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TRPM7 channels regulate glioma stem cell through STAT3 and Notch signaling pathways

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Abstract

Glioblastoma multiforme (GBM) is the most common and aggressive primary brain tumor in adults with median survival time of 14.6 months. A small fraction of cancer stem cells (CSC) initiate and maintain tumors thus driving glioma tumorigenesis and being responsible for resistance to classical chemo- and radio-therapies. It is desirable to identify signaling pathways related to CSC to develop novel therapies to selectively target them. Transient receptor potential cation channel, subfamily M, member 7, also known as TRPM7 is a ubiquitous, Ca²⁺ and Mg²⁺ permeable ion channels that are special in being both an ion channel and a serine/threonine kinase. In studies of glioma cells silenced for TRPM7, we demonstrated that Notch (Notch1, JAG1, Hey2, and Survivin) and STAT3 pathways are down regulated in glioma cells grown in monolayer. Furthermore, phospho-STAT3, Notch target genes and CSC markers (ALDH1 and CD133) were significantly higher in spheroid glioma CSCs when compared with monolayer cultures. The results further show that tyrosine-phosphorylated STAT3 binds and activates the ALDH1 promoters in glioma cells. We found that TRPM7-induced upregulation of ALDH1 expression is associated with increases in ALDH1 activity and is detectable in stem-like cells when expanded as spheroid CSCs. Finally, TRPM7 promotes proliferation, migration and invasion of glioma cells. These demonstrate that TRPM7 activates JAK2/STAT3 and/or Notch signaling pathways and leads to increased cell proliferation and migration. These findings for the first time demonstrates that TRPM7 (1) activates a previously unrecognized STAT3→ALDH1 pathway, and (2) promotes the induction of ALDH1 activity in glioma cells.

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Keywords

TRPM7; glioblastoma multiforme; cancer stem cell; Notch; STAT3; ALDH1

Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive primary brain tumor with median survival time of 14.6 months after surgery followed by radiotherapy and temozolomide¹. Despite decades of intensive research, GBM still have a poor prognosis and no effective treatment has been developed for GBM patients. Recent studies have shown that ion channels can regulate many malignant features of tumors, such as lack of differentiation, increased migratory and invasive phenotypes, and chemoresistance²⁻⁴. Transient receptor potential cation channel, subfamily M, member 7, also known as TRPM7, a subfamily member of Transient receptor potential (TRP) has a very high permeability for both Ca²⁺ and Mg²⁺⁵. In addition to its channel activity, TRPM7 contains a functional α -kinase domain within its C terminus⁶. Our previous studies have demonstrated a cell growth-promoting function of TRPM7 channels in FaDu and SCC25 cells, two common human head and neck squamous carcinoma cell lines⁷. Following that initial report, several groups found that TRPM7 play a role in the growth, proliferation and migration of several types of tumor cells, including breast cancer⁸, gastric cancer⁹, head and neck cancer⁷, nasopharyngeal carcinoma¹⁰, pancreatic cancer¹¹, prostate cancer¹², retinoblastoma¹³, and leukaemia¹⁴. Increased TRPM7 expression is found to be correlated with clinicopathological parameters, such as tumor grade, Ki67 proliferation index and patient survival¹¹.

Although cancer stem cell (CSC) population only represent a small fraction of cells within a tumor, including glioma, their super malignancy-initiating ability and resistance to classical chemo- and radio-therapies drives tumorigenesis, such as tumor proliferation and invasion¹⁵. Limited understanding of the molecular signaling pathways involved in their identity frustrate a potential effective treatment of glioblastoma, the most common primary brain tumor in adults. Just as CD133, which has long remained the most important tumor stem cell marker in glioma stem cells (GSC)¹⁶, aldehyde dehydrogenase1 (ALDH1), a cytoplasmatic stem cell marker in many types of malignancies, has recently been suggested to be a marker for the identification of tumor stem cells in human GBM¹⁶. Notch signaling pathway plays a crucial role in CSC in a variety of cancers including GSC by regulating survival, proliferation and the maintenance of stem cells¹⁷. Signal transducer and activator of transcription 3 (STAT3), a transcriptional factor which controls Notch pathway in GSC has been identified as a novel therapeutic target for the treatment of glioma¹⁷. In our pilot study, we found a strong evidence to support an involvement of TRPM7 channels in mediating the Ca²⁺-sensing current in A172 glioma cells. Our findings that suppression of TRPM7 expression significantly inhibited the growth, proliferation, migration and invasion of A172 cells indicates that TRPM7 channels may represent a novel and promising target for therapeutic intervention in malignant glioma (Leng et al. manuscript in preparation). However, the possible interactions among TRPM7, Notch, STAT3 and ALDH1 signaling molecules in the context of brain tumors remains unexplored. This study aimed to investigate the effects of TRPM7 pathway activation on glioma cells and stem-like cells

derived from them. In particular, we found that TRPM7 activates JAK2/STAT3 and/or Notch signaling pathways and leads to increased cell proliferation and migration. In addition, we found that TRMP7-mediated STAT3 activation directly regulates CSC marker, ALDH1.

Material and Methods

Cell culture

Human glioblastoma cell line, A172, was obtained from ATCC (Manassas, VA, USA). A172 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO) plus 10% fetal bovine serum (FBS), 50 units/ml penicillin, and 50 µg/ml streptomycin at 37°C.

