RESEARCH ARTICLE

Genes Associated with Fast Glioma Cell Migration In Vitro and In Vivo

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Identification of genes mediating glioma invasion promotes the understanding of glia motility and might result in biologically based therapeutic approaches. Most experimental studies have been performed in vitro, although glial cells typically undergo marked phenotypic change following placement into cell culture. To evaluate migration mechanisms operating in vitro versus in vivo, we used C6 rat glioblastoma cells for selecting highly migratory cells in a monolayer migration assay as well as in brains of nude mice, and analyzed in each paradigm the expression profiles of these "fast" cells versus those of the original "slow" cells using oligonucleotide microarrays comprising 8832 genes. In vitro, 516 (10.6%) of 4848 expressed genes were regulated (ie, differentially expressed in fast versus slow cells); 916 genes were expressed only in vitro, including 142 (15.5%) regulated genes. In vivo, 245 (6.1%) of 4044 expressed genes were regulated; 112 genes were expressed only in vivo, including 25 (22.3%) regulated genes, none of them having a known relation to glioma invasion. Of 730 regulated genes, only 31 (4.2%) were regulated in parallel in vitro and in vivo, most of them having a known relation to (glioma) invasion. Our data provide new molecular entry points for identifying glioma invasion genes operating exclusively in the brain. They further suggest that genes underlying glia cell motility are strikingly different in vitro and in vivo.

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INTRODUCTION

Glioblastoma is the most common and most malignant human brain tumor. One of its typical features is extensive invasion of single tumor cells into brain tissue, which prevents complete surgical resection and to some extent is responsible for the poor prognosis with median survival times of about 12 months (23). It is expected that understanding the molecular basis of this fatal diffuse invasion pattern will result in new, biologically based therapeutic approaches. Furthermore, based on the hypothesis of ontogenetically and phylogenetically conserved molecular mechanisms, data obtained on glial tumors can also promote the understanding of glia cell motility in general.

In the past few years a wide variety of genes have been described as being functionally involved in glioma invasion, such as proteases, adhesion molecules, extracellular matrix components and growth factors (13, 31, 36, 37). In addition, DNA microarray analysis studies have revealed a variety of genes associated with motility in glioma cell lines (27, 40). Since the vast majority of data have been obtained in vitro or after subcutaneous transplantation of tumor cells in vivo, they incompletely reflect the mechanisms operating in the brain. However, glioma cells undergo a marked phenotypic change upon placing them into cell culture, a process that has been termed mesenchymal drift (29). Specifically, the expression patterns of adhesion molecules and extracellular matrix components of glioma cell lines and primary glioblastoma cells are strikingly different from that of glioma cells in situ, ie, in the human brain (34, 35). Accordingly, global gene expression profiling of human glioma cells grown in vitro versus subcutaneously in vivo showed marked expression differences for genes encoding extracellular matrix components, adhesion molecules and metalloendopeptidases, among others (7). It is conceivable that changes in the expression patterns of those migration-related genes affect glioma cell motility, but differences and similarities in molecular mechanisms underlying glioma cell motility between in vitro and in vivo conditions are virtually unknown.

Here, we have selected highly motile ("fast") C6 rat glioma cells using several rounds of in vitro migration assays and compared their global gene expression profile to that of the original "slow" cells. Corresponding experiments were performed in vivo, where C6 cells stably transfected with green fluorescent protein (GFP) were selected for invasion of nude mouse brain using serial intracerebral transplantations. We found pronounced differences in motility-associated gene expression patterns between in vitro and in vivo selected cells, suggesting that the cellular environment exerts a great impact on motility mechanisms and that in vitro glioma invasion studies require cautious interpretation.

MATERIALS AND METHODS

Plasmids. The expression vector pMPSGGFP contains the hrGFP gene (humanized renilla reniformis green fluorescence protein, Stratagene, La Jolla, Calif) driven by the MPSV promoter (1). The plasmid pcDNA3.1/neo containing the neomycin resistance gene was purchased from Invitrogen (Groningen, The Netherlands).

Cell culture and transfection. The rat C6 glioma cell line (ATCC, Manassas, Va) was grown in Dulbecco's modified Eagle's minimal essential medium (DMEM), supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate and 4 mM L-glutamine (all cell culture reagents purchased from PAA, Linz, Austria). Cells were incubated under standard conditions (37°C in a 5% CO_2 humidified atmosphere).

To obtain stable expression of hrGFP the vectors pMPSGGFP and pcDNA3.1/neo were cotransfected using Effectene Trans-

fection Reagent according to manufacturer's instructions (Qiagen, Hilden, Germany) at a molar ratio of 5:1. After 2 weeks of selection with 100 µg/ml G418, clones were checked with an Olympus IX50 fluorescence microscope at 488 nm. Clones with strong and uniform hrGFP fluorescence were picked and expanded to cell lines. The clone chosen for all further experiments was named C6-SP-GFP (38).

Selection for migration in vitro. Highly migratory C6 rat glioma cells were selected using a modification of the technique reported by McDonough et al (28). Round coverslips of 10-mm diameter (Plano, Wetzlar, Germany) were attached to the center of each well in a 24-well cell culture plate (Nunc, Wiesbaden, Germany) using silicone (KAWO-SL 59, KAWO, Hildesheim, Germany). Immediately before seeding, each well was coated with 500 µl of Matrigel (BD Biosciences, Erembodegem, Belgium) solution containing 100 µg/ml in serum free DMEM. Sedimentation cylinders with an inner diameter of 1.2 mm were then placed in the center of the coverslips of each well and 2000 cells were seeded in a volume of one µl DMEM. Sedimentation cylinders were removed 16 hours after seeding, and cells were allowed to migrate over the border of the coverslips. Then the coverslips containing the slower cells were removed, and the faster cells in the wells of the culture plate were collected by trypsinization and used for another cycle of selection. A total of 18 rounds of selection were performed, and the resulting cells were designated C6fast. After the first cycle of selection the cells remaining on coverslips were also collected and called C6slow. The slower cells in the following cycles of selection were discarded. Since several passages in culture without any selection might cause transcriptional changes, parallel culture of C6slow cells was maintained without selection pressure during selection experiments. Thus, slow and fast cells that were compared using microarrays had undergone the same number of passages using identical experimental conditions.

