Transmembrane Protein 18 Enhances the Tropism of Neural Stem Cells for Glioma Cells

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Abstract

The failure of current glioma therapies is mainly due to the ability of the tumor cells to invade extensively the surrounding healthy brain tissue, hence escaping localized treatments. Neural stem cells (NSC) are able to home in on tumor foci at sites distant from the main tumor mass, possibly enabling treatment of scattered glioma clusters. To make the strategy more effective, we performed a cDNA expression library screening to identify the candidate genes that once overexpressed would enhance the tropism of NSCs for gliomas. Here, we show that a previously unannotated gene, the one encoding transmembrane protein 18 (TMEM18), is one such gene. Overexpression of TMEM18 was seen in the current study to provide NSCs and neural precursors an increased migration capacity toward glioblastoma cells in vitro and in the rat brain. Functional inactivation of the TMEM18 gene resulted in almost complete loss of the migration activity of these cells. Thus, TMEM18 is a novel cell migration modulator. Overexpression of this protein could be favorably used in NSC-based glioma therapy. [Cancer Res 2008;68(12):4614–22]

Introduction

Glioma cells misregulate the expression of growth factors, proteases, and extracellular matrix and cell surface proteins to gain their devastating invasion capacity (1, 2). Localized treatments are thus inefficient and comprehensive treatments are too damaging to the delicate brain. A solution is to find a treatment that can specifically locate the tumor cells. Neural stem cells (NSC) and neural precursor cells (NPC) have an intrinsic tropism for sites of brain injuries, including gliomas, and as shown first by Benedetti and colleagues (3) and Abodo and colleagues (4), engrafted primary and immortalized NSCs can be used in gene therapy of gliomas in animal models. These engrafted stem cells have been shown to spread through the existing migratory pathways in healthy brain as well as nontypical routes when gliomas are present (4, 5). Besides primary and immortalized NSCs/NPCs, embryonic stem cell–derived NPCs seem to have the same aptitude for glioma cell tracking (6). Moreover, NSCs are able to locate not only gliomas but also tumors of a nonneural origin, suggesting that there exist common regulators of cell trafficking probably composed of secreted factors from a tumor site and receptors present on NSCs (7, 8).

Candidate signals to attract NSCs to the sites of brain injuries and tumors have been studied. Among them are cytokines released from the immunoreactive microglial cells of the brain during inflammation (9), which also provide cues for NSC migration in brain development (10). Stromal cell–derived factor-1 (SDF-1) chemokine can attract NSCs too. When its receptor CX chemokine receptor 4 (CXCR4) is blocked, SDF-1 hinders the NSC migration to the site of injury (9, 11, 12). In addition, chemokine monocyte chemoattractant protein-1, the expression of which can be induced by tumor necrosis factor-α, can activate migration of NSCs (13). Cytokine stem cell factor, expressed by glioma cell lines and overexpressed in neurons at the sites of brain injury, is another possible contributing attractant for NSCs (14–16). Similar to cytokines and chemokines, growth factor–mediated signaling [e.g., vascular endothelial growth factor and epidermal growth factor (EGF) receptor] has been shown to regulate NSC and NPC migration (17, 18). Glioma invasion depends largely on the ability of the cell to modify the extracellular matrix, and interestingly, the extracellular matrix secreted from glioma cell lines is able to promote NSC motility (19).

The picture emerging from the above studies seems to support a model of the complex interaction of several factors in regulating NSC migration toward tumors. We hypothesized that other regulators are likely to exist and their genes can be identified through expression cloning based on gene function in influencing the tropism of NSCs toward glioma cells. We were particularly interested in the molecules that once overexpressed in NSCs and NPCs are able to enhance cell migration toward gliomas, as the manipulation of the expression of these molecules could then facilitate the use of NSCs/NPCs as gene therapy vectors to reach scattered glioma cells. We used Boyden chambers in the current study to select the cells that were primed by gene transfer of a tumor cDNA expression library. A novel gene encoding transmembrane protein 18 (TMEM18) emerged from the screen and was selected for extensive characterizations.

