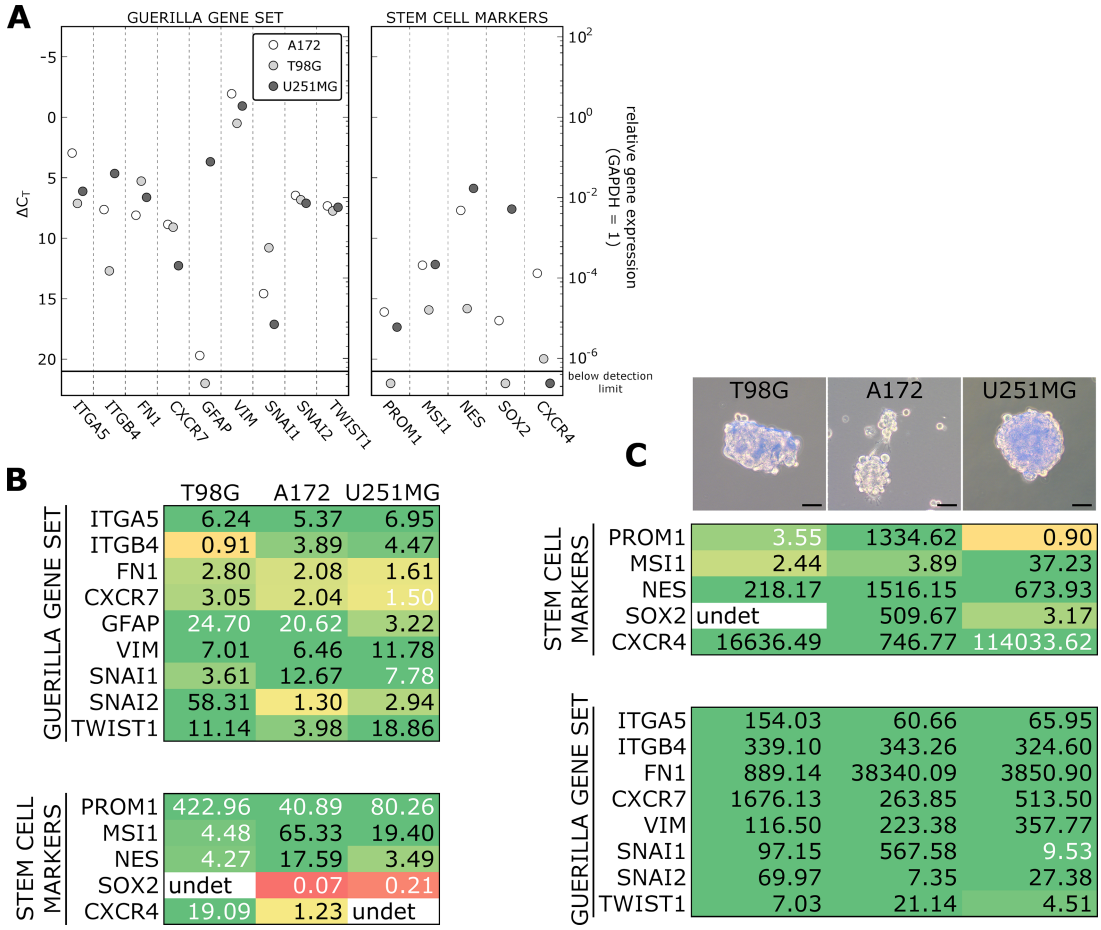


Figure 3. Analysis of (A) transcription of the "guerilla gene set" and "stem cell markers" in human glioblastoma cell lines, relative gene expression differences (B) between fast- and slow-migrating cells, and (C) between stem-like cells and native cells of these cell lines. (A) The commercially available glioblastoma cell lines A172 (white circles), T98G (light gray), and U251MG (dark gray) were analyzed by qRT-PCR concerning their basic mRNA expression levels of the "guerilla gene set" and the "stem cell markers" gene group. ΔCt values indicate the relative gene expression in comparison to the housekeeping gene GAPDH in a logarithmic plotting (left axis), which means a 3.33 lower ΔCt value denotes a 10-fold lower gene expression (depicted in a logarithmic scale on the right axis). All markers were detectable in the three cell lines, except for GFAP in A172 and T98G, PROM1 and SOX2 in T98G, and CXCR4 in U251MG cells. Obviously, for some genes, quite homogenous expression was observed (e.g., for SNAI2 and TWIST), while a broader variation was observed for others (e.g., GFAP). (B) Cell lines were subjected to the pore migration assay, and fast-migrating and slow-migrating cells were collected. Fractions were separately analyzed for the mRNA expression of the "guerilla gene set" and the "stem cell markers." The relative gene expression between fast-migrating and slow-migrating glioma cells is shown here in a color-coded way, implicating a higher expression of the gene of interest in fast-migrating glioma cells in increasing green shadings ($y > 1$), and lower expression in comparison to the slow-migrating fraction in increasing red shadings ($y < 1$). When expression was about equal in slow- and fast-migrating cells, the background color is yellow ($y \sim 1$). If the Ct value of one fraction was below the detection limit after 40 PCR