Selection for migration in vivo. C6-SP-GFP cells were transplanted into the right striatum of female CD1 nude mice (Charles River, Sulzfeld, Germany) aged 9 weeks using a stereotactic frame (Narashige, Tokyo, Japan) as previously described (39). The animals were anesthetized with an intraperitoneal injection of 150 µl of a solution containing 2% Ketanest and 0.3% Rompun in 0.9% NaCl. Two microliters cell suspension containing 4×10^4 cells was injected at 4 mm anterior to the interaural line, 2.5 mm lateral to the midline, in a depth of 3.5 mm into the brain with a Hamilton syringe fitted with a cannula of 0.15 mm inner and 0.72 mm outer diameter. After 2 weeks animals were killed, brains were removed and placed in ice-cold phosphate buffered saline (PBS, PAA). Then the cerebellum was trimmed, and the brain sectioned in the frontal plane on a vibratome. The resulting coronal slices of 500-µm thickness were examined using an Olympus IX50 fluorescence microscope and tumor growth, localization and hrGFP-fluorescence were verified. In the slices with greatest tumor extension, meninges were trimmed, slices were repeatedly washed in PBS, and the central compact tumor areas were dissected out under a dissection microscope (Figure 1A, B). The remaining slice containing diffusely invading glioma cells (Figure 1C) was finely minced with 2 scalpels and recultured in DMEM in a cell culture dish (10 cm, Nunc) under standard cell culture conditions overnight. Then the medium containing cell and tissue debris was aspirated and the plate was rinsed twice with PBS. The remaining adherent cells were checked for hrGFP fluorescence and used for another in vivo selection cycle. Altogether 4 in vivo selection cycles were performed. Selected cells were designated

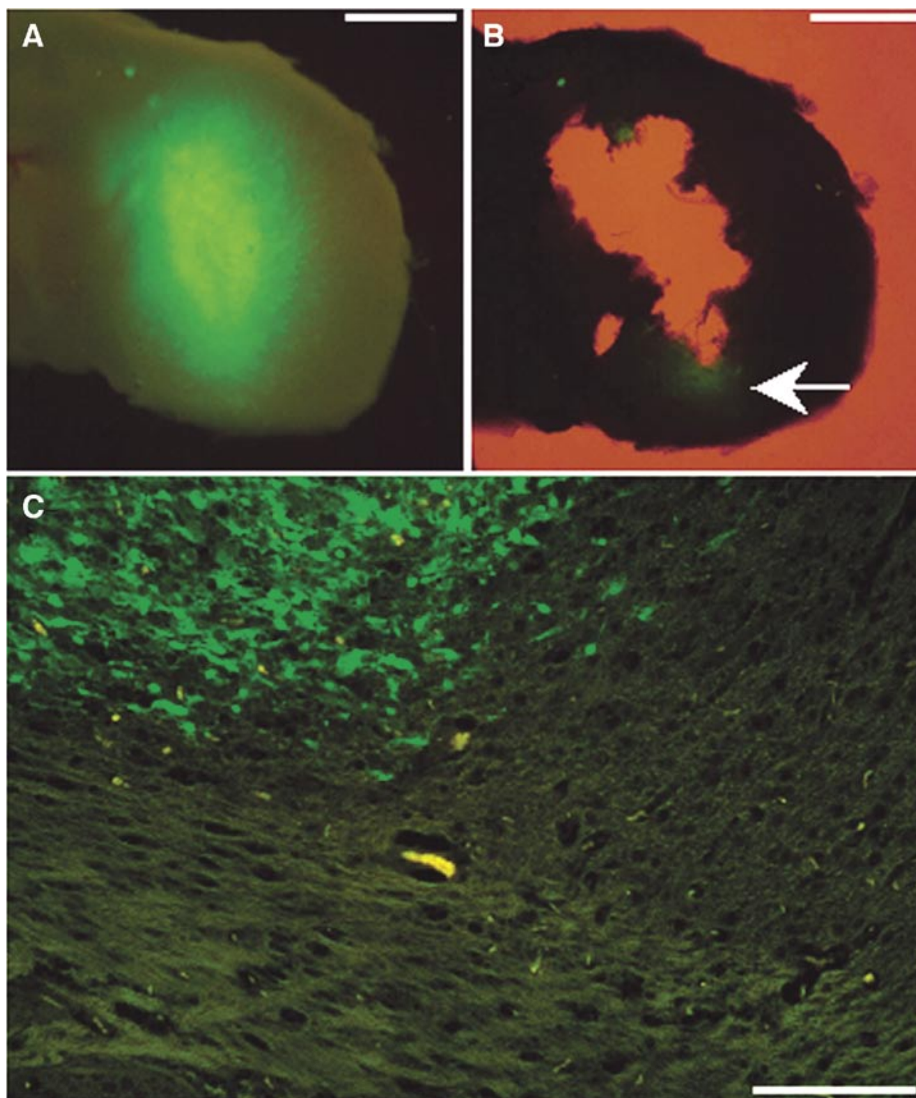

Figure 1. Selection of the most invasive C6-SP-GFP rat glioma cells in nude mice brain in vivo. After intracerebral establishment of GFP-positive tumors (**A**), the central compact tumor mass was excised (**B**), the invasive rim of the tumor (arrow in **B**) was prepared, and highly invasive tumor cells (**C**) were recultured. Length of scale bars represent one mm (**A, B**) and 100 µm (**C**).