Materials and Methods

Cells. NT2, U87MG, H4, and NIH3T3 cell lines were purchased from the American Type Culture Collection and HEK293FT cells were purchased from Invitrogen. All the cell lines were maintained in DMEM supplemented with 10% FCS (Life Technologies), penicillin-streptomycin (Life Technologies), normoxin (Invivogen), and nonessential amino acids (Life Technologies). C17.2 cells were kindly provided by Prof. E. Arenas (Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm, Sweden) and maintained in DMEM supplemented with 10% FCS, 5% horse serum (Life Technologies), penicillin-streptomycin, normoxin, and nonessential amino acids. The NIH Human Embryonic Stem Cell Registry listed human embryonic stem (hES) cell line, HES-1, and its feeder cell Kn, mouse embryonic fibroblasts were obtained from ES Cell International (ESI), Singapore. The HES cells were amplified and maintained according to the...
protocol provided by ESI. Embryoid bodies and neural spheres from hES cells were generated as previously described (20). Primary murine NSCs and NPCs were isolated from the embryonic forebrain of C57BL/6 mice. The treatment of animals was performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of our institution. Pregnant C57BL/6 mice at the specified gestational age of 14 d (E14) were killed via cervical dislocation and the uteri were aseptically removed. Fetuses were removed from the amniotic sac and transferred to a Petri dish containing ice-cold HBSS. Cortices were rapidly excised from the fetuses and mechanically dissociated by pipetting into a single-cell suspension. Cells were plated at a density of 2 × 10^5/mL into 10-cm culture dishes (Nunc) in DMEM/nutrient mixture F-12 (1:1) mixture medium (Invitrogen) containing B27 supplement (Invitrogen), 20 ng/mL basic fibroblast growth factor (Sigma-Aldrich), 20 ng/mL EGF (Invitrogen), and 1% penicillin-streptomycin (Invitrogen). Floating neurospheres with diameter range between 150 and 250 μm were passaged every 6 to 7 d. The multipotency of the cells was confirmed by immunocytochemical analysis after differentiation into three fundamental lineages in central nervous system (neurons, astrocytes, and oligodendrocytes).

cDNA expression library screening. Human Daudi cell cDNA library containing retrovirus supernatants was purchased from Stratagene and used as recommended by the supplier. One million of NT2 cells were infected with the cDNA library retrovirus supernatants to yield 20% infection efficiency to ensure a proper presentation of all the cDNAs in the library. Cells were allowed to recover for 4 d, after which they were selected in Transwell migration assays using Boyden chambers as described below. Migrating cells were collected and let to recover for 5 d before the next migration assay. After three rounds of the migration assays, both nonmigrating and migrating cells were collected.

For analysis of virus-imported cDNAs, chromosomal DNA was purified from nonmigrating or migrating cells using DNeasy kit (Qiagen) as recommended by the manufacturer. Retrovirus-imported sequences were recovered according to a PCR protocol suggested in ViraPort manual (Stratagene). Same amount of chromosomal DNA was used in PCR for nonmigrating and migrating cells. The success of the PCR was verified by running aliquots of the reactions on an agarose gel. PCR products were subsequently cloned into pDrive using TA Cloning kit (Qiagen). DH5α Escherichia coli cells were transformed with the cloning products and plated. After overnight incubation, bacterial clones were picked and plasmid DNA was isolated and then subsequently used in PCR using the same conditions as previously described to isolate individual sequences for sequencing.

On-transduction and gene silencing. TMEM18 was cloned from Human Daudi cell cDNA library infected cells by PCR using primers 5′-caccatgcgccttctctg and 5′-aaagtcttctttccttctcttc and subsequently cloned into pLenti6/V5-TOPO vector (Invitrogen) followed by sequencing to ensure that the cloned sequence was correct. TMEM18A virus containing one amino acid mutation from alanine to threonine at position 103, which did not seem to have any effect in later experiments. Empty and TMEM18 lentiviruses were produced using ViralPower Lentiviral Directional TOPO Expression Kit (Invitrogen). Virus transduction in NT2 and C17.2 cells to express TMEM18, cell selection for stable expression, and cell maintenance were carried out as recommended by the manufacturer (Invitrogen). The titer of lentivirus infection was controlled to have one virus per cell. For TMEM18 overexpression in primary murine NSCs/NPCs, neural spheres were dissociated on the day of transfection, and plasmid DNA pLenti/V5-TMEM18 was transfected into the cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. pLenti/V5-eGFP plasmid was used as a vector control. Thirty hours after the transfection, cells were collected for the migration assay and reverse transcription-PCR (RT-PCR) study described below.

