TROY (TNFRSF19) Is Overexpressed in Advanced Glial Tumors and Promotes Glioblastoma Cell Invasion via Pyk2-Rac1 Signaling

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Abstract

A critical problem in the treatment of malignant gliomas is the extensive infiltration of individual tumor cells into adjacent brain tissues. This invasive phenotype severely limits all current therapies, and to date, no treatment is available to control the spread of this disease. Members of the tumor necrosis factor (TNF) ligand superfamilly and their cognate receptors regulate various cellular responses including proliferation, migration, differentiation, and apoptosis. Specifically, the TNFRSF19/TROY gene encodes a type I cell surface receptor that is expressed on migrating or proliferating progenitor cells of the hippocampus, thalamus, and cerebral cortex. Here, we show that levels of TROY mRNA expression directly correlate with increasing glioma tumor grade. Among malignant gliomas, TROY expression correlates inversely with overall patient survival. In addition, we show that TROY overexpression in glioma cells activates Rac1 signaling in a Pyk2-dependent manner to drive glioma cell invasion and migration. Pyk2 communoprecipitates with the TROY receptor, and depletion of Pyk2 expression by short hairpin RNA interference oligonucleotides inhibits TROY-induced Rac1 activation and subsequent cellular migration. These findings position aberrant expression and/or signaling by TROY as a contributor, and possibly as a driver, of the malignant dispersion of glioma cells.

Introduction

Glioblastoma multiforme (GBM) is the most malignant form of all primary adult brain tumors in which median patient survival remains ~1 year (1). A characteristic feature of GBM is the propensity of glioma cells to invade the surrounding normal brain tissue (2-5). Invasion is a dynamic process dependent on the interplay between cell surface adhesion receptors and the cellular and extracellular matrix environments. The critical drivers of glioma invasion are not fully understood, yet several cell surface proteins including integrins (6, 7), L1CAM (8), and galectin1 (9-13) have been identified as important mediators of glioma invasion. Although some advancement toward GBM treatment has been made (14), these invasive cells still render complete surgical resection impossible and confer resistance to proapoptotic stimuli (2, 15) and far less to pro-autophagic stimuli (16). Thus, improved treatment of malignant glioma awaits a means to effectively target the dispersing tumor cells, and currently, no anti-invasive therapies are available.

Tumor necrosis factor (TNF) and TNF receptor (TNFR) superfamilies are involved in various physiologic and pathologic responses, including cell survival, programmed cell death, inflammation, and differentiation (17). Previously, gene expression profiling of glioma cells (migrating in vitro and invading in vivo) revealed several candidate genes involved in glioma cell invasion, as well as survival genes with a concordant decrease in proapoptotic genes (18, 19). Several candidates belonging to the TNFR superfamily (TNFRSF) emerged, including TNFRSF12A/Fn14 (18, 20). We previously showed that TNFRSF12A/Fn14 is highly expressed in GBM specimens and is upregulated during glioma cell migration in vitro and invasion in vivo (20, 21). Specifically, the Fn14 signaling axis has been implicated in GBM cell invasion (21) and survival (22). Similar to Fn14, our analysis of the glioma gene expression profile identified another type I transmembrane receptor member of the TNFRSF, TNFRSF19/TAJ/TROY, as a gene candidate highly overexpressed in GBM specimens as described in the present study.

TROY is an orphan member of the TNFRSF that is highly expressed in embryonic and adult central nervous system, and developing hair follicles (23-28). During mouse embryogenesis, TROY mRNA is detected in many developing tissues including the limb buds, eyelids, whiskers, mammary glands, epidermis, bronchial, tongue, dental and gastric epithelium, as well as the germinal zones of the central nervous system including the ventricular zone and subventricular...
zone. However, in adult animals, TROY expression changes
and is primarily restricted to hair follicles and neuron-like
cells in the cerebrum, cerebral cortex, and developing olfac-
tory system, including the dorsal root and retinal ganglion
neurons (23-28). In humans, TROY mRNA is primarily
expressed in the brain and also the prostate, whereas low
or undetectable levels are observed in the heart, lung, liver,
thymus, uterus, skeletal muscle, spleen, colon, testis, kid-
ney, and peripheral blood lymphocytes (29). In the periph-
ery nervous system, TROY functions as a coreceptor for the
ligand-binding Nogo-66 receptor 1 (NgR1) to form the
TROY/NgR1/LINGO complex that activates the RhoA
pathway to inhibit neurite outgrowth of dorsal root gangli-
on neurons in adult mice (24, 28). More recently, TROY
has been reported to be a possible factor in mediating the
switch of osteoblast versus adipocyte differentiation of hu-
mans multipotent mesenchymal stromal stem cells (30).

