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ORIGINAL ARTICLE

Microglia-derived TGF-β as an important regulator of glioblastoma invasion—an inhibition of TGF-β-dependent effects by shRNA against human TGF-β type II receptor

A Wesolowska[1], A Kwiatkowska[1], L Slomnicki[1], M Dembinski[1], A Master[1], M Sliwa[1], K Franciszkiewicz[2], S Chouaib[2] and B Kaminska[1]

[1]*Laboratory of Transcription Regulation, Department of Cell Biology, Nencki Institute of Experimental Biology, Warsaw, Poland and* [2]*Human Tumor Cytokines Laboratory, Institute Gustave Roussy, Villejuif, France*

The invasion of tumor cells into brain tissue is a pathologic hallmark of malignant gliomas and contributes to treatment failures. Diffuse glioblastomas contain numerous microglial cells, which enhance the progression of gliomas; however, factors responsible for invasion-promoting role of microglia are unknown. Transforming growth factor-β (TGF-β) can enhance tumor growth, invasion, angiogenesis and immunosuppression. Antagonizing TGF-β activity has been shown to inhibit tumor invasion *in vitro* and tumorigenicity, but a systemic inhibition or lack of TGF-β signaling results in acute inflammation and disruption of immune system homeostasis. We developed plasmid-transcribed small hairpin RNAs (shRNAs) to downregulate the TGF-β type II receptor (TβIIR) expression, which effectively inhibited cytokine-induced signaling pathways and transcriptional responses in transiently transfected human glioblastoma cells. Silencing of TβIIR abolished TGF-β-induced glioblastoma invasiveness and migratory responses *in vitro*. Moreover, tumorigenicity of glioblastoma cells stably expressing TβIIR shRNAs in nude mice was reduced by 50%. Microglia strongly enhanced glioma invasiveness in the co-culture system, but this invasion-promoting activity was lost in glioma cells stably expressing shTβRII, indicating a crucial role of microglia-derived TGF-β in tumor–host interactions. Our results demonstrate a successful targeting of TGF-β-dependent invasiveness and tumorigenicity of glioblastoma cells by RNAi-mediated gene silencing.
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Correspondence: Professor B Kaminska, Department of Cell Biology, Nencki Institute of Experimental Biology, 3 Pasteur Street, 02-093 Warsaw, Poland. E-mail: bozenakk@nencki.gov.pl
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Introduction

Malignant glioblastomas are among the most lethal and difficult tumors to treat due to infiltration of glioblastoma cells into the brain tissue, which makes surgical dissection difficult. Even the most intensive radio- and chemotherapy are not effective and yield only a modest impact on patient survival (Ohgaki and Kleihues, 2005). Thus, there is a major need for new drugs and therapeutic modalities. Therapies effectively targeting invasiveness of glioblastoma cells may significantly improve therapeutic outcome. Soluble factors released by tumor cells recruit inflammatory cells, including brain microglia, to the tumor site and transform them into tumor-supportive cells. However, diffuse gliomas contain numerous microglial cells (Badie and Schartner, 2001); defense functions of microglial cells are compromised by the tumor, for example through impaired surface expression of MHC class II antigens. Activated microglia release many factors, including cytokines and proteases, which directly or indirectly influence tumor progression (Watters *et al*., 2005). Recent studies demonstrated a strong ability of microglial cells to enhance tumor invasion in cell co-cultures and brain organotypic slices cultures (Markovic *et al*., 2005; Sliwa *et al*., 2007). Mechanisms and factors responsible for an invasion-promoting activity of microglial cells are largely unknown.

Transforming growth factor-β (TGF-β), a cytokine frequently expressed in malignant brain tumors (Gold, 1999; Piek *et al*., 1999; Kjellman *et al*., 2000), induces a survival advantage of tumor cells by enhancing cell growth, migration, invasion, angiogenesis and immune paralysis (Jennings and Pietenpol, 1998; Platten *et al*., 2001). Proteases secreted during glioma progression, such as matrix metalloproteinases (MMPs) and cathepsins, degrade extracellular matrix allowing glioma cells to spread and diffusely infiltrate the brain parenchyma (Platten *et al*., 2001; Rao, 2003; Lakka *et al*., 2004). TGF-β has been shown to induce MMPs expression, suppress tissue inhibitor of metalloproteinase (TIMP) expression, and promote the invasion of human glioma cells in a Matrigel invasion assay (Nakano *et al*., 1995; Wick *et al*., 2001). Therefore,

TGF-β signaling pathway is emerging as an attractive target in cancer and inhibitors of this pathway may affect tumor progression and improve overall survival. We have recently demonstrated that microglia-derived soluble factors stimulate proliferation and invasiveness of glioma cells (Sliwa *et al*., 2007). Since microglial cells are a major brain source of TGF-β1 under pathological conditions (Lindholm *et al*., 1992), we hypothesized that microglia-derived TGF-β1 may contribute to microglia promoting effect on glioma growth and invasion.

TGF-β signal propagates via specific TGF-β type II receptor (TβRII), which recruits and activates type I receptor (TβRI). Activated TβRI initiates cytoplasmic signaling pathways via phosphorylation of Smad proteins, the main downstream components of TGF-β signaling (Heldin *et al*., 1997; Attisano and Wrana, 1998; Derynck *et al*., 1998). Phosphorylated Smad proteins bind with the common Smad4 and translocate to the nucleus, where they interact with co-activators or co-repressors to mediate transcriptional regulation of target genes (Derynck *et al*., 1998). Many approaches targeting TGF-β pathway have been developed, including blockade of TGF-β action with a soluble Fc:TGF-β type II receptor fusion protein, specific inhibitors of TGF-β receptor kinases or intracellular mediators (Muraoka *et al*., 2002; Friese *et al*., 2004; Uhl *et al*., 2004). Anti-TGF-β strategies inhibit tumor cell viability, migration and metastases *in vitro* and in several animal models. However, a growing evidence suggests that a systemic inhibition of TGF-β signaling may have detrimental effects due to its importance in T-cell development, survival and homeostasis. (Li *et al*., 2006; Marie *et al*., 2006). TGF-β1 null mice develop a multi-organ inflammatory disease and die (Shull *et al*., 1992; Kulkarni *et al*., 1993). Similarly, though less severe, pathophysiology was observed in mice lacking a functional TβRII or Smad3 (Yang *et al*., 1999). Therefore, it would be essential to develop a cell-targeted strategy to inhibit TGF-β signaling.

RNA interference is sequence-specific process, initiated by double-stranded RNA known as small interfering RNAs (siRNAs; Zamore *et al*., 2000). Synthetic 21–22 RNAs mediate cleavage of target mRNA in mammalian cells (Elbashir *et al*., 2001). *In vitro* studies reported an effective, stable gene suppression by chemically synthesized siRNAs (Brummelkamp *et al*., 2002). Recently, researchers employed siRNAs transcribed from plasmid or viral vectors that efficiently deliver siRNAs *in vitro* and *in vivo* (Hommel *et al*., 2003; Pardridge, 2004).

We demonstrated that silencing of TβRII expression using plasmid-transcribed small hairpin RNA (shRNA) led to significant inhibition of TGF-β-induced signal transduction, glioblastoma invasiveness, migration and tumorigenicity in nude mice. An invasion-promoting activity of microglia was abolished in glioma cells deficient in TβRII. Our results implicate microglia-derived TGF-β as an important mediator of tumor–host interactions and a regulator of glioma invasion.

Results

Plasmid-transcribed hairpin siRNAs downregulate TβRII mRNA expression and TGF-β-dependent signaling in human glioblastoma cells

Four pairs of 57–59 oligonucleotides, including 19–21 bp sequence complementary to target TβRII mRNA and a loop sequence, were designed according to siRNA Design Guidelines (Ambion Inc., Austin, TX, USA) based on rules first described by Tushl (Figure 1a). Selected siRNA sequences have 30–70% GC content and contain no significant homology to non-TβRII sequences. pSTNeg coding for shRNA with no significant similarity to mouse, rat or human gene sequences, was used as a control. To verify shRNA effects, we measured the levels of *TβRII* mRNA in cells transfected with the plasmids encoding specific shRNAs (pST602, pST1208, pST1601, pST1119) 56 h after transfection. The level of *TβRII* mRNA in cells expressing specific shRNA was related to mRNA from T98G cells transfected with a pSTNeg plasmid. The level of *TβRII* mRNA was reduced by 30–67% in cells expressing specific shRNA (Figure 1b). The most significant reduction was 67% ($***P<0.001$). Immunoblot shows a significant reduction in TβRII protein expression in cells transfected with pST1119 plasmid (Figure 1c). We did not observe any effects on cell viability and morphology of transfected cultures within 72 h.