For short interfering RNA (siRNA)-mediated TMEM18 gene silencing, two sequences, 5′-tacattgtgctctgctgata and 5′-gtcagcgagagcgactgacta, were cloned into double-promoter siRNA expression vector pFIV-H1/6/-PURO (System Biosciences) as recommended by the manufacturer’s protocol. A siRNA sequence against luciferase provided in pFIV Vector cloning kit (System Biosciences) was used as a control. Cells were plated to reach 90% confluence on the day of transfection of the siRNA expression plasmids, and plasmid DNA was transfected with Lipofectamine 2000 according to the manufacturer’s protocol. Puromycin-resistant cells were selected for 4 d, after which they were used for migration assay and for RT-PCR study.

Reverse transcription-PCR. Cyttoplasmic RNA was collected with RNeasy kit (Qiagen) as recommended by the manufactures. The concentration and purity was verified before equal amounts of RNAs from all the samples were used to produce cDNAs by reverse transcription using oligo(T) priming of SuperScript III First-Strand Synthesis System (Invitrogen). PCR amplification for the produced cDNAs was carried out using HotStart Taq system (Qiagen) as suggested by the HotStart Taq manual. Real-time PCR was done using Power SYBR Green PCR master mix and protocol (Applied Biosystems), with primers for TMEM18 (5′-atgcgcgccttctctg and 5′-gtctttgcggatgtccacg), 5′-CCTGATACCGCTGATGTTGGTGGTG-T3′ (forward) and 5′-AGCTTTTG-ACGTGGCAGCCGAGAA-3′ (reverse). PCR products were separated by electrophoresis on a 2% agarose gel.

Immunostaining and Western blot analysis. Antibody against TMEM18 was produced in rabbits against peptides 122 to 135: C-DLKNAQERRKEKKR (BioGenes GmbH). This peptide is unique to TMEM18 based on BLAST search. The anti-TMEM18 serum was tested using Western blotting and detected one band with molecular mass of 18 kDa, which was blocked when the serum was incubated in the presence of the immunizing peptide. The serum was used in 1:200 for immunostaining and Western blot analysis. Antibody against β-actin was purchased from Sigma-Aldrich and used in 1:2000. Secondary horseradish peroxidase–linked antibodies were purchased from Bio-Rad. Primary antibody anti-β III tubulin (1:200; Promega), anti-GFAP (1:200; Sigma-Aldrich), and anti-O4 (1:100; Chemicon) were used in immunostaining of cells derived from NSCs/NPCs. 4,6-Diamidino-2-phenylindole (DAPI; Invitrogen-Molecular Probes) was used in concentration of 2 mmol/L.

In vitro cell migration assay. In vitro migration of NPCs toward glioma cells was examined using Boyden chamber assays. A migration kit from BD Falcon with 24-well cell culture plates was used. Each well of the plates was separated into two chambers by an insert membrane of 8-μm pores. One day before assays, 50,000 glioma cells were seeded into each lower chamber. The next day, cell culture medium in the lower chamber was removed and replaced with 500 μL of nonsupplemented DMEM. NSCs/NPCs (50,000 in 500 μL of nonsupplemented DMEM) were then seeded into the upper chamber. For the assay using a neutralization antibody to block cell migration, 40 μg/mL of anti-CXCR4 monoclonal antibodies (R&D Systems) were incubated with NSCs and NPCs for 30 min at room temperature before the cell seeding. After 12 or 24 h of incubation at 37°C, migrating cells on the bottom of the insert membrane and nonmigrating cells on the upper side of the membrane were dissociated by trypsinization. These cells were subsequently lysed and stained using a CyQUANT Cell Proliferation Assay kit (Invitrogen-Molecular Probes). Fluorescence was measured with a fluorescence plate reader (GENios Pro, Tecan). Values from 6 to 12 wells were expressed as the mean ± SD in percentage control. In most of in vitro migration assays, the migration of cells transduced with a vector control in response to serum-free DMEM was used as the basal migration rate. In those experiments without the use of a vector control, cell migration in response to serum-free DMEM was used as the basal migration rate. Statistical analyses were done using Student’s t test.