cycles, the relative expression was calculated using this detection limit, and mean data are displayed in white numbers. In case of both fractions being undetectable, this was marked "undet." As previously observed for the primary/secondary glioblastomas, fast-migrating cells from glioblastoma cell lines mostly showed higher expression of the "guerilla gene set" in comparison to slow-migrating cells. This result was closely mirrored in the gene set of "stem cell markers" when comparing fast-migrating to native cells of the cell lines A172, T98G, and U251MG, except for SOX2. However, the basal expression of SOX2 and other stem cell markers was quite low, so that frequently the detection in the fast-migrating cells was impossible. (C) Stem-like cells were generated from human glioblastoma cell lines T98G, U251MG, and A172 by sequential subcultivation in neurosphere medium. Top: In these conditions, the cells formed three-dimensional aggregates, as exemplarily shown (scale bars: 100 μm). Middle: Analysis of stem cell markers by qRT-PCR revealed a successful preparation of stem cell marker highly expressing cells. Shown are the relative expression/induction of stem cell markers in stem-like cell preparations in comparison to native cell lines. Bottom: Apart from the stem cell markers, also the genes of the "guerilla gene set" were dramatically induced in generated stem-like cells. However, the extent of upregulation was quite different depending on cell line and gene (e.g., low for TWIST and SNAI2 in comparison to CXCR7, FN1). Comparable to the presentation of fast- versus slow-migrating glioma cells, higher expression levels in stem-like cell preparations are assigned with a green background, about equal levels yellow. If a gene was undetectable in the stem-like cell preparation or in the native cell line, numbers are white; if both did not yield a signal, this is marked as "undet."