Since efficiency of gene silencing depends on delivery of siRNA into the cells, we evaluated the efficiency of transfection of human glioblastoma cells by flow cytometry. Routinely, 63% of human T98G glioblastoma cells transfected with pTracer–CMV2-encoding green fluorescent protein (GFP) displayed a green fluorescence 24 h after transfection (Figure 1d). Co-transfection with the plasmid coding for GFP-specific shRNA resulted in 85% reduction of the GFP fluorescence (Figure 1d).

Smad proteins are the major mediators of TGF-β-dependent signaling (Nakao *et al*., 1997). Consensus $(CAGA)_{12}$-box promoter, composed of multiple copies of the CAGA box (AG(C/A)CAGACA), is specifically recognized by activated Smad3–Smad4 complex (Dennler *et al*., 1998; Song *et al*., 1998). Thus, T98G glioblastoma cells were cotransfected with a Smad-dependent promoter and pST plasmids. A basal activity of $(CAGA)_{12}$-box promoter was detected in untreated cells cotransfected with pSTNeg, and stimulation with 0.1 ng/ml TGF resulted in sixfold induction of luciferase activity. The induction of Smad-dependent promoter was significantly reduced in cells cotransfected with TβRII-specific shRNA (Figure 2a).

Human plasminogen activator inhibitor-1 (PAI-1) is a gene that is potently induced by TGF-β in hepatocytes (Keeton *et al*., 1991; Westerhausen *et al*., 1991). The human PAI-1 gene promoter contains several Smad-binding sites, which confer TGF-β responsiveness upon this promoter (Dennler *et al*., 1998). We demonstrate a twofold induction of PAI-1 gene promoter in pSTNeg-transfected glioblastoma cells after

a

p*Silencer*™ plasmid	Hairpin siRNA insert	Position on TßRII gene sequence	Species
pST602	5'GATCC-GAATGACGAGAACATAACACT-GAGTACTG-AGTGTTATGTTCTCGTCATTC- TTTTTT-GGAA-A3'	602-624	human
pST1208	5'GATCC- GGACATCTTCTCAGACATC- GAGTACTG-GATGTCTGAGAAGATGTCC-TTTTTT- GGAA-A3'	1208-1226	human
pST1601	5'GATCC-GATACATGGCTCCAGAAGT- GAGTACTG-ACTTCTGGAGCCATGTATC-TTTTTT- GGAA-A3'	1601-1623	human
pST1119	5'GATCC- GCTGAAGCAGAACACTTCAGA- GAGTACTG-TCTGAAGTGTTCTGCTTAAGC-TTTTTT- GGAA-A3'	1119-1139	human/ rat

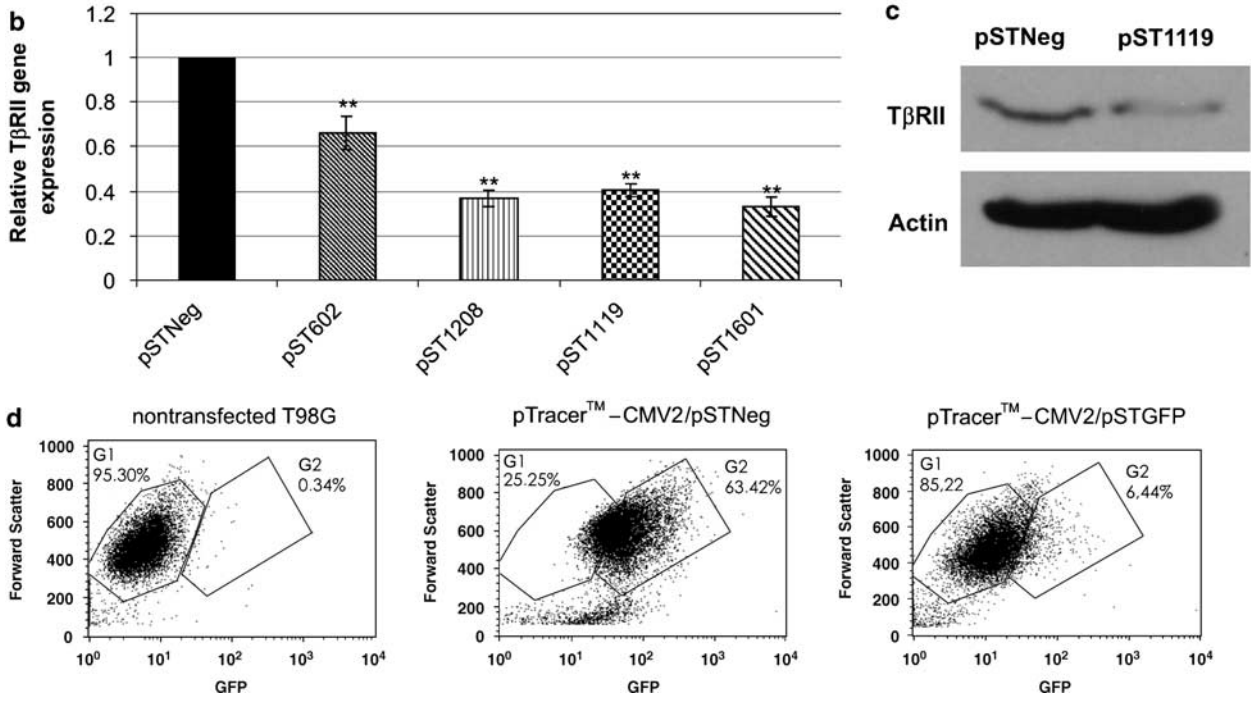

Figure 1 Plasmid-transcribed shRNAs against TβRII reduce mRNA level of TβRII in human T98G glioblastoma cells. (**a**) Hairpin siRNA sequences were originated from the coding sequence of human TβRII gene (M85079). Four pairs of 57–59 oligonucleotides including 19–21 bp sequence complementary to target TβRII mRNA and a loop sequence separating the two domains of hairpin siRNA insert were designed according to Tuschl algoritm (Ambion). (**b**) Real-time PCR evaluation of TβRII expression in transfected cells. Total RNA was isolated from cells transfected with pST602, pST1208, pST1601, pST1119 or pSTNeg plasmids 56 h after transfection. Following reverse transcription, the samples were analysed by real-time PCR using a set of primers, and a probe for *TβRII* and *GAPDH* (TaqMan Assay Reagents). Relative quantity of TβRII gene expression was estimated using the comparative C_T method. The bar graphs show the mean of *TβRII* transcript level in cells transfected with shRNA TβRII normalized to *GAPDH* and related to the *TβRII* expression level in cells transfected with pSTNeg plasmid. Data are expressed as means ± s.d. from three experiments (in each, RNA was isolated from triplicate cultures); $^{**}P<0.01$. (**c**) Immunoblot shows the levels of TβRII protein expression in transfected cells. Total lysates isolated from cells transfected with pSTNeg or pST1119 plasmid 56 h after transfection were analysed by western blotting with antibody recognizing TβRII (Cell Signaling). The membranes were probed with an anti-β-actin antibody to verify protein loading. (**d**) Efficiency of transfection was evaluated by measuring green fluorescence by flow cytometry in T98G cells mock-transfected, transfected with p*Silencer* 2.1–U6 plasmids or cotransfected with pTracer–CMV2 encoding Cyclo3GFP. Flow cytometry analysis revealed that 63% of cells expressed green fluorescence (gate G2) 48 h after transfection. A population of cells expressing green fluorescence decreased in cells transfected with pTracer–CMV2/pSTGFP. A representative experiment is shown. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; shRNA, small hairpin RNA; TβRII, TGF-β type II receptor.

treatment with 5 ng/ml TGF-β1 (Figure 2b). The induction of PAI-1 gene promoter was significantly reduced in T98G cells cotransfected with plasmids carrying TβRII-specific shRNA: pST602 and pST1119 (Figure 2b) ($^{**}P<0.01$). Our results demonstrate a significant inhibition of TGF-β-evoked transcriptional responses in T98G cells expressing TβRII-specific shRNA.