In vivo cell migration assay. Rat C6 glioma cells (1 million cells in 5 μL) were injected into the right striatum of the rat brain (AP +1.0 mm, ML +2.5 mm, and DV −5.0 mm from bregma and duram) using a 10 μL Hamilton syringe connected with a 30-gauge needle at a speed of 0.5 μL/min. Three days later, 1.25 million of green fluorescent DIO dye (Invitrogen)-labeled
TMEM18-overexpressing C17.2 cells were mixed with the equal number of red fluorescent Dil dye (Invitrogen)-labeled vector control C17.2 cells and injected into the contralateral side of the rat brain. The brain samples were collected 3 wk later for sectioning and examination. To quantify the number of migrating cells in the migration front, red and green fluorescent cells were counted in 10 sections, with dots of yellow color being considered as comigration of green and red cells. In the handling and care of animals, the Guidelines on the Care and Use of Animals for Scientific Purposes issued by the National Advisory Committee for Laboratory Animal Research, Singapore was followed. The experimental protocols of the current study were approved by the Institutional Animal Care and Use Committee, National University of Singapore, and Biological Resource Center, Agency for Science, Technology and Research (A*STAR), Singapore.

Nuclear localization assay. Green fluorescent protein (GFP) fusion protein expression plasmids were created as instructed in NT-GFP Fusion TOPO Expression kit manual (Invitrogen). The TMEM18 NH2 terminus with 15 amino acid residues was cloned by PCR using primers 5′-tcagtctttctcttcctcc and 5′-aagaatgcacaagagagaag. TAT coding sequence was formed by annealing oligos 5′-cagcgcaaaaaacgccgccagcgccgctaga and 5′-cagcgccagcgccgctaga. Plasmid constructs generated were sequenced to confirm GFP fusion and transfected into U87MG cell by Lipofectamine.

Results

TMEM18 is a novel modulator identified by cDNA expression library screening for the genes that promote glioma-directed stem cell migration. To identify genes that promote the migration of stem cells toward glioma cells, we performed cDNA expression library screening. A cDNA library derived from the Daudi Burkitt lymphoma cell line was used for expression cloning, in view of the capacity of the cells to invade locally and to metastasize via mechanisms similar to those developed by solid tumors (21, 22). Retrovirus vectors were used to transduce the cDNA library into human NPC line NT2. The transduced cells were evaluated subsequently for their glioma-directed migration ability in a Transwell cell migration assay using Boyden chambers. In the assay, nonmigrating cells stayed on the top of the membrane, whereas cells that were primed to migrate went through 8-μm pores into the opposite site of the Transwell insert membrane. Migrated cells were isolated and passed through two more rounds of the migration assay selection, after which virus-imported cDNAs in migrating cells were cloned by PCR and identified by sequencing.
The protocol is summarized in Supplementary Fig. S1A. Non-migrating cells were used as controls for the cDNA analysis. We sequenced 70 virus-imported cDNA clones from migrating cells and 46 clones from nonmigrating cells and identified several cDNAs that express proteins capable of enhancing the tropism of NT2 cells for glioma cells. Among the clones collected from the migrating cells for sequence analysis, two were found to encode TMEM18, whereas no clone from the nonmigrating cells had virus-imported TMEM18 gene sequence. TMEM18 was thus a promising candidate for further analysis for its role in regulating NSC migration.

Overexpression of TMEM18 enhances the glioma-specific migration ability of NSCs/NPCs. To verify the above finding, we investigated whether the overexpression of the TMEM18 cDNA in NT2 human NPCs, C17.2 murine NSCs, and primary NSCs/NPCs isolated from the mouse embryo forebrains at the 14th day would affect the migration. We used lentiviral vectors to create stable cell lines overexpressing TMEM18 in NT2 and C17.2 cells. Two populations of stable cell lines that express different levels of TMEM18 were selected from each type of NPCs. We also used transfection to overexpress TMEM18 in primary NSCs/NPCs. TMEM18 overexpression was confirmed using RT-PCR (Supplementary Fig. S2).

We used Boyden chamber assay to examine the movement of the above TMEM18-overexpressing cells toward human U87MG glioma cells, the same tumor cell line that was used in the cDNA expression library screening earlier. TMEM18-overexpressing NT2 cells, C17.2 cells, and primary NSCs/NPCs displayed significantly higher migration capacities when compared with their parental cells and empty vector controls (Fig. 1 for NT2 and C17.2 cells and Fig. 2 for primary NSCs/NPCs). These cells also responded to other glioma cell lines, H4 and C6, by displaying significant migration advantage over control cells (Figs. 1B and F and 2A).