In this study, we show that TROY mRNA is over-
expressed in advanced glial tumors and is associated with
poor prognosis. We provide evidence that increased TROY
expression stimulates glioma cell migration and invasion
via the Rac1 signaling pathway. In addition, we show that
TROY coimmunoprecipitates with the nonreceptor tyro-
sine kinase Pyk2 and that depletion of Pyk2 expression
or Pyk2 activity suppresses TROY-induced Rac1 activation
and subsequent glioma cell migration.

Materials and Methods

Expression profile data set of TROY/TNFRSF19.2 in
human gliomas and nonneoplastic brain

An expression microarray database consisting of 135
clinically annotated brain tumor specimens publicly avail-
able at the National Center for Biotechnology Information
(NCBI) Gene Expression Omnibus as data set GDS1962
was mined for TROY expression (TNFRSF19.1 and
TNFRSF19.2). Snap-frozen specimens from epileptogenic
foci [nonneoplastic brain (NB), n = 24] and tumor [29 low-
grade astrocytomas (LGA) and 82 GBMs] with clinical
information were collected at the Hermelin Brain Tumor
Center, Henry Ford Hospital (Detroit, MI) as previously
described (21). Gene expression profiling as described
previously (21) was conducted on all samples using Affyme-
trix U133 Plus 2 GeneChips according to the manufacturer’s
protocol at the Neuro-Oncology Branch at the National Cancer
Institute (Bethesda, MD). For analysis, gene expression data
were normalized in two ways: per chip normalization and
per gene normalization across all samples in the collection.
For per chip normalization, all expression data on a chip
were normalized to the 50th percentile of all values on that
chip. For per gene normalization, the data for a given gene
were normalized to the median expression level of that gene
across all samples. Gene expression differences were deemed
statistically significant using parametric tests, where variances
were not assumed equal (Welch ANOVA). Expression values
were then filtered for highly variable (differentially expressed)
genes (coefficient of variation > 30%) across samples, produc-
ing a list of 7,322 genes. Principal component (PC) analysis was
done to discern possible relationships between subgroups of
samples as previously described (21), and Kaplan-Meier survival
curves were developed for each PC cluster. One cluster had a
median survival time of 401 days (short-term survival) and the
other cluster had a median survival time of 952 days (long-term
survival). Box plots for TROY/TNFRSF19.2 expression level in
each cluster derived from PC analysis were graphed. Significance
between the two populations was tested with a two-sample t test.

Clinical samples and histology

Fresh human brain tumor tissues were obtained from 41
patients who underwent therapeutic removal of astrocytic
brain tumors under an Institutional Review Board–approved
protocol as previously described (31). Nonneoplastic control
brain tissues were identified from the margins of the tumors.
Histologic diagnosis was assessed under standard light
microscopic evaluation of the sections stained with H&E
based on the revised WHO criteria for tumors of the central
nervous system (32). The 41 astrocytic tumors consisted of
7 LGAs, 4 anaplastic astrocytomas, and 30 glioblastomas. All
of the tumor tissues were obtained at primary resection, and
none of the patients had been subjected to chemotherapy or
radiation therapy before resection.

Cell culture conditions

Human astrocytoma cell lines T98G, SNB19, U87, and
U118 were maintained in DMEM + 10% heat-inactivated
fetal bovine serum in a 37°C, 5% CO2 atmosphere.

Antibodies and reagents

Anti-HA epitope antibody was obtained from Cell Sig-
aling Technology, Inc. Anti-TROY antibody was obtained
from Abcam. The polyclonal anti-Pyk2 antibody was from
Upstate Biotechnology. Monoclonal antibodies to Rac1
were purchased from BD Transduction Labs. Monoclonal
antibody to α-tubulin was purchased from Millipore. Laminin
from human placenta was obtained from Sigma.

Expression constructs

The human TROY cDNA (clone ID: 5248745) was
purchased from Open Biosystems. The TROY coding
sequence (TNFRSF19.2) was amplified by PCR and
cloned in-frame upstream of a 3× HA epitope in pcDNA3.
For stable transduction of glioma cell lines, the HA
epitope–tagged TROY fragment was excised from
cDNA3 and ligated into the lentiviral transfer plasmid
pCDH (System Biosciences). pCDH contains a second
transcriptional cassette for the expression of a reporter gene [green fluorescent protein (GFP)]. An empty pCDH vec-
tor expressing only the GFP reporter was used as a control.
Recombinant lentiviruses were produced as described (33).
The kinase-deficient Pyk2 variant (Pyk2KD) containing
the substitution of K457A was generated using the Quik-
Change Site-Directed Mutagenesis kit (Stratagene).
The coding sequence of Pyk2KD was excised and subcloned
into the adenoviral shuttle vector pShuttle-CMV to prepare
recombinant adenoviruses using the Ad-Easy system as
described (34).
Small interfering RNA preparation and transfection
Small interfering RNA (siRNA) oligonucleotides specific for human Rac1 and nonmammalian GL2 luciferase were previously described (35). Validated siRNA sequences for human TROY (TROY-1, Hs_TNFRSF19_1; TROY-2, Hs_TNFRSF19_5) were purchased from Qiagen. Transient transfection of siRNA was carried out as previously described (35). All siRNA oligonucleotides were transfected at 25 nmol/L, and no cell toxicity was observed. Maximum inhibition of protein levels was achieved 72 hours after transfection. Small hairpin RNA (shRNA) specifically targeting human Pyk2 (36) was assembled in the lentiviral plasmid vector pLVTHM (Addgene), and recombinant lentiviruses were produced as previously described (37).