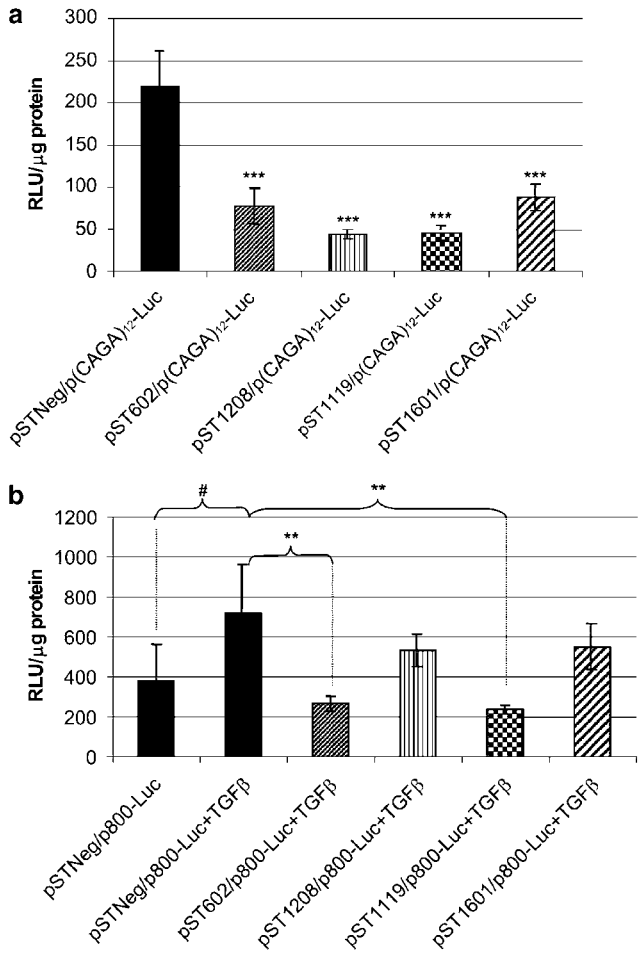

Figure 2 Inhibition of TGF-β-induced signaling by shRNAs against TβRII in human T98G glioblastoma. (**a**) Activity of TGF-β signal transduction pathway was analysed in human T98G glioblastoma cotransfected with a Smad-dependent promoter ($p(CAGA)_{12}$-Luc) and plasmids encoding control or gene-specific shRNA. Cells were transfected in 24-well plates and cultured for 24 h in DMEM/2% FBS. Subsequently, cells were stimulated with a recombinant TGF-β1 for 24 h, cells were lysed in a passive lysis buffer, and luciferase activities were evaluated using a luciferase assay (Promega). Luciferase activity in raw light units was normalized to the protein level in each sample. The bar graphs show the mean luciferase activity $\pm$ s.e.m. from three independent experiments (each in triplicate). Differences were statistically significant $^{**}P<0.01$. (**b**) TGF-β-dependent induction of PAI-1 gene promoter (p800-luc) in T98G cells. An addition of TGF-β1 led to at least a twofold induction of PAI promoter activity (# $P<0.05$). Two TβRII-specific p*Silencer* plasmids (pST602, pST1119) diminished TGF-β-dependent induction of PAI-1 promoter. Data are expressed as means $\pm$ s.e.m. from two independent experiments in triplicate. Statistically significant differences between luciferase activities in cells transfected with pSTNeg or shRNAs against TβRII were designated as $^{**}P<0.01$. Transfections and determination of luciferase activities were performed as described above. DMEM, Dulbecco's modified essential medium; FBS, fetal bovine serum; TGF-β, transforming growth factor-β; TβRII, TGF-β type II receptor; shRNA, small hairpin RNA.

Knockdown of TβRII expression affects invasiveness of human glioblastoma cells

We determined an invasion of transfected glioblastoma cells through the Matrigel matrix, an extract of non-crosslinked extracellular matrix macromolecules. T98G glioblastoma cells exhibit a high invasive potential and many cells migrating through Matrigel have been observed 24 h after seeding. In the absence of cytokine, there was no significant difference in the invasiveness of T98G cells transiently transfected with plasmids coding for either control or specific shRNA. An addition of TGF-β1 led to approximately a twofold increase of invasiveness when compared with a control, pSTNeg-transfected cells. In contrast, transfection of T98G cells with the pST1119 plasmid carrying TβRII-specific shRNA attenuated TGF-β-induced invasion. Among pST1119-transfected cultures, the number of cells migrating through the Matrigel matrix was similar in the absence and presence of the cytokine (Figures 3a and b).

Stable knockdown of TβRII affects migration of glioma cells

T98G glioblastoma cells do not form tumors after subcutaneous inoculation into nude mice. Since pST1119 plasmid (encoding shRNA against a region of the TβRII mRNA conserved between human and rat) effectively inhibited Smad signaling in the rat C6 glioma cells, we developed C6 glioma cells stably transfected with pST1119 or pSTNeg. Hygromycin B-resistant clones characterized by the presence of the insert in genomic DNA (not shown) have been selected for further analysis.

To evaluate effectiveness of TβRII silencing in C6 glioma clones stably expressing control or specific shRNA, we tested Smad-dependent signaling (Figure 4). An exogenous TGF-β1 (0.1 ng/ml) led to an increase of phospho-Smad 3 level in glioma cells stably expressing control plasmid, but not in cells expressing shRNA against TβRII (not shown). Accordingly, stimulation with TGF-β1 strongly activated Smad-dependent promoter in glioma cells stably transfected with pSTNeg plasmid, in contrast to a weak response in pST1119-transfected cells (Figure 4a).

As TGF-β is known to promote a scratch closure by activating cell motility, we evaluated migration of stable glioma clones. As shown in Figure 4b, pSTNeg-transfected clones re-populate the scratch area within 12–16 h. Addition of TGF-β significantly increases the number of cells migrating to the scratch area. A repopulation of the scratch area by glioma cells stably expressing shRNA against TβRII (1119/5) was significantly reduced ($^{**}P<0.01$) and TGF-β addition did not increase their migratory potential. Similar results were obtained for the second pair of clones. A repopulation of the scratch area was quantified by counting of 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI)-stained cell nuclei (Figure 4c). A stable knockdown of TβRII expression affects basal migration of glioma cells and blocks TGF-β-induced increase in cell motility.

Knockdown of TβRII impairs glioma growth in nude mice

Xenograft model was employed to determine whether stable knockdown of TβRII expression affects glioma growth in mice. A total of 5×10^6 of parental C6, Neg/9 or 1119/6 stably transfected glioma cells were injected into nude mice ($n=5$ mice in each group). There was no