Figure 2. Effects of TMEM18 overexpression on NSCs/NPCs. A, TMEM18 overexpression increases the migration activity of primary NSCs/NPCs in Boyden chamber. Columns, percentage of vector control; bars, SD. Statistical comparisons are calculated between cells overexpressing TMEM18 and vector controls using Student’s t test. *, P < 0.05; **, P < 0.01. B, glioma-tracking cells of TMEM18-overexpressing NSCs/NPCs in Boyden chamber assay are mainly astrocytic precursors. Top, phase-contrast and fluorescence images show the transfection efficiency of control plasmid pLenti/V5-eGFP; bottom, immunostaining using antibodies against β-tubulin, GFAP, and O4 (green). DAPI (blue) was used to counterstain nuclei. Note that the cells derived from migrating TMEM18-overexpressing NSCs/NPCs were stained strongly with anti-GFAP.

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migration capacities when nontumor cell lines, mouse fibroblast cell line NIH3T3 and human kidney cell line HEK293FT, were seeded in the bottom chamber in the assays (Fig. 1C and D). TMEM18 overexpression did not change the migration of primary NSCs/NPCs toward HEK293FT either. Moreover, the amount of cells migrating to plain DMEM cell culture medium remained similar between the TMEM18-overexpressing cells and the controls (Fig. 1). Hence, the preference of TMEM18-overexpressing cells for glioma cells implies a role for the protein in response to glioma-secreted factors.

Primary NSCs/NPCs isolated from E14 mouse forebrains are a heterogeneous population with multipotency, each of subpopulations capable of giving rise to specific type of neural cells. To understand whether TMEM overexpression has any specific effect on the migration of a particular subtype of NSCs/NPCs, we examined the differentiation ability of the primary cells, migrating TMEM18-overexpressing NSCs/NPCs collected on the bottom of the insert membrane of the Boyden chamber, and nonmigrating cells on the upper side of the membrane. Although there was no obvious difference in cell type distribution between the cells derived from primary NSCs/NPCs and the cells from nonmigrating TMEM18-overexpressing NSCs/NPCs, the cells derived from migrating TMEM18-overexpressing NSCs/NPCs displayed morphologic features typical of astrocytes and were strongly positive for GFAP, a marker for astroglial precursors and astrocytes (Fig. 2B). The cells derived from migrating NSCs/NPCs were negative for O4, a marker for oligodendrocytes, and only weakly stained with anti-β-III-tubulin, a marker for neurons. These results indicate that glioma-tracking populations of TMEM18-overexpressing NSCs/NPCs comprise largely of astrocytic precursors, which is consistent with previous observations from an in vivo study reporting that the majority of NSCs that migrated along with glioma outgrowths and satellites were astrocytic precursors (23).

Encouraged by the above in vitro results, we moved on to test whether overexpression of TMEM18 would improve the migration of C17.2 murine NSCs toward gliomas in the brain. In a rat C6 glioma xenograft model, green fluorescent dye–labeled TMEM18-overexpressing C17.2 cells and red fluorescent dye–labeled vector control C17.2 cells were mixed up and injected into the left side of the rat brain contralateral to a C6 glioma inoculation site (right). Three weeks later, the brains were sectioned for cell migration examination under a fluorescence microscope. A, migration of green fluorescent dye–labeled and red fluorescent dye–labeled cells shown at a low magnification. The squares on the right side in each image indicate the front of cell migration toward the tumor, which were shown at a high magnification in B. C, merge of the two images in B. Dots with yellow color present green fluorescent dye–labeled and red fluorescent dye–labeled cells moving together. Note many of green fluorescent dye–labeled TMEM18-overexpressing C17.2 cells (arrows) migrating alone. D, quantification of fluorescent dye–labeled C17.2 (red) and C17.2/TMEM18B (green) cells in the front of cell migration toward the C6 glioma inoculation site. *** P < 0.001.
around the front region were evaluated, where ~60% of the migrating cells were stained with the green fluorescent dye and 40% labeled with the red fluorescent dye. These findings suggest an improved migration of the TMEM18-overexpressing cells toward gliomas in the brain.