Quantitative reverse transcription-PCR
Total RNA from frozen tissues was extracted using RNeasy kit (Qiagen), following the manufacturer’s protocol. The quantity and quality of RNA were assessed with a 2100 Bioanalyzer (Agilent Technologies). cDNA was synthesized from 200 ng of total RNA as previously described (38). PCR analysis of TROY (sense, 5′-TGCTTCGACAGGATTCTTAGGAA-3′; antisense, 5′-GAGCCATCTCCAGGATTCTT-3′) and histone H3.3 (sense, 5′-CCACTGAACTCTGATTCGCC-3′; antisense, 5′-GCGTGCTAGCTGGATGCTT-3′) was conducted using a LightCycler 480 (Roche) with SYBR Green fluorescence signal detection after each cycle of amplification, and results were quantified and analyzed as described previously (20).

Rac activation assay, Western blotting, and immunoprecipitation assays
For the Rac activation assays, glioma cells were plated and cultured for 24 hours under normal conditions. Cells were cultured under serum-deprived condition for an additional 16 hours. Cells were washed twice with cold TBS and lysed in 50 mmol/L Tris (pH 7.2), 0.5% Triton X-100, and 10 mmol/L MgCl2 in a cocktail of protease inhibitors (Roche Diagnostics). Lysates were harvested and equal concentrations of lysates were assessed for Rac activity according to the protocol of the manufacturer (Pierce).

Immunoblotting and protein determination were done as described previously (22). For immunoprecipitation, equivalent amounts of cell lysates (500 μg) were preclotted and immunoprecipitated with the appropriate antibody, and the precipitates were washed twice with lysis buffer. Samples were then resuspended in 2× SDS sample buffer and boiled in the presence of 2-mercaptoethanol (Sigma), separated by SDS-PAGE, and transferred to nitrocellulose overnight at 4°C, and proteins were detected as described previously (22).

Immunofluorescence analysis for TROY-HA localization
Immunofluorescence was done using an anti-HA antibody (Cell Signaling Technology). Control cells or cells stably transfected with a lentiviral vector expressing TROY-HA were plated and allowed to attach onto a 10-well glass Teflon slide for 24 hours. Cells were then fixed for 5 minutes in 4% paraformaldehyde in PBS. Cells were then permeabilized with 0.5% Triton X-100 in PBS. After washing with PBS, cells were blocked with 2% bovine serum albumin and 1% goat serum, and incubated with anti-HA antibody for 1 hour at 25°C. Following washing with 0.1% bovine serum albumin in PBS and incubation with FITC-conjugated antimouse secondary antibody, cell nuclei were stained with 4′,6-diamidino-2-phenylindole in PBS for 15 minutes, washed, and mounted in 2% n-propyl gallate and 90% glycerol (pH 8.0). Cells were examined using a laser-scanning confocal microscope, equipped with helium, neon, and argon lasers (Zeiss) using the appropriate filters. Images were processed using Adobe Photoshop.

Radial cell migration assay
Cellular migration was quantified in a radial cell migration assay as previously described (21). For certain experiments, cells were transfected with siRNA targeting luciferase, TROY, or Rac1. After 24 hours, cells were plated onto 10-well glass slides precoated with 10 μg/mL of human laminin as previously described (21). Cell migration was determined over 24 hours.

Organotypic brain slice invasion assay
An ex vivo invasion assay into rat brain slices was done as described previously (21). Briefly, 400-μm-thick sections were prepared from brains of Wistar rats (Charles River Laboratories) floated on micropore membranes in culture media. Control T98G cells transduced with GFP or T98G cells transduced with TROY-HA (1 × 105) were gently deposited (0.5-μL transfer volume) onto the bilateral putamen of the brain slice and then incubated under standard conditions. Forty-eight hours after seeding the cells, glioma cell invasion into the rat brain slices was detected using a LSM 5 Pascal laser-scanning confocal microscope (Zeiss) to observe GFP-labeled cells in the tissue slice. Serial optical sections were obtained every 10 μm downward from the surface plane to the bottom of the slice, and for each focal plane, the area of fluorescent cells as a function of the distance from the top surface of the slice was calculated. The extent of glioma cell invasion was reported as the depth where the area of fluorescent tumor cells was half of the maximum area at the surface.

Matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry
For matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS), protein bands were stained with SYPRO Ruby protein staining kit (Bio-Rad) according to the manufacturer’s protocol. Prominent protein bands present in the anti-HA immunoprecipitate of lysates from TROY-HA–expressing cells were visualized under UV light and isolated. The samples of generated peptides were dissolved in 5 mL of 0.5% trifluoroacetic acid and measured by MALDI-TOF MS analysis on a Voyager reflector instrument (Applied Biosystems) and a Q-STAR mass spectrometer (Perceptive Biosystems).
in positive ion mode at the University of Arizona Proteomic Facility. Data searches were done using the NCBI protein data bank with a minimum matching peptide setting of four, a mass tolerance setting of 50 to 200 ppm, and a single trypsin miss cut setting.

Statistical analysis
Statistical analyses were done using the two-sample t test. P < 0.05 was considered significant.

Results
TROY/TNFRSF19.2 mRNA expression levels correlate with brain tumor grade and poor patient outcome
Members of the TNFRSF, most notably TNFR1, have been shown to play a role in inducing cell invasion and migration in several cancer types. In previous studies, we have reported that the fibroblast growth factor–inducible 14 (Fn14) receptor (TNFRSF12A) is overexpressed in advanced glial tumors and invading cells (20, 21). To determine whether other members of the TNFRSF are upregulated in glial tumors, we mined the expression microarray database containing 195 clinically annotated brain tumor specimens publicly available at NCBI Gene Expression Omnibus as data set GSE4290. Snap-frozen specimens from epileptogenic foci (NB, n = 24), LGA (n = 29), and GBM (n = 82) with clinical information were collected at the Hermelin Brain Tumor Center, Henry Ford Hospital (Detroit, MI) as previously described (21). Gene expression profiling done as described previously (21) was conducted on all samples using Affymetrix U133 Plus 2 GeneChips according to the manufacturer’s protocol at the Neuro-Oncology Branch at the National Cancer Institute (Bethesda, MD). For our analysis, gene expression data were normalized both per chip and per gene across all samples in the collection as described in detail in Materials and Methods. Gene expression differences were deemed statistically significant using parametric tests, where variances were not assumed equal (Welch ANOVA). Expression values were then filtered for highly variable (differentially expressed) genes (coefficient of variation > 30%) across samples producing a list of 7,322 genes. Of the TNFRSF genes analyzed, we found that expression of the TROY/TNFRSF19.2 isoform is significantly differentially expressed among brain specimens. In normal brain specimens, TROY expression is relatively low, but is increased with increasing tumor grade and is significantly higher in GBM samples (n = 82; Fig. 1A). To verify the results from the expression profiling, we did quantitative reverse transcription-PCR on independent nonneoplastic (n = 10), LGA (n = 6), anaplastic astrocytoma (n = 4), and GBM (n = 22) specimens. Normal brain specimens show relatively low mRNA levels for TROY compared with the brain tumor samples (P < 0.01). In GBM specimens, the mRNA level of TROY is significantly higher than in NB tissue (P < 0.01; Fig. 1B). Next, PC analysis was done to discern possible relationships between subgroups of samples as previously described (21), and Kaplan-Meier survival curves were developed for each PC cluster. One cluster had a median survival time of 401 days (short-term survival), and the other cluster had a median survival time of 952 days (long-term survival). Box-and-whisker plots for TROY expression level

![Gene expression profiling of TROY/TNFRSF19 across NB samples and brain tumor samples. A, TROY mRNA expression levels from NCBI Gene Expression Omnibus GDS1962 data set are presented as box-and-whisker plots. The box for each gene represents the interquartile range (25-75th percentile), and the line within this box is the median value. Bottom and top bars of the whisker indicate the 10th and 90th percentiles, respectively. Outlier values are indicated (closed circles). Significance between the indicated classes of brain specimens was tested using a two-sample t test assuming unequal variances. OL, oligodendrogliomas; Astro, low-grade astrocytomas. B, quantitative real-time PCR analysis of TROY expression in NB, grade 1 LGA, grade 2 to 3 astrocytomas, and GBM. Values were normalized to histone H3.3 reference gene. Data are presented as box-and-whisker plots. C, PC analysis of brain tumors from NCBI Gene Expression Omnibus GDS1962 data set revealed two groups differing by their survival and were denoted as long-term and short-term survival. Box-and-whisker plots for TROY expression in GBM specimens for each cluster are shown. Significance between the two populations was tested with a two-sample t test assuming unequal variances.](mcr.aacrjournals.org)
in each cluster derived from PC analysis were graphed. Significance between the two populations was tested with a two-sample t test assuming unequal variances. Analysis of the Affymetrix expression values for TROY in the GBM specimens for each cluster showed that patients with GBM in the short-term survival cluster had higher expression of TROY (10.5) than GBM patients in the long-term survival cluster (2.9; \( P < 0.01 \); Fig. 1C). These data suggest that high TROY expression correlates with poor patient outcome.