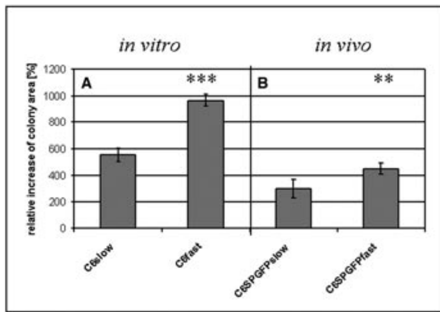

Figure 2. Results of monolayer migration assays used to verify increased migration following selection. Increase of colony areas at the end of the assay (72 hours) is expressed relative to colony area after removal of sedimentation cylinders. Bars represent means of 8-fold replicates with standard deviations. Increase of colony areas was different between slow versus fast cells in both C6 (***: t-test, $p<0.001$, **A**) and C6-SP-GFP cells (**: t-test, $p<0.01$, **B**).

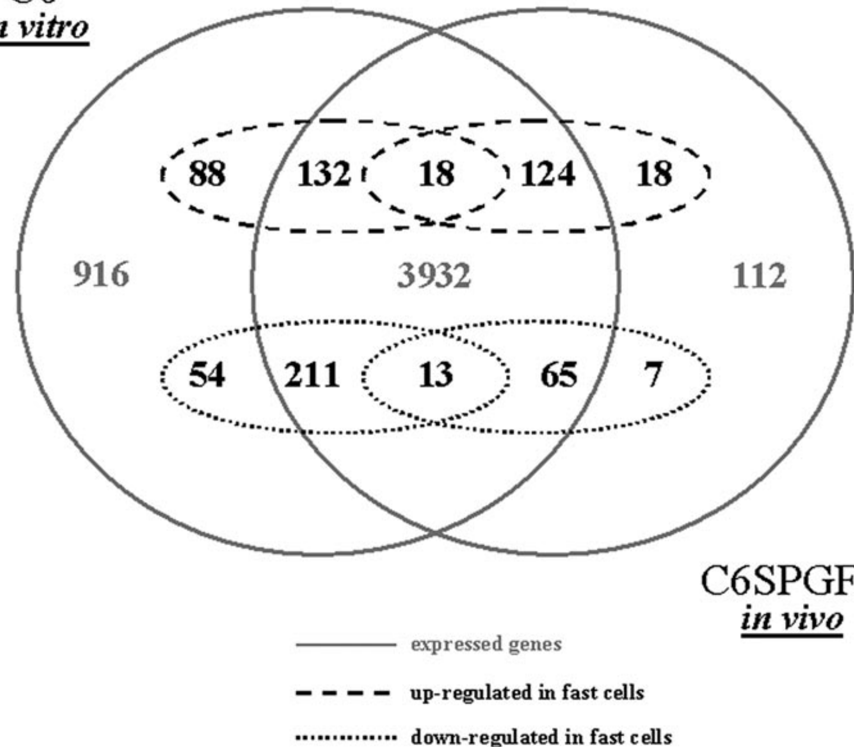

Figure 3. Venn diagram of expressed and regulated genes in C6 and C6-SP-GFP glioma cells.

C6-SP-GFPfast, while C6-SP-GFP cells maintained without selection pressure were named C6-SP-GFPslow. Since C6-SP-GFPfast cells used for microarray analysis consistently showed hrGFP fluorescence, we were able to verify that only tumor cells were analyzed, while non-neoplastic cells, such as neurons, vascular cells and mononuclear infiltrates, were absent.

In vitro monolayer migration assay. In vitro monolayer migration assays were performed on coated Permanox LabTek ChamberSlides™ with 8 chambers (Nunc) as described (3) with some modifications. Briefly, slides were incubated with 100 µl per chamber of Matrigel solution containing 100 µg/ml in serum free DMEM. After one hour incubation under normal culture conditions, chambers were washed 3 times with PBS and incubated with 100 µl of a BSA solution (one mg/ml in PBS, sterile filtered) for 30 minutes at room temperature. After additional washing with PBS, slides were used for migration assays. Sterile sedimentation cylinders (1.2-mm inner diameter, 7-mm outer diameter) were filled with medium and placed into the chambers. Two thousand cells were seeded in a volume of one µl DMEM. For sedimentation and adhesion the slides were incubated for 16 hours under standard culture conditions. After removing the sedimentation cylinders medium was gently aspirated and replaced by 500 µl DMEM per chamber. At this time cells had formed round colonies and started migrating on the substrate. Colonies were fixed with 3.7% formaldehyde/PBS directly after removing the sedimentation cylinders ($t=0$) and after 72 hours. Cells were stained with Coomassie blue (0.05% w/v in 50% methanol, 10% acetic acid). Experiments were performed as 8-fold replicates.

Morphometry. Distance of migration was determined by using an Olympus BX50 microscope at 25-fold magnification, a digitizing CCD camera Olympus DP10 (Olympus, Hamburg, Germany), and Sigma Scan Pro 4 image analysis software (SPSS Science, Chicago, Ill). Colonies were surrounded by using the distance and area measurement tool and colony areas were automatically determined. Data were exported to an .cel database and statistically evaluated.