**Endogenous TMEM18 is critical for the migration of NSCs/NPCs.** To determine the effect of endogenous TMEM18 in regulating NSC migration, we used RNA interference approach to reduce the expression of TMEM18 in NT2 cells. Puromycin-resistant siRNA expression vector was constructed to express siRNA sequences against TMEM18 and against the luciferase gene (as a siRNA control). Two different transductions with two siRNA constructs against TMEM18 in NT2 cells yielded four populations with different levels of reduction of TMEM18 mRNA expression, ranging from 31%, 35%, 37% to 65% of the original endogenous TMEM18 mRNA level (Fig. 4A). In a 24-h Boyden chamber assay, siRNAs against TMEM18 displayed strong inhibitory effects on the migration of NT2 cells when compared with cells transfected with plasmids expressing siRNA against the luciferase gene (Fig. 4B). Reduction in the amount of TMEM18 mRNA to 60% of the normal levels lowered the number of cells migrating toward glioma cells to ~50% of the control. Further reduction of TMEM18 mRNA expression to 31% of the normal level in NT2 cells almost abolished the cell migration ability completely (Fig. 4B). Moreover, down-regulation of TMEM18 comparably reduced cell migration toward plain DMEM as well (Fig. 4B), suggesting that TMEM18 is an important factor regulating general cell motility.

The effect of endogenous TMEM18 on the movement was also studied in hES cell-derived NPCs. Along with the differentiation of hES cells into embryoid body and neural sphere, TMEM18 mRNA expression was visible in parental NT2 and C17.2 cells and against the luciferase gene as a siRNA control. Two different transductions with two siRNA constructs against TMEM18 in NT2 cells yielded four populations with different levels of reduction of TMEM18 mRNA expression, ranging from 31%, 35%, 37% to 65% of the original endogenous TMEM18 mRNA level (Fig. 4A). In a 24-h Boyden chamber assay, siRNAs against TMEM18 displayed strong inhibitory effects on the migration of NT2 cells when compared with cells transfected with plasmids expressing siRNA against the luciferase gene (Fig. 4B). Reduction in the amount of TMEM18 mRNA to 60% of the normal levels lowered the number of cells migrating toward glioma cells to ~50% of the control. Further reduction of TMEM18 mRNA expression to 31% of the normal level in NT2 cells almost abolished the cell migration ability completely (Fig. 4B). Moreover, down-regulation of TMEM18 comparably reduced cell migration toward plain DMEM as well (Fig. 4B), suggesting that TMEM18 is an important factor regulating general cell motility.

The effect of endogenous TMEM18 on the movement was also studied in hES cell-derived NPCs. Along with the differentiation of hES cells into embryoid body and neural sphere, TMEM18 mRNA expression increased (Fig. 4C). This increase was accompanied with an enhanced migration of cells in neural sphere toward glioma U87MG cells (Fig. 4D). Taken together, these findings suggest that the expression of endogenous TMEM18 at a physiologic level is crucial to the migration capacity of NSCs/NPCs.

**Up-regulation of CXCR4 by TMEM18 mediates the glioma-specific migration capacity of NSCs/NPCs.** In view of the importance of the CXCR4 that governs the migration of stem cells toward gliomas (24), we investigated whether TMEM18 would affect its expression in NSCs/NPCs. We observed that, although weak CXCR4 expression was visible in parental NT2 and C17.2 cells as well as their vector controls, overexpression of TMEM18 seemed to raise its expression levels in NT2 cells, C17.2 cells, and primary NSCs/NPCs (Fig. 5A). Using an antibody against CXCR4 to block cell surface CXCR4 receptors on these cells, we further observed that the tropism of these TMEM18-overexpressing cells toward U87MG cells was inhibited drastically in Boyden chamber assays and the number of migrating cells went down to a level close to that of basal cell migration in response to serum-free DMEM (Fig. 5B). These results suggest that up-regulation of CXCR4 in TMEM18-overexpressing cells might be one possible mechanism underlying the augmented glioma tropism of these cells.

**The NLS sequence of TMEM18 is sufficient for nuclear targeting.** To uncover the cellular localization of TMEM18, a polyclonal antibody against TMEM18 COOH-terminal peptide was produced. The activity and specificity of the antibody were examined using cellular immunostaining and Western blot analysis (Supplementary Fig. S2). In NT2 cells, preimmunization serum produced almost no signal, whereas the serum against TMEM18 gave a strong immunofluorescence. Western blot analysis with the