TROY is expressed in glioblastoma cell lines, and siRNA-mediated depletion of TROY suppresses glioblastoma cell migration

We next examined the expression of TROY protein in four different cultured glioblastoma cell lines. Expression of TROY was high in U118 cells, moderate in U87 cells, and lower in T98G and SNB19 cells (Fig. 2A). To determine the role of TROY in glioma cell migration, we used RNA interference (RNAi) to knockdown the expression of TROY in the four glioma cell lines and examined the migratory behavior of the cells on human laminin using a two-dimensional radial cell migration assay (21). siRNA-mediated knockdown of TROY expression in the glioma cell lines with two independent siRNA oligonucleotides was \( \sim 80\% \) to 90\% effective and has no effect on the expression of another TNFRSF member, Fn14. Representative results are shown for U118 cells (Fig. 2B). Knockdown of TROY expression resulted in a significant inhibition of cell migration in all four cell lines relative to control cells expressing siRNA targeting luciferase (\( P < 0.05 \); Fig. 2C).

Overexpression of TROY in glioma cells stimulates cell migration and invasion, and TROY expression localizes to the leading edge of cell migration

To further examine the function of TROY signaling in glioma cell migration, we overexpressed HA epitope-tagged TROY in T98G glioma cell lines by lentiviral transduction (Fig. 3A). Overexpression of HA-tagged TROY in T98G glioma cells resulted in a 2-fold increase in cell migration rate (Fig. 3B). To examine the role of TROY in glioma cell invasion in the context of the authentic brain microenvironment, we used an \textit{ex vivo} organotypic rat brain slice model. Overexpression of TROY in T98G glioma cells resulted in a 2-fold increase in the depth of cell invasion after 48 hours relative to control cells transduced with GFP alone (Fig. 3C). Immunolocalization of TROY using an anti-HA antibody revealed that TROY was localized near the cell periphery and was enriched in lamellipodia (Fig. 3D, b).

Pyk2 associates with TROY and mediates TROY-induced glioblastoma migration

To determine possible mechanisms by which TROY regulates cell migration, we sought to identify potential binding partners of TROY using immunoprecipitation experiments coupled with MALDI-TOF MS analysis. T98G cells expressing HA-tagged TROY or control T98G cells expressing GFP alone were lysed and immunoprecipitated with anti-HA antibodies, and the immunoprecipitates were resolved by SDS-PAGE. Prominent protein bands present in the immunoprecipitates of TROY-expressing cells but absent in the immunoprecipitates of control cells were recovered from the gel. Proteins were eluted and trypsin digested, and MALDI-TOF and MS-MS analysis of the trypsin digests were done. The nonreceptor protein tyrosine kinase Pyk2 was a candidate sequence identified by MS in the TROY immunoprecipitate. Association of TROY with Pyk2 was verified by coimmunoprecipitation. T98G cells transfected with HA-tagged TROY or cotransfected with HA-tagged TROY and Pyk2 were immunoprecipitated with anti-HA antibodies, and the precipitates were immunoblotted with anti-Pyk2 antibodies (Fig. 4A). Both endogenous Pyk2 and transfected Pyk2
coimmunoprecipitated with TROY, substantiating the intracellular interaction between TROY and Pyk2.

To determine whether the association with Pyk2 was required for TROY-induced stimulation of glioma migration, we examined the effect of depletion of Pyk2 expression by shRNA in TROY-overexpressing T98G cells. Knockdown of Pyk2 expression significantly inhibited TROY-stimulated glioma cell migration (Fig. 4B). Moreover, TROY-stimulated migration of glioma cells required Pyk2 activity (Fig. 4C). Expression of a kinase-inactive variant of Pyk2 (Pyk2KD) significantly inhibited the migration of the control T98G cells, consistent with our previous results (36). Expression of Pyk2KD also significantly inhibited the migration of TROY-overexpressing T98G cells, indicating that Pyk2 lies downstream of TROY. Together, these results indicate that TROY-mediated glioma cell migration is dependent on Pyk2 function.

**TROY regulates Rac1 activity, and depletion of Rac1 expression by siRNA suppresses TROY-induced cell migration and invasion**

Because Rac1 plays a central role in cytoskeletal rearrangement associated with cell migration and invasion, we investigated the effect of TROY expression on Rac1 function. To examine the effect of TROY on Rac1 activity, we compared the activation of Rac1 in glioma cells overexpressing TROY relative to the activation of Rac1 in untransfected cells. Overexpression of TROY resulted in a ~2-fold induction of Rac1 activation relative to untransfected cells (Fig. 5A). Because Pyk2 interacts with TROY and mediates TROY-induced migration, we also tested whether Rac1 activation induced by TROY expression is dependent on Pyk2. shRNA-mediated depletion of Pyk2 in TROY-overexpressing glioma cells suppressed TROY-induced Rac1 activity to the level of that in control cells (Fig. 5A). This suggests that the