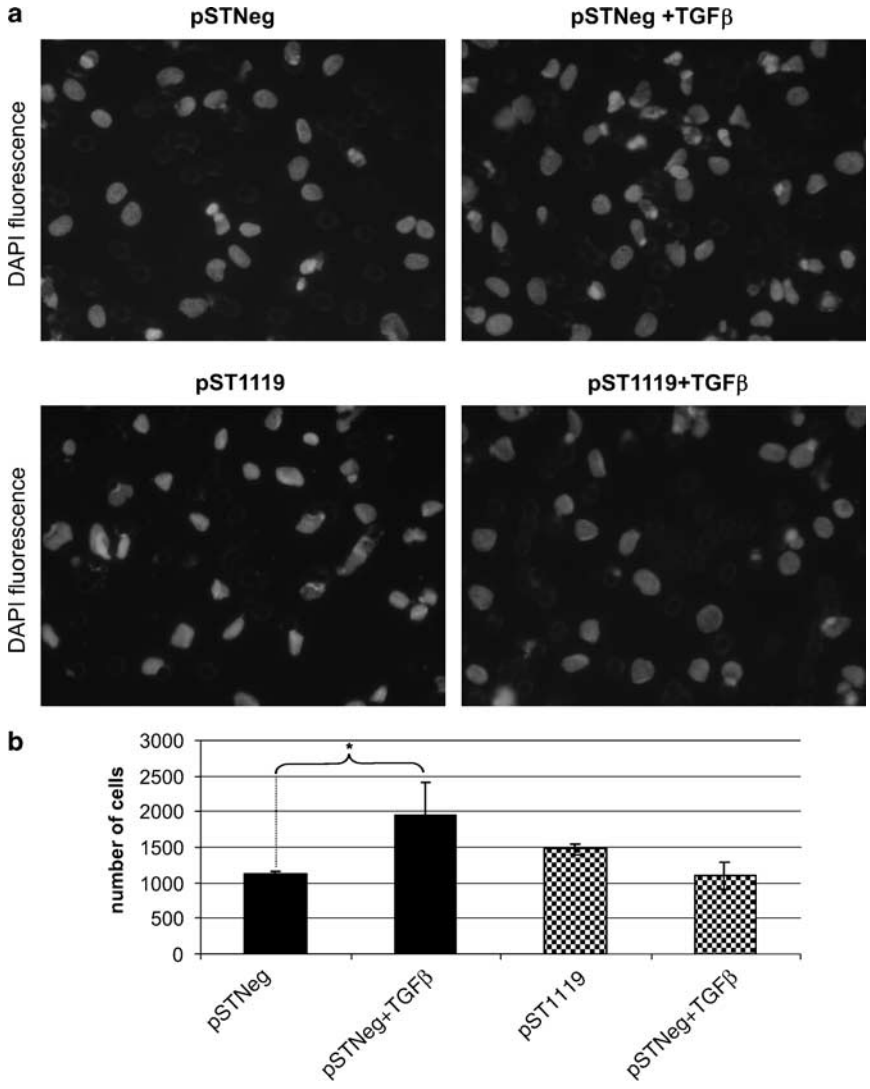

Figure 3 TGF-β-induced invasiveness of T98G glioblastoma cells is reduced by shRNA against TβRII. (**a**) T98G cells transfected with pST1119 or pSTNeg plasmids were detached by trypsinization 24 h after transfection, collected by centrifugation and counted. Equal number of cells was seeded in 24-well transwell coated with the Growth Factor Reduced Matrigel Matrix and cultured in DMEM/2% FBS in the presence or absence of 5 ng/ml TGF-β1 for 24 h. The cells invading through Matrigel Matrix were fixed in ethanol, stained with DAPI and counted using a fluorescent microscope. Fluorescent images of stained cell nuclei from a representative experiment are shown. (**b**) Bar graphs show the number of invading cells among pST1119 or pSTNeg-transfected cells in the absence or presence of TGF-β1 (5 ng/ml). A statistically significant increase of Matrigel invading cells was observed after TGF-β treatment in pSTNeg-transfected cells (*$P<0.05$ when compared to untreated cells). Data are presented as mean counts of cells in the entire microscopic field from duplicate wells in the representative experiment. Similar results were observed in three independent experiments. DAPI, 4′,6-diamidino-2-phenylindole, dihydrochloride; DMEM, Dulbecco's modified essential medium; FBS, fetal bovine serum; TGF-β, transforming growth factor-β; TβRII, TGF-β type II receptor; shRNA, small hairpin RNA.

difference between growth of a wild-type or Neg/9 glioma cells (Figure 5, upper panel). A significant decrease of 1119/6 glioma growth was apparent 21 days after glioma inoculation. Tumors arising from Neg/9 glioma cells were double in volume than tumors of 1119/6 cells. In the second experiment, 2×10^6 of Neg/9 or 1119/6 glioma cells were injected into nude mice ($n = 10$ or 15, respectively). Subcutaneous tumors formed by glioma cells stably expressing TβRII-specific shRNA appeared later, and the corresponding tumors grew more slowly than those formed by Neg/9 cells. Again, tumors arising from Neg/9 glioma cells were twice the size than tumors of 1119/6 cells (Figure 5, lower panel). It demonstrates that siRNA-mediated knockdown of TβRII expression abrogates glioma growth in nude mice.

Microglia promoting effects on glioma invasiveness is mediated by TGF-β1

Even though recent studies demonstrated an invasion-promoting role of microglia (Teicher, 2001; Markovic *et al*., 2005; Sliwa *et al*., 2007), microglia-derived factors responsible for its invasion-promoting effects are still unknown. Since the results presented above demonstrated that an exogenous TGF-β1 plays a critical role in regulation of glioma invasion and migration, we sought