Oligonucleotide microarray analysis. The Affymetrix Rat Genome U34 A Array (RG U34 A, Affymetrix, Santa Clara, Calif) is complementary to 8832 genes and ESTs. Affymetrix GeneChip analysis was performed according to the instructions of the Expression Analysis Technical Manual (Affymetrix). Cells were grown in 6-cm cell culture dishes with (C6 cells) or without (C6-SP-GFP cells) Matrigel. Total RNA was extracted at about 80% confluency using the RNeasy kit (Qiagen, Hilden, Germany). Double-stranded cDNA was synthesized from total RNA using Gibco BRL Superscript Choice System (Invitrogen, Karlsruhe, Germany) and a T7-$(dT)_{24}$ primer (5′-GGCCA GTGAA TTGTA ATACG ACTCA CTATA GGGAG GCGG-$(dT)_{24}$-3′). After phenol/chloroform extraction of double-stranded cDNA, synthesis of biotin-labeled cRNA was performed using the ENZO BioArray™ HighYield™ RNA Transcript Labeling Kit (ENZO Life Sciences, Farmingdale, NY). After purification with RNeasy kit (Qiagen), ethanol precipitation and quantifying, the biotinylated cRNA was fragmented by alkaline treatment. Fifteen micrograms of adjusted fragmented cRNA in a hybridization cocktail containing eukaryotic hybridization controls were hybridized to a GeneChip Probe Array for 16 hours. After washing and staining Probe Arrays were scanned in an Affymetrix GeneChip scanner. Data (Excel-files) were exported to a Cobi database and analyzed using GeneData Expressionist software (GeneData, Basel, Switzerland). Duplicate experiments were performed. To analyze and identify possible functional groups, genes were grouped into Gene Ontology (GO) Consortium classes of GO categories of "biological process,"

"molecular function" and "cellular component" (*http://www.geneontology.org*). In order to reduce false positive results, we applied stringent criteria for identifying differentially expressed genes, ie, the t-test at $p<0.05$ plus differences in expression levels of more than 1.5-fold.

RT-PCR. To verify the GeneChip data with an independent technique, RT-PCR was performed to check the transcripts of PSMB8, LAMA3, ID3 and MGP in comparison to GAPDH. Template cDNA was synthesized out of 2 µg total RNA using the Omniscript™ Reverse Transcriptase Kit (Quiagen) and the T7-$(dT)_{24}$ primer following manufacturer's instructions. Forward and reverse primers were 5´-AATGG CGCAT GGCAC AA-3´ and 5´-GCCGG GAGGA TGTCT GC-3´ for PSMB8, 5´-GCAGA TGGCC GTGTT GTCT-3´ and 5´-TATCC AGTTC TAGAT GCAGG ATGC-3´ for LAMA3, 5´-AGGAG CCGCT TAGCC TCTTG-3´ and 5´-CTCCA GGTCC AGCCT CGAG-3´ for ID3, 5´-TATGA ATCTC ACGAA AGCAT GGAA-3´ and 5´-TCATT ACTTT CAACC CGCGG-3´ for MGP, 5´-GCCAT CACTG CCACC CAGAA GAC-3´ and 5´-CATAC CAGGA AATGA GCTTG ACAAA-3´ for GAPDH. RT-PCR was performed using Hot Star Taq Polymerase Kit (Qiagen) following manufacturer's instructions with one µl of cDNA template. Cycling conditions were 95°C for 15 minutes, followed by 25 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for one minute. PCR products were run on 1.5% agarose gels stained with ethidium bromide and analyzed using Gel-Pro Analyzer™ Software (Version 3.0, Media Cybernetics Inc., Silver Spring, Md).

Gene Description	Symbol	GenBank	Score	p
Genes up-regulated in C6fast				
Nucleoside diphosphate kinase 7	NDK7	AI112173	31.7	0.0012
Laminin-5 alpha 3	LAMA3	AA946108	19.2	0.0055
Sialophorin	SPN	AI045440	13.0	0.0023
Genes down-regulated in C6fast				
Small inducible cytokine A2	SCYA2	X17053	24.6	0.0065
EST, moderately similar to g1-related zinc finger protein [M. musculus]	-	AA891810	16.7	0.0066
Agrin	AGRN	M64780	9.1	0.0010
Biglycan	BGN	U17834	8.7	0.0203
Fatty acid binding protein 5, epidermal	FABP5	S69874	8.7	0.0100
Integrin, alpha 1	ITGA1	X52140	7.8	0.0029
EST	-	AA800786	7.8	0.0108

Table 1. Expressed genes with highest regulation in C6fast versus C6slow cells. Score represents fold up-regulation or down-regulation of expression in fast versus slow cells. p values are levels of statistical significance using t-test.

Gene Description	Symbol	GenBank	Score	p
Genes up-regulated in C6-SP-GFPfast				
EST, highly similar to SMRT2 metallothionein II -[R.norvegicus]	-	AI176456	9.8	0.0016
Follistatin	FST	AA858520	4.5	0.0007
Transferrin	TF	D38380	4.4	0.0018
Gonadotropin releasing hormone receptor	GNRHR	S59525	4.0	0.0099
Diaphorase (NADH/NADPH)	NQO1	J02679	3.7	0.0299
Small inducible cytokine A2	SCYA2	X17053	3.5	0.0051
Complement component 1, s subcomponent	CLS	D88250	3.3	0.0046
Genes down-regulated in C6-SP-GFPfast				
EST	-	AA892986	12.3	0.0008
Matrix gla protein	MGP	AI012030	6.3	0.0034
Gephyrin	GPHN	X66366	4.6	0.0031

Table 2. Expressed genes with highest regulation in C6-SP-GFPfast versus C6-SP-GFPslow cells. Score represents fold up-regulation or down-regulation of expression in fast versus slow cells. p values are levels of statistical significance using t-test.

RESULTS

Establishment of fast cell clones. C6fast cells, which had undergone 18 cycles of selection for in vitro migration, were compared to C6slow cells, which were passaged without selection pressure. In a monolayer migration assay the colony area of C6fast cells was 1.7-fold greater after 72 hours migration as compared to C6slow cells ($p<0.001$, t-test; Figure 2A). The colony area of C6-SP-GFPfast cells, which had undergone 4 cycles of in vivo selection, was 1.5-fold larger than that of parental C6-SP-GFPslow cells which were passaged without selection pressure ($p<0.01$, t-test; Figure 2B). These data indicate that selection, both in vitro and in vivo, resulted in cell populations with an increased migratory phenotype. In proliferation assays no differences were revealed between C6slow versus C6fast or between C6-SP-GFPslow versus C6-SP-GFPfast cells (data not shown).