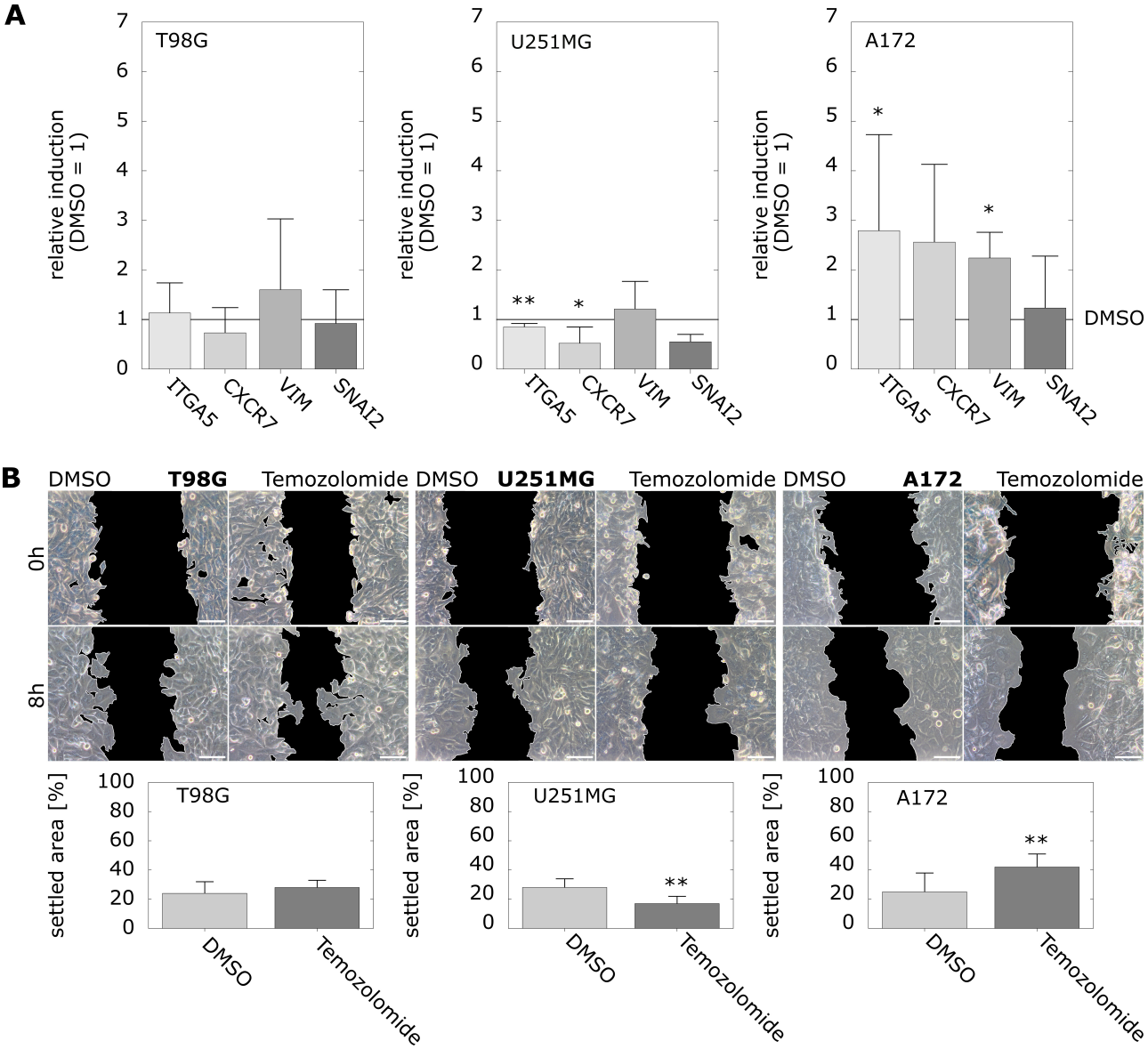


Figure 4. Temozolomide affects (A) the expression pattern of selected "guerilla genes" in fast- and slow-migrating T98G, U251MG, and A172 cells, and (B) the migratory potential of these human glioblastoma cell lines. (A) Glioblastoma cell lines were treated for 10 days with a sublethal dose of temozolomide (500 µM) or solvent control [0.2% (v/v) DMSO = 1] and subjected to a pore migration assay. Relative gene expression patterns of selected "guerilla genes" (ITGA5, CXCR7, VIM, and SNAI2) were analyzed by qRT-PCR between fast- and slow-migrating cell fractions and normalized to the regulation patterns detected for the DMSO control. Here we observed that the cell lines T98G, U251MG, and A172 exhibited cell line-specific reactions on the temozolomide treatment. While the expression differences in slow- and fast-migrating T98G cells were hardly affected by prior temozolomide treatment, U251MG cells showed significantly less induction of ITGA5 and CXCR7 in fast-migrating cells after temozolomide treatment, contrasted by A172 cells that showed induced regulation patterns with significant changes for ITGA5 and VIM ($n = 5$ independent experiments; error bar indicates standard deviation). (B) Cells were treated with 500 µM temozolomide for 10 days [controls: 0.2% (v/v) DMSO] and seeded confluently to culture dishes. Migration into a scratch made with a pipet tip was time resolved analyzed (here exemplarily shown after 8 h; scale bars: 100 µm). Migratory potential of temozolomide-treated cells in comparison to control (DMSO treated) cells was cell line dependent. T98G cells showed equal migratory potential, irrespective of the treatment. Temozolomide-treated U251MG cell migrated less than control cells, while temozolomide-treated A172 cells showed higher migration rates than control cells. Micrographs show examples of scratches after 0 and 8 h, diagrams show mean settled area measurements in percentage of $n = 6$ individual experiments, and error bar indicates standard deviation. *$p < 0.05$, **$p < 0.01$.