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**Figure 4.** Endogenous TMEM18 expression affects cell migration. A, siRNAs against TMEM18 transcripts reduced the expression of TMEM18 transcripts in NT2 cells, as quantified by real-time PCR. Two different sequences of siRNAs against TMEM18 were tested in NT2 cells and yielded four different knockdown levels of the TMEM18 mRNA. siRNA against luciferase was used as a control. B, silencing endogenous TMEM18 expression reduced the migration activity of NSCs/NPCs toward both DMEM and U87MG. C and D, neuronal differentiation of hES cells was accompanied by progressive increase in TMEM18 mRNA expression and cell migration toward glioma cells. Columns, percentage of control; bars, SD. Statistical comparison is calculated between cells with down-regulated TMEM18 and controls using Student’s t test. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
serum revealed both endogenous and overexpressed TMEM18 with molecular mass of 18 kDa. The bands disappeared when the serum was incubated in the presence of the immunizing peptide (data not shown).

The TMEM18 antibody stained the cytoplasm of NT2 cells, with intensive staining in the perinuclear area (Fig. 6A and B). In comparison with cytoskeletal structures stained with an α-tubulin antibody (Fig. 6B, green), TMEM18 was localized only partly with the areas of the tubulin network (Fig. 6B, yellow). Several structures within the nucleus were also positively stained (Fig. 6B). With a closer look, the TMEM18 antibody recognized a ring structure superimposed on the nucleus, presumably the nuclear membrane (Fig. 6B). This structure became even clearer when the TMEM18 immunofluorescence staining was overlaid with nuclear staining (Fig. 6B, blue).

As TMEM18 contains a putative NLS sequence (Supplementary Fig. S1C), we tested whether it was effective in directing nuclear location in U87MG cells using a GFP fusion protein approach. Transfection of the control GFP plasmid in U87MG cells led to green fluorescent signals all over the cells, in both the nucleus and the cytoplasm (Fig. 6C, GFP). This was expected because the small size of GFP (30 kDa) permits diffusion between the nucleus and the cytoplasm. Noticeably, transfection with a plasmid vector encoding a hybrid protein composed of GFP linked to the putative NLS at the COOH terminus of TMEM18 (KED) resulted in significant accumulation of fluorescent signals in the cell nucleus (Fig. 6C, GFP-KED). The TMEM18 KED peptide seemed as effective as TAT peptide (Fig. 6C, GFP-TAT), a well-established nuclear localization signal peptide, in directing GFP into the nucleus. In addition, diffuse fluorescence signals were detectable in these U87MG cells transfected with the fusion genes.

Discussion

We have described in this report the identification and characterization of a novel cell motility modulator TMEM18. TMEM18 was first identified in a screen to discover membrane spanning proteins (25). There have been no other publications on this protein since. The functional importance of this protein is highlighted in the strong amino acid conservation throughout mammals and even reaching to lower forms of multicellular eukaryotes. Furthermore, according to the profile of TMEM18...
expressed sequence tags (EST), the protein is transcribed in embryonic developmental states and in many of the adult human tissues (National Center for Biotechnology Information’s EST expression profile viewer). Clearly, TMEM18 has a crucial biological function. Based on our results, this function would probably be linked to cell mobility. In particular, with respect to tumor therapy, TMEM18 can be used as a specific enhancer for glioma-directed migration of NSCs.

The results that TMEM18-overexpressing cells and control cells migrated at the same rate in plain cell culture medium or toward nonglioma cells (NIH3T3 mouse fibroblast and 293T human embryonic kidney cells) suggest that TMEM18 overexpression provided no beneficial effects on the general movement of NSCs. However, knockdown of endogenous TMEM18 expression with RNA interference had enormous inhibitory effects on the overall movement of NSCs. The effect seemed much stronger than that induced by TMEM18 overexpression. It is worthy of noting that, as determined by our quantitative real-time PCR experiments, TMEM18 mRNA is low-abundant transcripts and could be functionally sensitive to siRNA-mediated gene silencing. Furthermore, along with the increase of TMEM18 expression from an undetectable level to an easily detectable level when hES cells differentiated into NPCs, these NSCs/NPCs displayed an increased capacity of cell migration (Fig. 4C and D). These findings indicate a crucial role of the basal, physiologic level expression of TMEM18 for cell movement, which is well consistent with the highly conserved and ubiquitously expressed pattern of TMEM18.