Oligonucleotide microarray analysis. Among the 8832 genes and ESTs comprised by Affymetrix RG U34 A array, 3432 were present in all 4 (slow and fast in duplicate each) and 4848 in at least 2 of the C6 samples, while 3363 were detected in all 4 and 4044 in at least 2 of the C6-SP-GFP samples (Figure 3). Nine hundred sixteen (18.9%) of 4848 "in vitro genes" were expressed only in C6 cells ("in vitro only" genes), ie, they were absent in vivo, while 112 (2.8%) of 4044 "in vivo genes" genes genes were expressed only in C6-SP-GFP cells ("in vivo only" genes), ie, they were absent in vitro. A total of 3932 genes were expressed both in vitro and in vivo (Figure 3). Analysis of Gene Ontology classes revealed that "signal transducer" genes (molecular function category) were overrepresented among "in vitro only" genes as compared to "in vivo only" genes (18.3% versus 3.2%), while "defense/immunity" genes (molecular function category, 0.8% versus 9.7%) and "cell communication" genes (biological process category, 21.3% versus 33.3%) were overrepresented among "in vivo only" genes.

For revealing genes that were regulated, ie, differentially expressed between slow and fast cells, we first applied the t-test

Gene Description	Symbol	GenBank	Score	p
Genes up-regulated in C6-SP-GFPfast and not expressed in C6				
Transferrin	TF	D38380	4.4	0.0018
EST, Moderately similar to I37421 glutaminyl-peptide cyclotransferase (EC 2.3.2.5) - [H.sapiens]	-	AA859661	2.6	0.0297
Metallothionein 3	MT3	AA924772	2.0	0.0088
Arginosuccinate synthetase 1	ASS	X12459	n.c.	n.c.
Elastin	ELN	J04035	n.c.	n.c.
Parathymosin	PTMS	AA892800	n.c.	n.c.
Synuclein, gamma	SNCG	X86789	n.c.	n.c.
Rattus norvegicus S100A1 gene, exon 1	S100A1	U26356	n.c.	n.c.
Caspase 1	CASP1	U14647	n.c.	n.c.
Glycoprotein 38	GP38	U92081	n.c.	n.c.
Neural F box protein 42	NFB42	AF098301	n.c.	n.c.
Caveolin 3	CAV3	AI043968	n.c.	n.c.
Gonadotropin releasing hormone receptor	GRHR	S68578	n.c.	n.c.
Cartilage link protein 1	CRTL1	M22340	n.c.	n.c.
6-Pyruvoyl-tetrahydropterin synthase	PTS	M77850	n.c.	n.c.
Growth associated protein 43	GAP43	L21192	n.c.	n.c.
Rattus norvegicus Nclone10 mRNA	-	U31866	n.c.	n.c.
EST	-	S68944	n.c.	n.c.
Genes down-regulated in C6-SP-GFPfast and absent in C6				
Growth arrest specific 7	GAS7	AJ003148	1.774	0.0185
Protein kinase C beta	PRKCB1	X04139	n.c.	n.c.
Kynurenine 3-hydroxylase	KMO	AF056031	n.c.	n.c.
Calcium/calmodulin-dependent serine protein kinase	CASK	Y08769	n.c.	n.c.
Myelin protein zero (Charcot-Marie-Tooth neuropathy 1B)	MPZ	K03242	n.c.	n.c.
Low density lipoprotein receptor-related protein associated protein 1	LRPAP1	AA892810	n.c.	n.c.
Rattus norvegicus (clone REM4) ORF mRNA, partial cds	-	L41686	n.c.	n.c.

Table 3. Differentially expressed (regulated) genes in C6-SP-GFP glioma cells in vivo and absent from C6 glioma cells in vitro. Score represents fold up-regulation or down-regulation of expression in fast versus slow cells. p values are levels of statistical significance using t-test. Score and p could not be calculated (n.c.) for genes that were expressed in fast cells but absent in slow cells (or vice versa).

($p<0.05$), resulting in 430 genes in C6 cells and 404 genes in C6-SP-GFP cells. Then all genes that differed in expression levels by at least 1.5-fold in mean intensity were selected. Furthermore, genes that were absent in fast cells and present in slow cells (or vice versa) were added, resulting in a total of 516 regulated genes for C6 (10.6% of in vitro genes) and 245 regulated genes for C6-SP-GFP (6.1% of in vivo genes). In vitro, downregulation was more common (53.9%), while upregulation predominated in vivo (65.3%).

Table 1 (C6) and Table 2 (C6-SP-GFP) show 10 genes (expressed in both fast and slow cells) with the highest regulation. Regulation scores in C6 cells were below 10-fold for most genes. Five genes had scores higher than 10, ie, genes encoding nucleoside diphosphate kinase 7 (NDK7), laminin-5 alpha 3 chain (LAMA3), sialophorin (SPN), small inducible cytokine A2 (SCYA2) and an EST, moderately similar to g1-related zinc finger protein (Table 1). In C6-SP-GFP cells, scores were generally lower than in C6 cells, with only one EST having a score of higher than 10 (Table 2). Among 916 "in vitro only" genes, 142 (15.5%) were regulated, while 25 (22.3%) of 112 "in vivo only" genes were regulated (see Figure 3). These 25 genes associated with fast in vivo migration and possibly related to brain invasion are listed in Table 3. The percentages of Gene Ontology classes ("biological process," "molecular function" and "cellular component" categories) among upregulated or downregulated genes was not different between C6 and C6-SP-GFP cells, except for the "signal transducer" class which included 14.9% of regulated genes in vitro but only 7.0% in vivo.