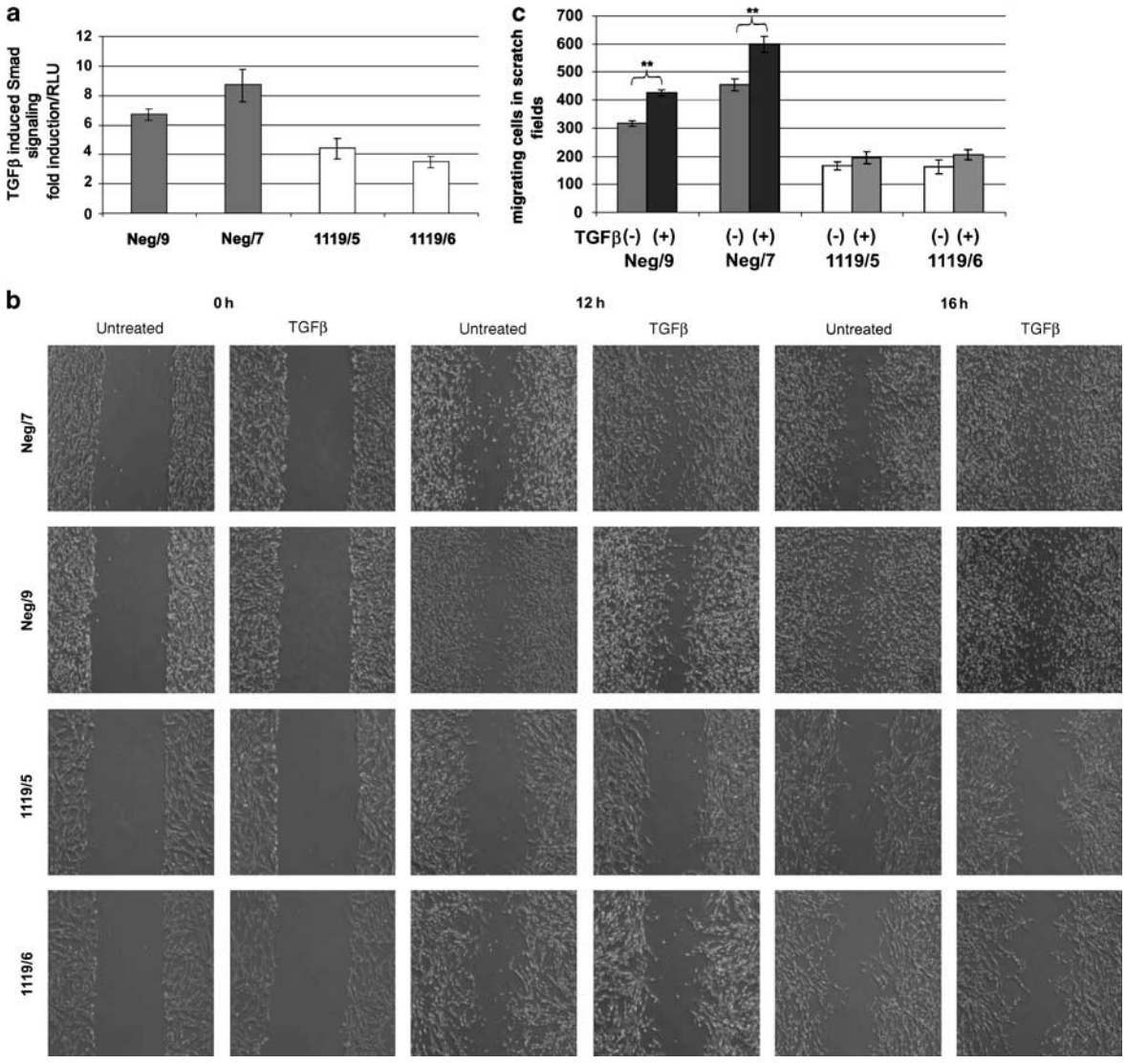

Figure 4 Stable knockdown of TβRII with shRNA reduces TGF-β1 signaling and glioma cell motility. (**a**) Activity of TGF-β signal transduction pathway was analysed in C6 glioma cells stably expressing control or shRNA against TβRII. Cells were transfected with $(CAGA)_{12}$-Luc plasmids, cultured for 24 h in DMEM/2% FBS, treated with TGF-β1 for 24 h and luciferase activities were evaluated as described above. The data are shown as means ± s.d. ($n = 3$) from a representative experiment (one out of three). (**b**) Cell motility of stably transfected C6 glioma was evaluated using a scratch assay. For a scratch assay glioma cells were seeded in duplicate on 60-mm culture dishes at 1.5×10^6 cells per dish. A scratch through the plate was gently made using a pipette tip when cells were 80% confluent. After washing with PBS, cells were incubated in DMEM with 2% FBS without or with TGF-β (5 ng/ml). Migration of the cells into the scratch was observed at time points of 12 and 16 h. (**c**) Quantitative evaluation of motility/migration of two stably transfected C6 glioma cell lines. A staining of cell nuclei with DAPI (0.01 mg/ml, Sigma-Aldrich) for 10 min at room temperature was performed. Cells from at least three representative fields between scratch edges at 16 h time point were counted under the fluorescence microscope with the excitation at 330–380 nm. Results represent means ± s.d. from two independent experiments each in triplicate. DAPI, 4′,6-diamidino-2-phenylindole, dihydrochloride; DMEM, Dulbecco's modified essential medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; TGF-β, transforming growth factor-β; TβRII, TGF-β type II receptor; shRNA, small hairpin RNA.

to determine whether this cytokine mediates an invasion-promoting effect of microglia. The level of TGF-β1 released by microglia alone and microglia co-cultured with glioma cells was measured by enzyme-linked immunosorbent assay (ELISA). Rat primary microglial cultures secrete a considerable amount of TGF-β1 (81 pg/ml) and this level increases 230% in microglia co-cultured with glioma cells.

Invasiveness of Neg/9 or 1119/6 glioma cells in the presence or absence of primary microglial cell cultures was evaluated using the Matrigel matrix invasion assay. After 36 h, glioma cells invading through the Matrigel-coated membrane were fixed, stained with DAPI and counted using iCys Research Imaging Cytometer (Figures 6a and b). The knockdown of TβRII expression abolished microglia-promoting effects on glioma invasiveness. While the number of Neg/9 glioma cells was doubled in the co-culture with microglial cells, the number of 1119/6 glioma cells invading the Matrigel matrix was similar in the absence or presence of

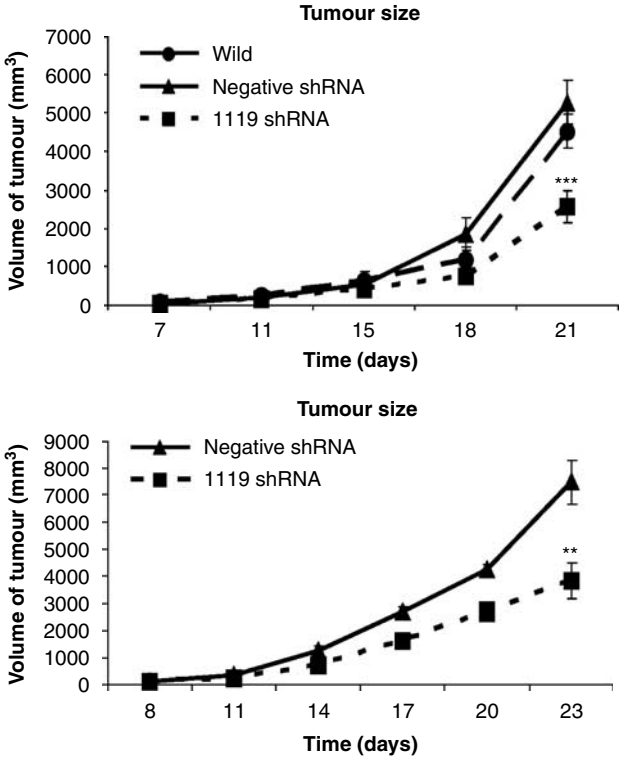

Figure 5 Stable knockdown of TβRII expression affects glioma growth in nude mice. A total volume of 50 μl containing 2×10^6 or 5×10^6 cells was subcutaneously injected into 6–8-week-old male nude mice. Injected mice were examined every 2–4 days for tumor apparition. Palpable tumors developed within 6–10 days. The volumes of tumor were calculated. Upper panel: A wild-type C6 glioma, pSTNeg- or pST1119-transfected glioma cells were injected into nude mice ($n = 5$ mice). Statistical significance was determined by Newman–Keuls analysis. A significant abrogation of pST1119 glioma growth was apparent 21 days after tumor cell inoculation; $^{***}P < 0.001$. Lower panel: 2×10^6 pSTNeg or pST1119 glioma cells were injected into nude mice ($n = 10$ or 15, respectively). A significant abrogation of pST1119 glioma growth was apparent 23 days after tumor cell inoculation; $^{**}P < 0.01$. TβRII, TGF-β type II receptor.