**FIGURE 3.** Overexpression of TROY induces glioma cell migration and invasion. A, lysates of T98G cells transduced with empty lentiviral vector (V) or lentiviral vector encoding HA epitope–tagged TROY immunoblotted with anti-HA antibody. B, migration rate analysis of TROY-HA expressing glioma cells and control transduced cells. Cell migration was assessed over 48 h. Data represent the average of three independent experiments. *, P < 0.01; **, P < 0.05. C, T98G cells stably expressing GFP or T98G cells expressing HA-tagged TROY and GFP were implanted into the bilateral putamen on rat organotypic brain slices. Depth of invasion 48 h after seeding was calculated from Z-axis images collected by confocal laser-scanning microscopy. The mean value of the depth of invasion was obtained from six independent experiments. *, P < 0.01. D, immunofluorescent staining for TROY in T98G (a) and T98G-TROY-HA (b) cells using an anti-HA antibody. c, T98G-TROY-HA stained with secondary antibody alone. Arrow represents TROY staining at the membrane periphery and cellular extension.
TROY-mediated regulation of Rac1 activation is dependent on Pyk2. To determine the role Rac1 plays in TROY-induced glioma migration, we silenced Rac1 expression in T98G cells overexpressing the TROY receptor. siRNA-mediated depletion of Rac1 expression in T98G was ∼90% effective and resulted in significant inhibition of TROY-mediated cell migration (Fig. 5B).

**Discussion**

In this study, we showed that the TNFRSF member TROY is overexpressed in glioblastoma and that its expression inversely correlates with patient survival. We further showed that knockdown of TROY expression by RNAi significantly inhibited glioblastoma cell migration in vitro. Conversely, increased expression of TROY enhanced glioblastoma cell migration in vitro and invasion ex vivo. TROY-induced stimulation of glioma cells correlated with increased Rac1 activation and was dependent on Pyk2 function. These results support a role for aberrant expression and/or signaling by TROY in the malignant dispersion of glioma cells.

TROY is an orphan member of the TNFRSF, and unlike the classic TNFRSF members, TROY lacks a death domain (23). During embryonic development, TROY is widely expressed, but its expression in the postnatal organism is restricted (23-28). The strict control of TROY expression indicates that aberrant expression may be unfavorable. Indeed, it has been recently reported that TROY is highly expressed in primary and metastatic melanoma cells, but not in melanocytes found in normal skin biopsies (39). Similarly, we report that the levels of expression of TROY in glial tumors correlate with tumor grade, with advanced GBM showing the highest expression of TROY. Similar to our previous finding with Fn14 (21), TROY expression seems to be an indicator of poor outcome. PC analysis indicated that GBM patients in the short-term survival group expressed TROY at significantly increased levels compared with GBM patients with long-term survival. Thus, the aberrant reexpression of TROY in GBM may play an important role in GBM progression, specifically cell invasion, and possibly serves as a good predictor of survival outcome complimenting other prognostic indicators.

The human TNFRSF19 gene has two transcripts: TNFRSF19.1, which encodes a 423-amino acid polypeptide, and TNFRSF19.2, which encodes a 417-amino acid polypeptide. The sequence of both transcripts is identical with the exception of the COOH terminus of cytoplasmic domain. TNFRSF19.2 contains a major TNFR-associated factor (TRAF)–binding consensus sequence at amino acid 413 to 416, whereas this sequence is absent in TNFRSF19.1. The lack of the TRAF-binding sequence in TNFRSF19.1 suggests that these two TROY variants may signal differently, although that remains to be determined. We identified that the TROY variant TNFRSF19.2 was highly overexpressed in glioblastoma specimens, whereas no changes were observed in the mRNA expression of TNFRSF19.1. Elevated NF-κB activity has been observed in GBM (40, 41) and has been implicated in contributing to cellular resistance to cytotoxic intervention and promotion of expression of genes involved in cell survival and invasion (42). As TROY-mediated activation of NF-κB has been reported in experimental systems (43, 44), it is possible that TROY overexpression in GBM may contribute to NF-κB hyperactivation and subsequently cell invasion.
Our data showed that overexpression of TROY in glioma cells results in the inhibition of RhoA (data not shown) and an increased activation of Rac1, and thus results in the enhancement of cell migration. However, it is unclear how the TROY receptor is activated in glioma cells. It is possible that TROY signaling in GBM cells is dependent on the NgR1-LINGO complex, which uses the myelin-associated glycoprotein (MAG), Nogo-A, and oligodendrocyte myelin glycoproteins (OMgp) as ligand factors. Interestingly, p75 has also been shown to be upregulated in the invasive glioma cells and regulates glioma invasion (45, 46). Therefore, it is possible that in glioma, TROY can also replace p75 in the NgR1-LINGO complex to sustain glioma cell invasion in vitro.