Among a total of 730 genes that were differentially expressed between slow and fast cells in vitro or in vivo (Figure 3), only 31 (4.2%) were regulated in parallel both in vitro and in vivo, with 18 genes showing higher and 13 genes lower expression in fast cells (Figure 4; Table 4).

RT-PCR. Four genes regulated both in vivo and in vitro were arbitrarily chosen to corroborate the microarray data by RT-PCR. cDNAs from C6slow, C6fast, C6-SP-GFPslow and C6-SP-GFPfast samples were analyzed for the expression of LAMA3, ID3, PSMB8 and MGP in comparison to GAPDH expression levels. GAPDH expression did not differ among the 4 samples. In accordance with the microchip data, mRNA levels of LAMA3, ID3 and PSMB8 were higher in fast cells as compared to slow cells (LAMA3, 24.9-fold in C6fast, absent in C6-SP-GFPslow; ID3, 1.8-fold in C6fast, 5.2-fold in C6-SP-GFPfast; PSMB8, 1.9-fold in C6fast, 2-fold in C6-SP-GFPfast) as determined by analyzing band intensities, whereas the mRNA level of MGP was higher in slow cells (9.2-fold in C6slow, 9-fold in C6-SP-GFPslow) (Figure 5).

DISCUSSION

We have established highly motile C6 glioma cells by selection for fast migration on a Matrigel coated surface in vitro (C6fast cells) and for diffuse invasion of nude mouse brain in vivo (C6-SP-GFPfast cells). Comparison of global gene expression patterns between the original ("slow") and the fast populations revealed differently expressed ("regulated") genes, ie, genes related to and possibly underlying migratory velocity. We have found *i)* 25 genes regulated in vivo but absent in vitro, possibly representing processes specific to brain invasion; *ii)* 142 genes regulated in vitro but absent in vivo, possibly reflecting in vitro mechanisms of minor relevance to brain invasion; *iii)* 31 genes which were regulated both in vitro and in vivo and thus may hint at general mechanisms of glioma migration irrespective of assay and environment; and *iv)* little overlap between regulated genes in

vitro and in vivo (4.2% of regulated genes), suggesting that fast migration in vitro and in vivo involves different molecular mechanisms.

Genes regulated both in vitro and in vivo (Table 4). The group of 31 genes regulated in both experimental paradigms includes several genes that have been directly or indirectly associated with glioma invasion before, while for others expression and/or function in gliomas are entirely unknown so far. Proteins that have been shown to be expressed in glioma biopsy specimens and to promote in vitro migration of glioma cells include laminin-5 (11), galectin-3 encoded by LGALS3 (5), and insulin-like growth factor-1 (4, 17), all of them being upregulated in fast cells here. Conversely, plasminogen activator inhibitor 1 (PAI1) and platelet-derived growth factor-AA (PDGFA), being downregulated in fast cells here, are expressed in gliomas and inhibit in vitro glioma invasion (18, 32) or have no effect on glioma motility (4). Other proteins regulated here have been found in glioma cell lines and/or glioma biopsy specimens and shown to mediate migration of other, non-glial cell types such as T-cells, endothelial cells and carcinoma cells, while functional data on glioma cells are missing so far; these proteins include sialophorin (CD43) (6, 10), ephrin-B1 (16, 19), inhibitor of DNA-binding 3 (ID3) (26, 43) and met-enkephalin and its precursor preproenkephalin (9, 15). Likewise, thymidylate synthase is overexpressed in malignant gliomas as compared to non-neoplastic brain (2), and in a variety of cancer types its activity is related to dissemination (33). Munc-18-1 (n-Sec1, rbSec1, p67), which is the human ortholog of rat STXBP1, as well as matrix GLA protein are overexpressed in gliomas (21, 42), while functional studies on motility are missing. Expression of 2′,3′-cyclic nucleotide 3′-phosphodiesterase in C6 cells has been reported and considered an indicator of oligodendroglial differentiation (14) but, again, effects on motility are unknown. Conversely, the expression of proteasome subunits such as β8 has not been examined in gliomas, but one study found decreased glioma infiltration in vitro following application of proteasome inhibitors (25). No expression or functional data on UDP-glycosyltransferase, the src-like tyrosine kinase Frk/Rak and the subtilisin/kexin type proprotein convertase-3 are available in gliomas, while related enzymes, ie, α2,6-sialyltransferase, other src kinase family members, and the proprotein convertase furin, may affect glioma cell invasion via sialylation of integrins (44), integrin- and PDGF-based glioma invasion mechanisms (8) and activation of precursor metalloproteases (30), respectively. Finally, several genes have not at all been examined in gliomas, and these include SRPX, TCRG, GSTsu8, LOT1, MSX2, GPR30, TST and LIPA. Taken together, the majority of genes regulated both in vivo and in vitro in our study, or relatives of these genes, have been associated with invasion before and thus support the validity of our approach for identifying invasion genes, while a minority has not been analyzed yet in gliomas and represent new molecular entry points. Interestingly, a link to glioma invasion is completely missing in 5 of 10 downregulated genes, but in only 3 of 15 upregulated genes, suggesting that more migration-suppressing genes than invasion-promoting genes remain to be revealed.