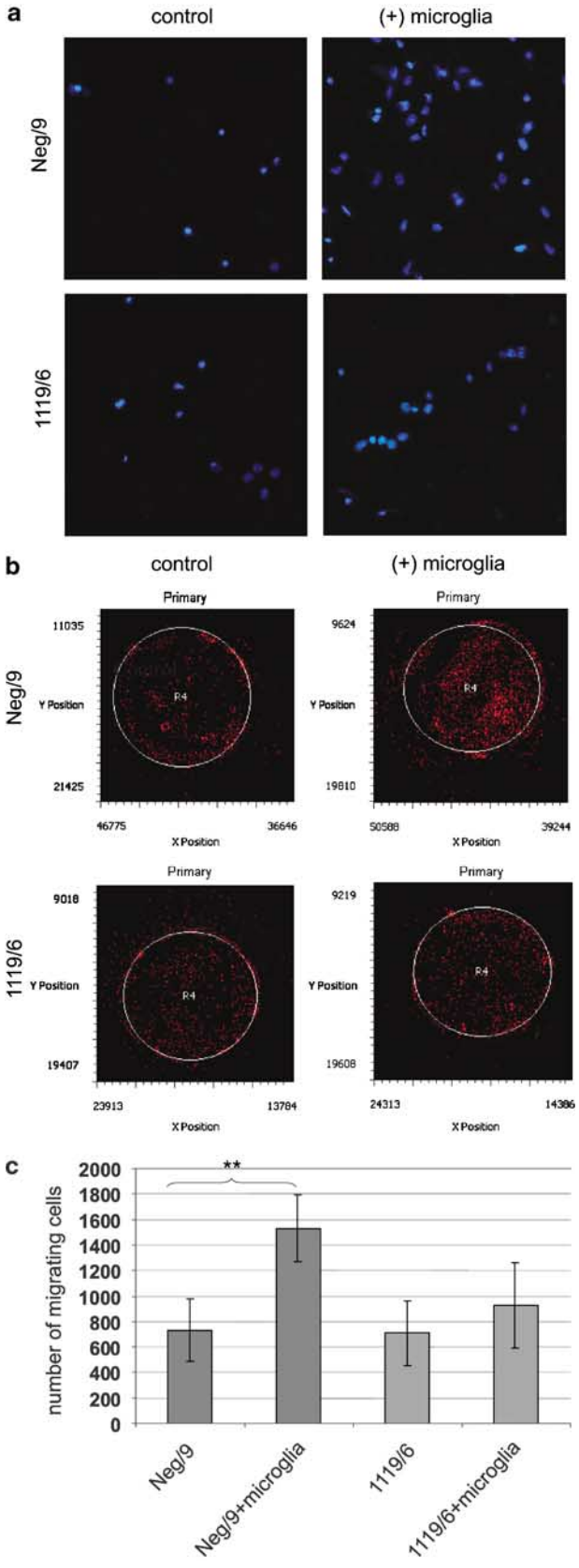

Figure 6 Microglia-promoting effect on glioma invasiveness is mediated by TGF-β1. (**a**) and (**b**) Invasiveness of pSTNeg- or pST1119-expressing glioma cells in the presence or absence of microglial cells was evaluated in the Matrigel matrix invasion assay. Glioma cells were seeded at a density of 4×10^4 per insert in a 24-well Transwell coated with the Growth Factor Reduced Matrigel Matrix. In the lower compartment a medium alone (DMEM/2% FBS) or microglial cells were seeded. After 36 h, glioma cells from the top of the filter were removed, and the cells invading through the coated membrane were fixed in methanol, stained with DAPI (**a**) and counted using iCys Research Imaging Cytometer with microscope unit Olympus IX-71 with a bright-field lamp. Scanned inserts from a representative experiment (1 out of 3) are presented (**b**). (**c**) Bar graphs show the number of invading glioma cells in the absence or presence of microglia. Results are expressed as means ± s.d. from three independent experiments (independently derived microglia primary cultures), each in triplicate. A statistical significance of differences between experimental conditions was determined using Newman–Keuls analysis; $^{***}P < 0.001$ for glioma cells co-cultured with microglia or medium alone. DAPI, 4′,6-diamidino-2-phenylindole, dihydrochloride; DMEM, Dulbecco's modified essential medium; FBS, fetal bovine serum; TGF-β1, transforming growth factor-β1.

microglia (Figure 6c). In additional experiments, a neutralizing antibody against TGF-β1 has been used to interfere with the cytokine-dependent effects. The enhanced invasiveness of C6 glioma cells in the Matrigel matrix in the presence of microglial cells was decreased significantly by co-incubation with a neutralizing antibody against TGF-β1 (Figure 7). Those results indicate that TGF-β1 released by microglia is mostly responsible for the invasion-promoting activity of microglial cells.

Discussion

*Inhibition of TGF-*β *signaling, invasiveness and* in vivo *tumorigenicity of glioblastoma cells by plasmid-transcribed shRNA against human TGF-*β *type II receptor*

In the present study, we demonstrate that a knockdown of TβIIR by plasmid-transcribed shRNA efficiently blocks TGF-β-induced signaling, transcriptional responses and invasiveness of human glioblastoma cells. A stable knockdown of TβRII expression impairs glioma growth in nude mice. It is a first demonstration of effective blocking of TβIIR expression with the plasmid-transcribed shRNA in human and rat glioblastoma cells.

TGF-β1 is often elevated in the plasma of breast, liver, lung and prostate cancer, hepatocellular carcinoma and glioblastoma patients. A loss of growth-inhibitory response to TGF-β and an increased expression of TGF-β have been associated with malignant conversion and progression (Yamada *et al*., 1995). Since TGF-β often upregulates the expression of its receptors, TβIR and TβIIR showed stronger expression in tumors when compared to normal tissues (DaCosta Byfield *et al*., 2004; Hjelmeland *et al*., 2004). Therefore, TGF-β signaling pathway emerged as an attractive target in cancer. Inhibitors of TGF-β expression or TGF-β signaling pathway were effective in blocking tumor invasiveness *in vitro* and in animal models (Friese *et al*., 2004; Uhl *et al*., 2004; Yingling *et al*., 2004; Schlingensiepen *et al*., 2006). Downregulation of TGF-β expression by RNA interference has been shown to inhibit glioma migration, invasiveness and to restore anti-tumor immune response in the mouse model of glioma (Friese *et al*., 2004). Retroviral overexpression of dominant-negative TβRII retarded tumor growth of EpRas cells and metastatic mesenchymal mouse colon carcinoma cells (Oft *et al*., 1998). Tumorigenicity of mouse thymoma was suppressed by a soluble TβRII (Won *et al*., 1999). Retrovirus-mediated introduction of

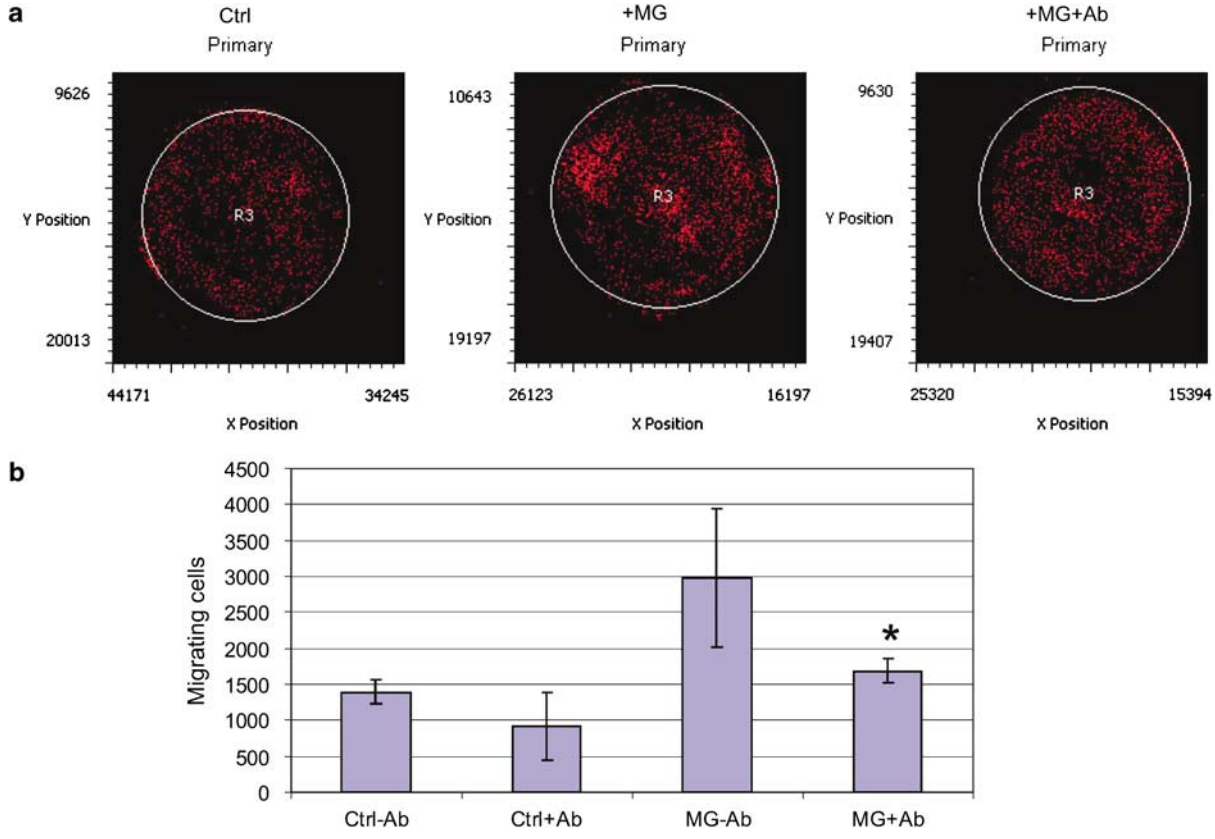

Figure 7 Invasion-promoting effects of microglia are abrogated with a neutralizing antibody against TGF-β1. (**a**) Representative fields of invading glioma cells, scanned and counted using iCys Research Imaging Cytometer. (**b**) Bar graphs show the number of invading glioma cells in the absence or presence of microglia after addition of anti-TGF-β1 mAb (56E4, 1:100, Cell Signaling). Results are expressed as means$\pm$s.d. from three independent cultures in a representative experiment (out of two independently derived microglial primary cultures). TGF-β1, transforming growth factor-β1.

a dominant-negative TβRII to bone marrow cells led to the generation of leukocytes capable of potent anti-tumor response and suppression of metastasis in melanoma and prostate cancer model (Shah *et al*., 2002).