Our studies identified Pyk2 as a cytoplasmic binding partner of TROY. Pyk2 is highly expressed in the nervous system and uniquely located within cells to transduce information from interactions with the extracellular matrix and soluble mediators through cell surface integrins, receptor tyrosine kinases, and G protein-coupled receptors into the activation of intracellular signaling pathways that modulate cell growth and migration (47, 48). Pyk2 expression is elevated in glioma, and expression is increased with advancing WHO grade (49). Moreover, Pyk2 expression is differentially upregulated in invasive glioma cells relative to cells in their cognate tumor cores in glioblastoma tumor samples (19), and phosphorylation of Pyk2 mediates heregulin-stimulated glioma invasion (50). In vitro studies with glioma cell lines indicated that migratory potential correlated positively with endogenous Pyk2 activity (51) and that silencing Pyk2 expression inhibits glioma cell migration (36). Moreover, knockdown of Pyk2 expression (37) or inhibition of Pyk2 activity (33) extended survival in a glioma xenograft model. In this study, we showed that knockdown of Pyk2 expression inhibited TROY-stimulated glioma cell migration and Rac1 activation. TROY-stimulated glioma cell migration required Pyk2 activity, as expression of a kinase-deficient Pyk2 blocked the effect of increased TROY expression, suggesting that Pyk2 is a proximal signaling effector for TROY. Understanding the mechanisms that underlie Pyk2 regulation of TROY-induced Rac1 activation is an area of current investigation.

Rac1 has been shown to play a role in various malignant carcinoma cells and in gliomas (52, 53). In glioma, inhibition of Rac1 expression by RNAi strongly inhibits cell migration and invasion (35, 54). Recently, studies have shown that certain members of the TNFRSF can regulate the activation of Rac1 (21, 55, 56). Notably, activation of Fn14 results in the activation of Rac1 to drive glioma invasion and migration (21). Because the activation of Rac1 occurs through the exchange of bound GDP for GTP catalyzed by one of multiple Rac guanine exchange factors (GEF), it is likely that TROY may modulate cytoskeleton dynamics by influencing one or more of the Rac GEF proteins involved in Rac1 activation, possibly through Pyk2.

**FIGURE 5.** TROY signaling activates Rac1 and induces cell migration via the Rac1 signaling pathway. A, T98G, T98G-TROY-HA, and T98G-TROY-HA cells expressing a shRNA targeting Pyk2 were cultured under serum-free medium for 16 h. Cellular lysates were then analyzed for activation of Rac1. B, depletion of Rac1 expression by siRNA suppresses TROY-induced glioma cell migration. T98G and T98G-TROY-HA cells were transfected with a siRNA oligonucleotide targeting Rac1. Cell migration was assessed over 24 h using a radial migration assay on 10 μg/mL laminin substrate. *, P < 0.01. Western blot validation of Rac1 depletion and TROY-HA expression is shown.

**FIGURE 6.** Schematic model of TROY-Pyk2-Rac1 signaling pathway in GBM cells. Activation of the TROY receptor by possible interaction with NgR1-LINGO complex elicits a signaling pathway to recruit Pyk2 to the TROY receptor complex. Pyk2 functions to induce Rac1 activation potentially by influencing a Rac GEF to drive glioma cell migration and invasion.
Importantly, several Rac GEFs, including Ect2, VAV3, TRIO, and Dock180, have been shown to be overexpressed in GBM specimens, and depletion of the expression of these GEFs by RNAi reduced Rac1 activation and inhibited glioma cell migration and invasion (38, 57). Interestingly, Pyk2 has been shown to modulate the activity of several Rac GEFs, including VAV1 and VAV2 (58, 59). A model of TROY signaling to mediate cell migration is shown in Fig. 6.

In summary, we have identified an important role for the TNFRSF member TROY in glioma cell migration and invasion. Increased expression of TROY stimulates in vitro glioma cell migration and invasion, is associated with increased activation of Rac1, and correlates with poor patient outcome. Furthermore, the results indicate that TROY-stimulated glioma cell migration and Rac1 activation are regulated by Pyk2. Future studies will seek to substantiate the current in vitro results implicating the importance of TROY in glioma invasion in a preclinical animal model. Overall, these observations suggest that TROY may represent a novel target for therapeutic intervention to inhibit invasion in malignant glioma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