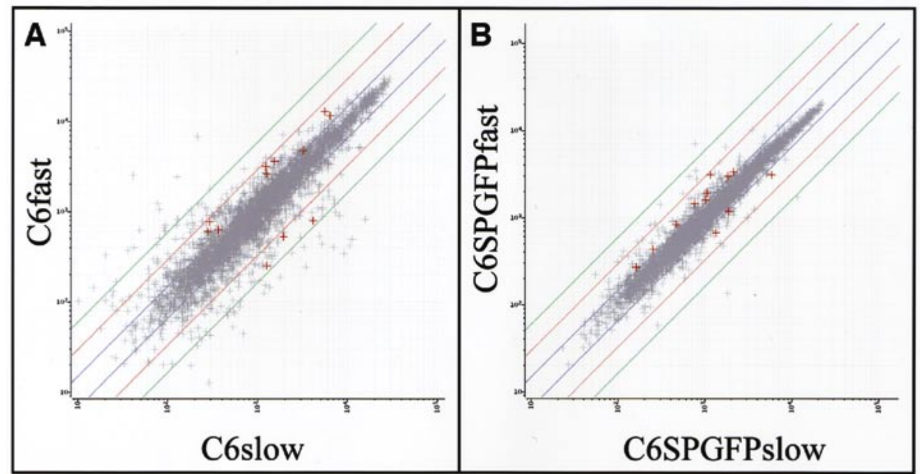

Figure 4. Logarithmic scatter plot showing all expressed genes in C6 (**A**) and C6-SP-GFP (**B**) cells. Diagonal pairs of lines indicate 1.5-fold (blue), 3-fold (red) and 6-fold regulation (green). Genes that are differentially regulated in both C6 and C6-SP-GFP cells are indicated by red crosses (corresponding to the 12 genes with scores for both C6 and C6-SP-GFP cells in Table 4).

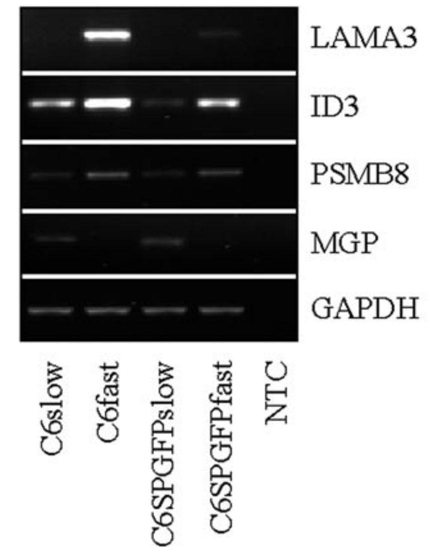

Figure 5. RT-PCR analysis of four genes (LAMA3, ID3, PSMB8, MGP) found to be regulated by GeneChip analysis (see Table 4) and the GAPDH housekeeping gene in C6slow, C6fast, C6-SP-GFPslow and C6-SP-GFPfast cells. NTC represents no template control.

Genes regulated in vivo only (Table 3). Among 112 genes expressed only in vivo, 25 (22.3%) were regulated. This percentage is higher than that of regulated "in vitro only genes" (15.5%) and considerably higher than the percentages of regulated genes among all genes expressed in vitro (10.6%) or in vivo (6.1%), suggesting that invasion genes are overrepresented among genes that are only expressed in glioma cells growing in their natural environment, ie, the brain. These 25 genes are of particular interest, because they might represent genes specifically mediating diffuse brain invasion, while being turned off in vitro. They are therefore easily missed in cell culture invasion studies such as horizontal migration or Boyden chamber assays, but might represent the most relevant genes from the therapeutical and biological points of view.

In contrast to the genes regulated both in vitro and in vivo (discussed above), most of the 25 genes regulated in vivo only have not been analyzed in gliomas with respect to expression or function so far; while some

Gene description	Symbol	GenBank	Score C6	Score C6-SP-GFP	p C6	p C6-SP-GFP
Genes up-regulated in fast migrating glioma cells						
Laminin 5 alpha 3	LAMA3	AA946108	19.2	n.c.	0.0055	n.c.
Sialophorin	SPN	AI045440	13.0	n.c.	0.0023	n.c.
EST, moderately similar to T46465 hypothetical protein DKFZp434A0530.1- human	-	AA874943	7.6	n.c.	0.0016	n.c.
Preproenkephalin, related sequence	PENK-rs	S49491	2.3	2.6	0.0056	0.0067
Glutathione transferase, subunit 8	GSTsu8	X62660	2.6	1.6	0.0178	0.0221
UDP glycosyltransferase 1 family, polypeptide A6	UGT1A6	J05132	2.6	1.7	0.0436	0.0002
Proteasome subunit, beta type, 8	PSMB8	D10729	2.4	1.8	0.0228	0.0457
EST	-	AI639331	2.2	1.8	0.0489	0.0183
Syntaxin binding protein 1	STXBP1	AA800015	2.1	1.5	0.0023	0.0370
Inhibitor of DNA-binding 3	ID3	AI171268	1.8	1.6	0.0476	0.0049
Thymidylate synthase	TYMS	L12138	1.7	1.6	0.0498	0.0451
EST,weakly similar to A54756 isocitrate dehydrogenase	-	AA891785	1.5	1.5	0.0022	0.0139
Insulin-like growth factor 1	IGF1	M81183	n.c.	1.5	n.c.	0.0145
Lectin, galactose binding, soluble 3	LGALS3	J02962	n.c.	n.c.	n.c.	n.c.
Sushi-repeat-containing protein	SRPX	D78359	n.c.	n.c.	n.c.	n.c.
T-cell antigen receptor, gamma subunit	TCRG	AI176307	n.c.	n.c.	n.c.	n.c.
Ephrin B1	EFNB1	U07560	n.c.	n.c.	n.c.	n.c.
Src related tyrosine kinase	FRK	U09583	n.c.	n.c.	n.c.	n.c.
Genes down-regulated in fast-migrating glioma cells						
Matrix gla protein	MGP	AI012030	n.c	6.3	n.c.	0.0034
2',3'-cyclic nucleotide 3'-phosphodiesterase	CNP2	L16532	5.0	1.8	0.0117	0.0017
Lipase A, lysosomal acid	LIPA	S81497	4.8	1.8	0.0273	0.0071
Platelet-derived growth factor A chain	PDGFA	D10106	3.5	1.5	0.0115	0.0170
Plasminogen activator inhibitor-1	PAI1	M24067	n.c	3.2	n.c.	0.0012
EST	-	AA893743	n.c	2.0	n.c.	0.0068
G protein-coupled receptor 30	GPR30	U92802	n.c	2.0	n.c.	0.0152
Proprotein convertase subtilisin/kexin type 3	PCSK3	X55660	1.8	n.c.	0.0401	n.c.
EST	-	AA893984	n.c	1.7	n.c.	0.0001
Lost on transformation 1	LOT1	U72620	n.c	1.5	n.c.	0.0094
Msh homeo box homolog 2	MSX2	U12514	n.c	n.c.	n.c.	n.c.
Thiosulfate sulfurtransferase	TST	X56228	n.c	n.c.	n.c.	n.c.
EST, moderately similar to ras GTPase activating protein-related protein	-	AA800671	n.c	n.c.	n.c.	n.c.