U251MG, and A172 cells treated with temozolomide in comparison to DMSO-treated control cells, we next performed a scratch assay and measured the settled areas after 8 h of migration time (Fig. 4B). Indeed, in relation to DMSO-treated control cells, for T98G, we did not observe an influence on migration, whereas U251MG cells treated with temozolomide migrated slower and A172 cells treated with temozolomide migrated faster (U251MG: DMSO, 28±6; temozolomide, 17±5; A172: DMSO, 25±13; temozolomide, 42±9) (Fig. 4B). Thus, temozolomide clearly influenced the migration properties of different GBM cell lines in a distinct way, which is reflected by the cell lines' individual "guerilla gene set" expression profile.

In summary, our results indicate that "evolutionary-like" expression changes occurred during glioma progression when comparing slow- and fast-migrating cells of freshly obtained surgical human gliomas. Further, a close relation became evident between migratory and stemness properties. Regulation patterns in gene expression could also be identified in GBM cell lines, not only regarding fast- and slow-migrating cells but also with respect to temozolomide-treated and untreated cells. These differences coincided with the expression of stem cell markers and the migration potential in the respective cell lines.

DISCUSSION

The analysis of the invasion potential of gliomas has been examined in many studies previously[12]. In this topic, Hans-Joachim Scherer was one of the pioneers describing the invasion morphology of diffuse gliomas[25]. Afterward, several independent studies showed that, for example, cell–cell and cell–extracellular matrix crosstalk are indispensable factors in glioma invasion[3,26] and demonstrated that hypoxia might play an important role because of its relation to the mesenchymal shift in GBM, which leads to an elevated migratory potential[27]. In addition, some investigations used genome-wide screening approaches deepening insights into genes, RNAis, and molecular pathways involved in the migration processes of GBMs[5–8]. In these studies, GBM cell lines or human glioma stem cell lines were used and deployed for several different migration assays like Matrigel invasion combined with wound healing assays[6], radial cell migration combined with organotypic brain slice invasion assays[5,7], as well as human stem cell xenograft models to separate fast-migrating GBM cells[8]. In these set-ups, several different gene groups and networks involved in EMT, cell polarity, neurophysiological processes, signaling, cell cycle, stemness, and cell adhesion were identified[8,12]. However, in most cases, GBM cell lines were investigated, and, to date, comprehensive investigations focusing on progression-dependent characteristics of migration processes do not exist. Thus, in our approach, we isolated tumor cells from fresh human glioma samples of different malignancy grades according to their migratory potential and characterized them with regard to transcription of typical genes involved in migration and invasion (ITGA5 and ITGB4), tumor progression (CXCR7 and GFAP), stemness (PROM1, MSI1, NES, SOX2, and CXCR4), and EMT (FN1, VIM, SNAI1, SNAI2, and TWIST1).

We were able to show that a progression-dependent expression and regulation pattern of investigated genes revealed an "evolutionary-like" process and a close relation between migratory and stemness properties of glioma cells. A multitude of genes were often expressed at lower levels in fast-migrating cells of astrocytomas of grades II and III compared to slow-migrating cells. These genes were elevated in fast-migrating cells of glioblastomas and decreased in fast-migrating cells of GBM recurrences. In addition, when transferring the results to commercial GBM cell lines, isolated fast-migrating and also stem-like cells of GBM cell lines showed migration-associated gene expression profiles similar to those of fresh surgical glioblastoma samples.

When reviewing these results in light of recent literature, several connections become evident. For example, integrins were previously identified to be overexpressed in gliomas, promoting invasion and migration[14,28]. Also, integrin α3 was shown to be overexpressed in $CD133^+$ neurospheres compared to $CD133^-$ neurospheres and could be detected in situ in tumor cells localized in the perivascular stem cell niche[14]. In accordance with this, our study showed an upregulation of two representative integrins (ITGA5 and ITGB4), observed in fast-migrating glioma cells. Moreover, as stated in our study and proven by expression analysis of stem cell markers in fast-migrating cells as well as of migration-associated genes including ITGA5 in stem-like cells, a connection of migration and stemness is quite likely.