A systemic inhibition of TGF-β signaling may result in detrimental effects. TGF-β1 null mice developed a multi-organ inflammatory disease and did not survive; less severe pathophysiology was observed in mice lacking a functional TGF-βRII or Smad3 (Shull *et al*., 1992; Kulkarni *et al*., 1993; Yang *et al*., 1999). Recent studies demonstrated that mice with T cell-specific TGF-βRII deficiency developed a lethal inflammation, autoimmune-associated lesions, because many aspects of T-cell biology, such as T-cell development, survival and homeostasis, all depend on intact TGF-β signaling (Li *et al*., 2006; Marie *et al*., 2006). Therefore, a systemic and long-lasting inhibition of TGF-β1 signaling would produce disruption of the immune system homeostasis and serious side effects.

We developed plasmid vectors coding for shRNA, capable of specific silencing of human TβRII mRNA and protein expression in glioblastoma cells. However, the efficiency of different shRNA to block TβRII expression at the mRNA level varied; two shRNA were highly effective, particularly in blocking TGF-β-induced transcriptional responses in human glioblastoma cells. TGF-β-dependent activation of Smad-dependent promoter and the human PAI-1 gene promoter were significantly reduced in human glioblastoma cells expressing shRNA against TβRII (Figure 2). PAI-1 is an inhibitor of tissue-type and urokinase-type plasminogen activator (uPA). High expression of PAI-1 and uPA characterizes malignant astrocytic tumors (Yamamoto *et al*., 1994). Antisense against uPA or transfer of the PAI-1 gene reduced the invasiveness of glioma cells *in vitro* and decreased tumorigenicity (Praus *et al*., 1999).

Disruption of a cell monolayer induced a cell migration of control glioma cells, probably due to an activation of TGF-β production. In contrast, glioma cells expressing shRNA against TβRII exhibited a decreased motility under such conditions. An addition of exogenous TGF-β1 led to the enhancement of glioma cell migration, but migration of glioma cells expressing shRNA against TβRII was not altered, demonstrating how a lack of cytokine responsiveness impairs tumor cell motility. Our study demonstrates that a knockdown of TβRII inhibits both basal and cytokine-induced glioblastoma motility in a scratch-wound healing assay.

Surprisingly, silencing of TβRII expression did not affect a basal invasiveness of human and rat glioblastoma cells (Figures 3 and 6), but it abolished TGF-β1-stimulated invasiveness. T98G glioblastoma cells produce a significant amount of TGF-β (unpublished), but a low, basal activity of Smad-dependent promoter (Figure 2a) suggests that this cytokine is not active under basal conditions. On the other hand, an exogenous TGF-β1 has been shown to promote an invasion of human glioma cells in a Matrigel invasion assay by inducing MMPs expression and suppressing TIMP expression (Nakano *et al*., 1995; Wick *et al*., 2001). Our preliminary studies show that silencing of TβRII expression inhibits TGF-β1-induced expression of *MMP-9* mRNA levels in transiently transfected T98G cells (not shown).

Taking advantage of having a stably transfected glioma cells, we demonstrated that shRNA-mediated knockdown of TβRII significantly reduced (by 50%) glioma growth in nude mice. Thus, blocking of TGF-β-dependent responses, in particular its effects on migration/invasiveness of human malignant glioblastoma cells, would be an effective approach to affect tumor growth.

Few examples of inhibition of TGF-β1 signaling in cancer cells by RNA interference have been described recently. A simultaneous knockdown of Smad2, Smad3 and Smad4 proteins in human HaCaT keratinocyte cells, affected TGF-β-dependent responses in invasion, wound healing and apoptosis (Jazag *et al*., 2005). RNAi-mediated Smad4 silencing suppressed growth significantly and induced apoptosis of human rhabdomyosarcoma cells (Ye *et al*., 2006). A knockdown of Smad4 in MDA-MB-231 breast cancer cells, using shRNA-expressing vectors inhibited bone metastasis in nude mice and increased survival (Deckers *et al*., 2006). However, even though such approach efficiently blocked Smad-dependent responses, it would leave unaffected Smad-independent responses, which are important but underestimated elements of TGF-β signaling network (Yu *et al*., 2002; Derynck and Zhang, 2003). Furthermore, Ogorelkova *et al*. (2006) demonstrated that adenovirus-delivered shRNAs against human TβIIR, but not antisense RNA, were able to silence endogenous TβIIR in A549 and Hela cells, indicating a superiority of siRNA approach. The possibility of silencing of TβIIR with shRNA delivered by plasmid vectors, provides an additional instrument of control for tissue- and cell-specific siRNA expression.

Knockdown of TβRII expression in glioma cells abrogates microglia-promoting effects on glioma invasiveness

There is increasing clinical and experimental evidence that in the tumor microenvironment, tumor-associated macrophages convert towards a phenotype, which is oriented to the promotion of tissue remodeling and repair. Tumor-educated macrophages may promote tumor progression and metastasis (Pollard, 2004; Mantovani *et al*., 2006). Microglial cells and blood-born macrophages are abundant within and around gliomas. Microglia accumulation in diffuse glial tumors does not represent a non-specific reaction to tissue injury or an ineffective response of the innate immune system, but rather reflects an active participation of these cells in supporting and promoting the invasive phenotype of gliomas. Microglia can release many factors, including extracellular matrix proteases and cytokines, which may directly or indirectly influence tumor migration/invasiveness and proliferation (Lindholm *et al*., 1992; Lehrmann *et al*., 1998; Rao, 2003; Markovic *et al*., 2005; Watters *et al*., 2005).

Activated microglia and blood-borne macrophages are the major source of TGF-β mRNA after brain injury

and in experimental rat gliomas (Lindholm *et al*., 1992; Kiefer *et al*., 1994). However, a role of microglia-derived TGF-β in regulation of migratory and invasive potential of gliomas has not been evaluated. Recent studies, including ours, demonstrate a crucial role of microglia in supporting growth and invasiveness of gliomas, thus it is important to elucidate molecular mechanisms underlying glioma–microglia interactions. The present study is the first demonstration that the invasion promoting effect of microglia is mostly mediated by TGF-β. We demonstrate that cultured microglial cells secrete TGF-β1 and the exposure to glioma cells strongly enhances cytokine secretion. It implies that microglia might be an abundant source of cytokine in diffuse brain tumors. Since microglia are most abundant at the periphery of the tumor, it would result in highest levels of the active cytokine at the sites where the tumor cells invade the brain tissue. We demonstrated that siRNA-mediated knockdown of TβRII expression in glioblastoma cells or the presence of a neutralizing antibody against TGF-β1 completely abolished the microglia-promoting effects on glioma invasion. TGF-β-mediated interactions with tumor infiltrating macrophages and/or tumor microenvironment may be responsible for the observed decrease of pST1119 glioma growth in nude mice. Altogether, our findings demonstrate an important role for microglia-derived TGF-β1 in supporting and promoting the invasive phenotype of gliomas. Efficient silencing of human TβIIR with shRNA delivered by plasmid vectors to glioblastoma cells provides a novel tool for the development of glioma gene therapy.

Materials and methods

Cell cultures and treatment

Human T98G glioblastoma cells and rat C6 glioma cells were from American Type Culture Collection (ATCC, Rochville, MD, USA). Cells were cultured in Dulbecco's modified essential medium (DMEM) with a heat-inactivated 10% fetal bovine serum (FBS) and antibiotics in a humidified atmosphere of CO_2/air (5/95%) at 37°C as described (Ciechomska *et al*., 2003; Zupanska *et al*., 2005). A recombinant human TGF-β1 was from R&D Systems (Wiesbaden, Germany). Primary rat glial cultures were obtained from 1-day-old Wistar rat pups as described (Zawadzka and Kaminska, 2005; Sliwa *et al*., 2007). Microglial cells were isolated by a mild shaking from glial cultures and incubated for 48 h in a culture medium before experiments as described (Sliwa *et al*., 2007). More than 96% of the adherent cells were positive for isolectin B_4, a specific microglial marker.

Constructs, cell transfection and flow cytometry

Four pairs of 57–59 oligonucleotides including 19–21 bp sequence, complementary to the target TβRII mRNA (M85079) and a loop sequence, were designed according to Tuschl algoritm (Ambion). Hairpin siRNA-encoding oligonucleotides were cloned into *Bam*HI- and *Hin*dIII-digested p*Silencer* siRNA expression vector (Ambion). The negative control and GFP-targeting inserts (components of Ambion siRNA Expression Vector Kit) were subcloned in parallel. A *Sca*I restriction site in the loop sequence was used for the restriction digestion. All plasmids have been sequenced.