Most interestingly, TMEM18 overexpressing cells respond strongly to glioma cell–secreted cues in both in vitro Transwell assays and an in vivo migration experiment. Although both RT-PCR and Western blot yielded unidirectional results in the increase of TMEM18 expression in the current study, mismatch in terms of increase level is obvious. A discrepancy between transcription and translation is not an uncommon phenomenon. A recent article has indicated that the correlation between mRNA and protein expression is moderately or weakly positive, with correlation coefficients ranging from 0.2 to 0.6 (26). This can be most plausibly explained by the observation that mRNA and protein are synthesized with independent mechanisms and degraded by different pathways. In Transwell assays, cells will have to force themselves through holes in Boyden chamber membrane that are smaller than the normal size of a cell body. It could be even more difficult for cells to migrate to a target site in the brain where cell migration needs to overcome numerous extracellular interactions. We thus conclude that TMEM18 overexpression increases the sensitivity of NSCs to appropriate signals that stimulate cell migration. In other words, without appropriate cues, TMEM18 overexpression will have undetectable effects on the movement of NSCs.

TMEM18 is expected to be a transmembrane protein. But it did not seem to be located to outer cell membrane, as would be expected for example for chemokine receptors, nor was it spread to cover cytoskeletal structures that would affect cell movement directly. TMEM18 is predicated to possess a NLS sequence at the COOH terminus. NLS sequences occur in a subset of soluble nuclear proteins that are imported into the cell nucleus by transport receptors. Membrane proteins with NLS-like sequences are found in the majority of mammalian inner nuclear membrane (INM) proteins (27). A recent study in budding yeast shows that NLS sequences are essential for passage of integral membrane proteins through the nuclear pore complex and receptor-mediated transport of the proteins to the INM (28). Further studies are warranted to assess whether TMEM18 is really located along the inside of the nuclear envelope.

Cell motility is a highly complex process and involves several factors, from sensing of environmental cues, restructuring the cytoskeleton, dynamic regulation of cell attachment and detachment to extracellular matrix, to signaling between all these processes to coordinate the movements. Hence, there are many steps downstream of chemoattractant receptor signaling that can affect cell movement and migration. Bioinformatics searches for protein domains did not reveal any informative features related to

Figure 6. Cellular localization of TMEM18 and the function of its NLS. A and B, cellular localization of TMEM18 protein. NT2 cells were stained against TMEM18 (A and B, red), α-tubulin (B, green), and DNA (B, blue). C, superimposition of TMEM18, DNA, and α-tubulin. The immunostaining reveals the nuclear membrane (a ring structure around the nucleus in A and B) recognized by the TMEM18 antibody. C, GFP fusion protein with NH2 terminus of TMEM18 is localized to the nucleus. U87 cells were transfected with plasmid vectors expressing GFP, TMEM18 NH2-terminal–linked GFP (GFP-KED), or HIV TAT-linked GFP (GFP-TAT). Left, light microscope pictures of the cells; right, fluorescence microscope pictures of the cells.
a possible biochemical function of TMEM18. Our preliminary data on the up-regulation of CXCR4 in TMEM18-overexpressing cells and inhibiting cell migration by antibodies against CXCR4 suggest an enhanced effect of the SDF-1/CXCR4 axis by TMEM18. Further research is necessary to define the basic mechanisms underlying the effects of endogenous TMEM18 on general cell migration and the effects of overexpressed TMEM18 to enhance cell response to migration-stimulating signals. An adequate understanding of these mechanisms could have important implications for effective cellular delivery of therapeutic agents for brain tumor therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 9/13/2007; revised 3/28/2008; accepted 3/31/2008.


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We thank the lab members for helpful discussion and support.

References


Announcements

MEETING OF THE RADIATION RESEARCH SOCIETY

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22–24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on "The Effects of Radiation on Aqueous Solutions," which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is "Physical Measurements for Radiobiology" and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray's lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

ERRATUM

The following correction should be made in the article by Beck and Valentine, "The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration," November, 1952, page 891; substitute for the last paragraph:

The data in Table S permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O2 consumption, it is seen that the amount of glucose "cleavage products" exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if \( M = +0.27 \) and \( L = -0.16 \) and the normal differential is 65 per cent M and 35 per cent L, then

\[
0.65 (+0.27) + 0.35 (-0.16) = +0.12
\]

a figure identical to the observed +0.12 for normal leukocytes.
Transmembrane Protein 18 Enhances the Tropism of Neural Stem Cells for Glioma Cells
Jaana Jurvansuu, Ying Zhao, Doreen S.Y. Leung, et al.