In addition, EMT transition is well known to be correlated to an increased migratory behavior[29,30]. In fact, it was shown that ZEB1 and ZEB2 are responsible for disrupting cell–cell contact inhibition. TWIST and SNAI family members augment glioblastoma cell motility and invasiveness, and β-catenin and miR-21 enhance the extracellular matrix cleavage, suggesting that EMT is a major molecular event in glial tumors[31]. Nevo et al.[8] showed that several genes involved in EMT (e.g., STAT3, CHI3L1, and CD44) were downregulated in a human glioma stem cell xenograft model to explore gene expression changes in situ in invading glioma cells compared to the tumor core. In contrast, Balasubramaniyan et al.[32] described that, during differentiation of cancer stem-like cells, mesenchymal gene expression signatures were induced. We were able to show that fast-migrating cells of primary GBMs are characterized by higher expression of the EMT markers TWIST1, SNAI1, and SNAI2, while

these markers were expressed lower by fast-migrating cells of recurrent tumors. In addition, expression analysis of the "stem cells markers" including MSI1 yielded a higher expression in primary samples. This is in accordance with our own previously published results, showing that in comparison to matched primary samples, solid tumors of recurrent GBMs expressed these genes at a significantly lower level[23,33]. Thus, when comparing either fast-migrating cells of primary and recurrent GBMs or matched primary and recurrent samples of the same patient, TWIST1, SNAI1, SNAI2, and the "stem cell markers" including MSI1 expression were strongly upregulated in primary samples, respectively.

Further, it is known that expression of several stem cell markers (PROM1, MSI1, NES, and SOX2) increases during glioma progression[24]. In accordance with this, we now demonstrated that these stem cell markers were found at lower expression levels in fast-migrating cells of astrocytomas of grades II and III and were overexpressed in GBM fast-migrating cells. Vice versa, glioma stem-like cells were characterized by an increased migration-associated gene expression profile. All these results clearly point to a progression-dependent expression of migration-associated genes and stem cell markers and to a close connection between stemness and migratory properties. As described previously, CXCR7 expression increased with glioma malignancy, and CXCR7 is expressed in solid samples of GBM recurrences at a lower level[16,22]. Thus, comparing the expression pattern of either fast-migrating cells or solid tumors during glioma progression and recurrence, similar expression profiles of CXCR7 become evident.

However, this assumption does not fit for all investigated genes. For example, in solid tumors, CXCR4 expression did not change during glioma progression or recurrence[16,22], and EMT markers FN1 and VIM, as well as the intermediate filament GFAP and the stem cell marker SOX2, were similarly expressed in matched solid primary and recurrent samples[23,33], but were found to be expressed in a progression-dependent manner in fast-migrating glioma cells.

Finally, the influence of temozolomide on the expression of migration-associated genes in different GBM cell lines was examined. Here variations in gene expression could also be identified in GBM cell lines treated with temozolomide, and differences coincided with the expression of the "stem cell markers" and the migratory potential in the respective cell lines. An increased expression of ITGA5 and CXCR7 was observed, which accounts for an enhanced resistance of in vitro-isolated fast-migrating cells, which is also supported by the discoveries of Bruyère et al.[34].

In summary, it seems that "evolutionary-like" expression changes occurred during glioma progression when comparing slow- and fast-migrating cells of fresh human gliomas. Furthermore, a close relation between migratory and stemness properties seems to be most likely, which is proven by overexpression of migration-associated genes in glioma stem-like cells and, vice versa, of stem cell markers in fast-migrating cells. In addition, temozolomide affects the migratory properties of different GBM cell lines in a distinct way, which is reflected by an individual expression profile of migration-associated genes.

CONCLUSIONS

The expression of migration-related genes in fast-migrating glioma cells was not only regulated in a progression-dependent manner, but fast-migrating cells also harbored specific stem cell-like features. Therefore, the identification of the stem likeness of fast-migrating cells may provide new strategic approaches for treatment applications.

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