For transfection, cells were cultured at density $1–5 \times 10^5$ cells/well for 24 h and transfected with 1 μg p*Silencer* plasmids encoding Negative or specific shRNA using Lipofectamine 2000 (Invitrogen, Paisley, UK). For reporter gene analysis, 0.5 μg Smad-dependent promoter $(CAGA)_{12}$-Luc (from Dr S Souchelnytskyi) or p800-Luc containing PAI-1 promoter fragment ($+71$ to -800) (from Dr CS Cierniewski) were used in cotransfection. After transfection, cells were cultured in DMEM/2% FBS for 24 h and treated with TGF-β1 for additional 24 h. Subsequently, cells were harvested by scraping, lysed in 50 μl of a passive lysis buffer (Promega, Madison, WI, USA) and the luciferase activities were measured. The protein concentration was determined by BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

The efficiency of transfection and silencing of GFP expression was evaluated by flow cytometry. Glioblastoma cells cotransfected with a plasmid encoding GFP gene (pTracer™CMV2, Invitrogen) and p*Silencer*™ encoding the GFP-specific or control shRNA were collected 48 h after transfection. Reduction of GFP fluorescence in transfected cells was measured by flow cytometry using Cell Quest Software (Becton Dickinson).

Real-time PCR

Total RNA from 5×10^5 glioblastoma cells was collected 56 h after transfection. Cells were homogenized with 0.6 ml of RLT reagent (Qiagen, Valencia, CA, USA) and cDNAs were synthesized by an extension of $(dT)_{15}$ primers with 200 units of SuperScript II reverse transcriptase (GIBCO BRL, Gaithersburg, MD, USA). PCR amplifications were performed with TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), specific primers and the fluorescence probe designed for exon VIII of *TβRII* sequence. The primers and probe were designed using PRIMER EXPRESS software: P1: TGAAGCGTTCTGCCACACA and P2: ACCACCA GGGCATCCAGAT and TaqMan probe 6-FAM-CGTGG TCCCAGCACTCAGTCAACGT-TAMRA. For normalization, quantitative real-time PCR with pre-developed TaqMan Assay for human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was performed simultaneously. Relative quantity of TβRII gene expression was estimated with ABI PRISM 7700 Sequence Detection System using comparative C_T method.

Immunoblot analysis

Fifty micrograms of the total protein extract from transiently transfected cells were loaded onto 10% polyacrylamide gel, electrophoresed and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Blots were probed overnight at 4°C with an antibody specific for TβIIR (1:1000, Cell Signaling Technologies, Hitchin, UK) and a monoclonal anti-β-actin antibody (1:2000, Oncogene). Secondary anti-rabbit (Cell Signaling Technologies) or anti-mouse (Oncogene Research Products, San Diego, CA, USA) antibodies linked to horseradish peroxidase were used. Immunocomplexes reaction was developed with the enhanced chemiluminescence system (ECL; Amersham Corp.).

Matrigel invasion assay

The invasion assay was a modification of a published protocol (Albini *et al*., 1987). Tissue culture inserts (12 μm pore size Transwell, Corning, NY, USA) coated with Growth Factor Reduced Matrigel Matrix (BD Biosciences, San Diego, CA, USA) were used. Hundred microliters of the Matrigel Matrix (1 mg/ml) in distilled water was dried under sterile conditions (37°C) for 5–6 h and reconstituted for 15 min in 50 μl of serum-free medium. T98G glioblastoma cells were transfected with

TβRII-specific or control p*Silencer*™ plasmids. Cells were detached by trypsinization 24 h after transfection, counted and seeded at a density of 10^5 cells/insert. 1500 μl DMEM/2% FBS with or without TGF-β1 (5 ng/ml) was added to the lower compartment. After 24 h of incubation, Matrigel Matrix was removed, the cells on the membranes were fixed with 95% methanol/5% phosphate-buffered saline (PBS) solution and a staining of cell nuclei with DAPI (0.01 mg/ml, Sigma-Aldrich, St Louis, MO, USA) for 10 min was performed. The membranes from Transwell inserts were cut out and all cells were counted independently by two researchers under a fluorescence microscope (excitation at 330–380 nm).

Selection of C6 clones stably expressing shRNA against TβRII
Stable clones were selected by culturing transfected cells in medium containing 500 μg/ml hygromycin. Colonies resistant to Hygromycin B appeared within 16 days, the surviving cells were collected and seeded by a serial dilution to develop independent cell lines. Genomic DNA from the parental C6 and transfected cell lines was isolated with DNAzol Reagent (GIBCO BRL) and plasmid integration was confirmed by amplification of 100 ng DNA using primers: A, 5′-GCGTCCT TTCCACAAGATATATAA-3′; B, 5′-GCTTCTCCTCCCTT TAGTGA-3′ in the following 30 cycles: 94°C, 10′; (94°C, 30″; 59°C, 30″; 72°C, 30″) and 72°C, 60″.

Scratch assay
A total of 1.5×10^6 stably transfected cells were seeded on 60-mm culture dishes in duplicate. A scratch was gently made using a pipette tip when cells were 80% confluent. Cells were washed with PBS, and cultured in DMEM/2% FBS with and without TGF-β1 (5 ng/ml) for 12 and 16 h. Cells were fixed and stained with DAPI (0.01 mg/ml, Sigma-Aldrich) for 10 min at room temperature. Cells from $\geqslant 3$ representative fields were counted.

Tumorigenicity of C6 glioma clones in nude mice
All of the animal experiments were carried out under the conditions established by the European Community (Directive 86/609/CCE). Glioma cells were trypsinized, washed twice, and harvested by centrifugation at 1000 *g* for 5 min. Cell pellets were re-suspended in sterile PBS. A total volume of 50 μl containing 2 or 5×10^6 cells was subcutaneously injected into 6–8-week-old male nude mice (Institute Gustave Roussy, Villejuif, France). Injected mice were examined every 2–4 days for tumor apparition. Palpable tumors developed within 6–10 days. The volumes of tumor were calculated using formula: volume $V = L \times W \times 0.52$, where L is the length and W is the width of the tumor (in millimeters). *P*-value less than 0.05 were considered as significant. All mice were autopsied.

Invasiveness of glioma cells stably expressing shRNA TβRII in co-cultures with microglia
PSTNeg- or pST1119-expressing glioma cells were seeded at a density of 4×10^4 per insert in 24 wells Transwell coated with the Growth Factor Reduced Matrigel Matrix. Inserts containing glioma cells were transferred to a 24-well plate with microglia in the lower compartment. Under control conditions, only DMEM/2% FBS was present in the lower compartment. After 36 h, glioma cells from the top of the filter were removed, and the cells invading through the coated membrane were fixed with 95% methanol/5% PBS solution, stained with DAPI and counted using iCys Research Imaging Cytometer with microscope unit Olympus IX-71 with a bright-field lamp. Fluorescence was measured using argon laser (488 nm), objective $\times 20$ and integral pixel correction.

In additional experiments, inserts containing glioma cells were transferred to a 24-well plate with or without microglia in the lower compartment. An invasion of glioma cells through the Matrigel Matrix has been evaluated in the presence of a neutralizing antibody against TGF-β1 (rabbit anty-TGF-β1 mAb (56E4), 1:100 dilution, Cell Signaling). Invading cells were fixed with 95% methanol/5% PBS solution, stained with DAPI and counted using iCys Research Imaging Cytometer as described above.

Determination of TGF-β1 level by ELISA
The level of TGF-β1 was determined in the conditioned medium from control microglial cultures and microglial cells exposed to glioma cells using ELISA kit (R&D Systems). The level of TGF-β1 in C6-conditioned medium and DMEM/FBS was subtracted from measurements in each sample. Experiments were performed on three independently derived microglial cultures; each in triplicate.

Statistical analysis
Statistical analyses were performed using Statistica (ver. 7.1 StatSoft Inc., OK, USA) software. Statistical significance was determined by Newman–Keuls analysis.

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