Table 4. Genes differentially expressed in C6 and C6-SP-GFP rat glioma cells. Score represents fold up-regulation or downregulation of expression in fast versus slow cells. p values are levels of statistical significance using t-test. Score and p could not be calculated (n.c.) for genes that were expressed in fast cells but absent in slow cells (or vice versa).

have been found in glioma cell lines and/or biopsy specimens, including TF, MT3, ELN, SNCG, S100A1, CASP1, GAP43, PRKCB1, KMO and LRPAP1 without addressing effects on motility. However, functional data obtained in other cell types make some of these genes reasonable and promising candidates for further functional studies, such as MPZ, KMO and SNCG. First, expression in C6 cells of P0 (myelin protein zero), the major myelin protein component of peripheral nerve myelin encoded by MPZ, promotes homophilic cell adhesion (45), suggesting that P0 downregulation in glioma cells (as observed here in fast cells) might induce single cell dispersion leading to diffuse invasion. Second, since *i)* downregulation of the KMO gene product kynurenine 3-hydroxylase (as detected here at the mRNA level in fast cells) shunts kynurenine metabolism toward kynurenic acid formation (41), *ii)* kynurenic acid represents the only naturally occurring NMDA receptor antagonist, *iii)* migration of nerve cells is inhibited by NMDA receptor agonists (22), and *iv)* glioma cells express functional glutamate receptors of NMDA and non-NMDA types (24), increased kynurenic acid levels due to decreased activity of kynurenine 3-hydroxylase might promote migration of glioma cells. Third, synuclein-gamma is overexpressed in glioblastomas as compared to other astrocytic gliomas (12), and overexpression of synuclein-gamma (also called BCSG1) in breast cancer cells led to a significant increase in motility and invasiveness in vitro and a profound augmentation of metastasis in vivo (20), making it conceivable that synuclein-gamma (overexpressed here in fast cells) bestows a higher migratory phenotype on glioma cells.

Three details of our in vivo methods should be considered in interpreting and generalizing the data. First, we have used

xenotransplantation of rat cells, because the histological features of this model closely resemble human gliomas (39), whereas we are not aware of a corresponding syngenic mouse model with representative histopathology. Thus, due to the difference in species between glioma cells (rat) and hosts (mouse), some relevant molecules involved in tumor-host interactions might have been missed. Second, putting explanted cells into culture before microarray analysis might change their transcriptome, so that the microarray data might not entirely reflect the in vivo gene expression pattern. However, the strikingly different microarray results of our in vitro versus in vivo approaches suggest that the brief period of culturing explanted tumor cells did not introduce major transcriptional changes typical of in vitro conditions. Third, some changes in gene expression might have been caused by intracerebral transplantation per se, because slow cells had not been passaged in the brain. However, the number of regulated genes was lower in vivo as compared to in vitro (245 versus 516), whereas noticeable effects of xenotransplantation should lead to higher expression differences in vivo. Since we could demonstrate similar amounts of migration stimulation between our in vitro and in vivo paradigms, these figures argue against marked effects of transplantation upon gene expression. As an alternative for isolating fast and slow cells in vivo, we considered taking non-migratory cells within the xenografts as slow cells or the use of laser capturing of invading tumor cells from tissue sections. However, these approaches would introduce the disadvantage that fast cells showing diffuse (single cell) brain invasion are surrounded by non-neoplastic cerebral cells such as neurons and vascular cells, while most non-migratory cells in the center of the tumor are surrounded by other tumor cells. The respective cellular interactions would lead to altered gene expression patterns, making these approaches less useful. Thus, each experimental design has its own limitations which need to be carefully considered in interpreting the data.

Differences between in vitro and in vivo mechanisms of glia motility. We found marked differences between regulated genes in vitro versus in vivo. While gene ontology classes were not significantly different (except for "signal transducer" genes which were more commonly involved in vitro), indicating operation of similar biological processes, the identity of individual regulated genes was strikingly different. Thus, only 31 (4.2%) of 733 regulated genes were regulated in parallel in vitro and in vivo (Figure 3; Table 4), and the only gene included among both the in vitro and the in vivo list of 10 genes with highest regulation factors (Tables 1, 2) was even downregulated in vitro but upregulated in vivo. While most of the genes regulated both in vitro and in vivo (Table 4) have already been associated with (glioma cell) migration in previous studies, the genes expressed and regulated in vivo only (Table 3) have not been analyzed with respect to glioma cell motility so far, presumably because they are not expressed in vitro where the vast majority of experiments have been performed. To conclude, the molecular mechanisms of fast glioma cell migration are strikingly different in vitro and in vivo, so that one has to be very cautious in simply extrapolating glia motility data obtained in vitro to processes occurring inside the brain.

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