Kojima T, Morikawa Y, Copeland NG, et al. TROY, a newly identified
Pahl HL. Activators and target genes of Rel/NF-κB.
Lipinski CA, Tran NL, Viso C, et al. Extended survival of Pyk2 or FAK
Lipinski CA, Tran NL, Menashi E, et al. The tyrosine kinase pyk2
Salhia B, Tran NL, Chan A, et al. The guanine nucleotide exchange
Weaver KD, Yeyeodu S, Cusack JC, Jr., Baldwin AS, Jr., Ewend
Spanjaard RA, Whren KM, Graves C, Bhawan J. Tumor necrosis factor
He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. A sim-
Chuang YY, Tran NL, Rusk N, Nakada M, Berens ME, Symons M.
33. Eby MT, Jasmin A, Kumar A, Sharma K, Chaudhary PM. TAJ, a novel
Qiu W, Hu Y, Andersen TE, et al. Tumor necrosis factor receptor
309. Gao C, Blystone SD. A Pyk2-Vav1 complex is recruited to
58. Ruan W, Lee CT, Desbarats J. A novel juxtamembrane domain
56. Chan AY, Coniglio SJ, Chuang YY, et al. Roles of the Rac1 and
55. Tanabe K, Bonilla I, Winkles JA, Strittmatter SM. Fibroblast growth
34. He TC, Zhou S, da Costa LT, Yu J, Kindler KW, Vogelstein B. A simpl-
33. Loftus JC, Yang Z, Tran NL, et al. The Pyk2 FERM domain as a target
32. Salhia B, Tran NL, Vaso C, et al. Extended survival of Pyk2 or FAK
31. Lipinski CA, Tran NL, Menashi E, et al. The tyrosine kinase pyk2
promotes migration and invasion of glioma cells. Neoplasia 2005;7:
45–45.
30. Lipinski CA, Tran NL, Vaso C, et al. Extended survival of Pyk2 or FAK
29. Eby MT, Jasmin A, Kumar A, Sharma K, Chaudhary PM. TAJ, a novel
member of the tumor necrosis factor receptor family, activates the
C-Jun N-terminal kinase pathway and mediates caspase-independent
superfamily member 19 (TNFRSF19) regulates differentiation fate
of human mesenchymal (stromal) stem cells through canonical Wnt
27. Nakada M, Drake KL, Nakada S, Niska JA, Berens ME. Ephrin-B3
ligand promotes glioma invasion through activation of Rac1. Cancer
of tumors of the nervous system. J Neuropathol Exp Neurol 2002;61:
813–70.
24. Chuang YY, Tran NL, Rusk N, Nakada M, Berena ME, Symons M.
Role of synaptotagmin 2 in glioma cell migration and invasion. Cancer
Res 2004;64:8271–5.
22. Salhia B, Tran NL, Chan A, et al. The guanine nucleotide exchange
factors trio, Ect2, and Vav3 mediate the invasive behavior of glioblas-
receptor superfamily member TROY is a novel melanoma biomarker
20. Weaver KD, Yeyeodu S, Cusack JC, Jr., Baldwin AS, Jr., Ewend
MG. Potentiation of chemotherapeutic agents following antagonism
17. Wang L, Rahn JJ, Lun X, et al. y-Secretase represents a therapeutic
target for the treatment of invasive glioma mediated by the p75
16. Girault JA, Costa A, Derkinderen P, Studer JM, Toutsant M. FAK and
PYK2/CAKβ in the nervous system: a link between neuronal activity,
15. Avraham H, Park ST, Schinkmann K, Avraham S. RAFTK/Pyk2-
14. Gutenberg A, Bruck W, Buchfelder M, Ludwig HC. Expression of
tyrosine kinases FAK and Pyk2 in 331 human astrocytomas. Acta
13. van der Horst EH, Weber I, Ulrich A. Tyrosine phosphorylation of
PYK2 mediates heregulin-induced glioma invasion: novel heregulin/
HER3-stimulated signaling pathway in glioma. Int J Cancer 2005;
113:689–98.
12. Lipinski CA, Tran NL, Bay C, et al. Differential role of proline-rich
tyrosine kinase 2 and focal adhesion kinase in determining glioblas-
11. Fritz G, Just I, Kaina B. Rho GTPases are over-expressed in human
Rho GTPase expression in tumourigenesis: evidence for a significant
9. Chan AY, Coniglio SJ, Chuang YY, et al. Roles of the Rac1 and
Rac3 GTPases in human tumor cell invasion. Oncogene 2005;24:
7821–9.
8. Tanabe K, Bonilla I, Winkles JD, Strittmatter SM. Fibroblast growth
factor-inducible-14 is induced in axotomized neurons and promotes
7. Ruan W, Lee CT, Desbarats J. A novel juxtamembrane domain in
tumor necrosis factor receptor superfamily molecules activates
Rac1 and controls neurite growth. Mol Biol Cell 2008;19:
3192–202.
Rac1 guanine nucleotide exchange factor, promote human glioma
5. Gao C, Blzystone SD, A Pyk2/Vav1 complex is recruited to J3-adhesion
4. Gakhids MA, Cullere X, Olson T, et al. Vav GEFs are required for β2
integrin-dependent functions of neutrophils. J Cell Biol 2004;166:
273–82.
TROY (TNFRSF19) Is Overexpressed in Advanced Glial Tumors and Promotes Glioblastoma Cell Invasion via Pyk2-Rac1 Signaling

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