Enrichment for glioma stem cells

For neurospheroid culture, A172 cells cultured in conventional tissue culture media aforementioned were grown to confluence and dissociated using 0.1% trypsin, and dispersed by pipetting with a 23-gauge needle. After checking for single cell, the cells were pelleted and suspended in sphere enrichment medium, specifically, human neurobasal medium supplemented with B27, 20ng/ml EGF and 20ng/ml FGF-2 (Invitrogen, Carlsbad, CA), and 5µg/ml heparin (Sigma-Aldrich, St. Louis, MO). These cells were then plated in ultra-low attachment surface tissue culture plates (Corning). Following overnight incubation at 37°C with 5% CO₂, the distinct non-adherent human glioma stem cells were apparent in culture. These spheres were collected, then gently centrifuged at low speed (1000 rpm), passaged and maintained for growth in the sphere enrichment medium for future use.

SiRNA transfection and retroviral infection

STAT3 siRNA was purchased from Santa Cruz (Santa Cruz, CA), TRPM7 siRNA was purchased from Dharmacon, Thermo Scientific (Lafayette, CO). A scrambled siRNA, with no homology to any known sequence was used as control. A172 cells were transfected with 50 nM of each specific siRNA or control siRNA using Lipofectamine™ reagent in serum free OptiMEM-1 medium (Invitrogen, Carlsbad, CA) according to the manufacture's instruction. After six hours of transfection, cells were grown for further 48h or 72h in growth medium as indicated in each experiment before utilization. All studies were done in triplicates. A172 cells were transduced with the different MSCVpuro STAT3 vectors respectively as previously described¹⁸

Reporter plasmid construction

To assay ALDH1 gene promoter activity, the 5'-flanking region of the human ALDH1 gene was inserted into the firefly luciferase reporter vector, pGL3-Basic (Promega, Madison, WI), which has no eukaryotic promoter or enhancer element as described previously¹⁹. The strategy for cloning of the fragment of the ALDH1 gene promoter into pGL3-basic vector was as follows: PCR was performed using the PCR2.1 Topo cloning plasmid which contains the ALDH1 gene promoter fragment as a template and 5'- and 3'-primer pairs (the newly synthesized XhoI and HINDIII sites in the primers are underlined), 5'-ATC GCTCGAGAA GAA CTT GAA TTG TTT GGA AGC-3', 5'-ATC GAA GCT TCG TGC CTG AGG ATG

ACA TTT'-3'. The PCR product was then cloned into pGL3-Basic vector. The correct orientation and sequences of plasmid construct were verified by DNA sequence analysis. The unaltered plasmid, pGL3-Basic, was used as a control, and the plasmid, pGL3-SV40 (Promega, Madison, WI) contained the firefly luciferase gene driven by the SV40 promoter as a positive control.

MTT assay

A172 cells were seeded at 1×10^4 cells in 100 μ l of medium per well into 96-well plates and were transfected with 50 nM specific siRNA or control using Lipofectamine™ for indicated times. 10 μ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma-Aldrich, St. Louis, MO, the ratio of MTT reagent to medium is 1:10) was added into each well and incubated in the dark at room temperature for 2 to 4 h. Absorbance at 570nm was measured using 690 nm as reference filter using a CytoFluor™ 2300 plate reader.

RNA isolation, real-time RT-PCR, Western blotting and immunofluorescence microscopy

Total RNA isolation, cDNA synthesis and PCR amplification for the following genes, immunoblotting and quantification of immuno-detectable bands were performed as previously described²⁰. Primary antibodies used include: anti-pSTAT3/tSTAT3, anti-pJAK2(Tyr1007/1008)/tJAK2, anti-pAKT/tAKT (Cell Signaling Technology, Danvers, MA), anti-TRPM7 (Abcam, catalog number: ab85016, Cambridge, MA), anti-CD133, anti-ALDH1, anti-Hey2, anti-Survivin and anti- β -actin (Sigma-Aldrich, St. Louis, MO). PCR primers used for TRPM7 (5'-3') were: GCCACCAAGGAGGTACACAT and CTTCCAGGGCCGTGTAGATA, ALDH1 (5'-3') were: CCCTTTGGTGATTCAAGAT and TTGAAGAGCTTCTCTCCACTCTT, CD133 (5'-3') were, CAGAAGGCATATGAATCCAAAA and ATAAACAGCAGCCCCAGGAC, Notch1 (5'-3') were, CAC TGT GGGCGGGTCC and GTTGATTGGTTTCGGCACCAT, Survivin (5'-3') were, GCCCAGTGTTTCTTCTGCTT and CCTCCCAAAGTGCTGGTATT, Hey2 (5'-3') were, AAAAAGCTGAAATATTGCAAATGA and GTACCGCGCAACTTCTGTT, Jagged1(5'-3') were, GACTCATCAGCCGTGTCTCA and TGGGGAACACTCACACTCAA, WNT1(5'-3') were GCGCTTCCTCATGAACCTT and GTGCATGAGCCGACATC, Notch2(5'-3') were, AATCCCTGACTCCAGAACG and TGGTAGACCAAGTCTGTGATG AT, Sox11(5'-3') were, GACCCAGACTGGTGCAAGAC and GCCCAGCCTCTTGGAGAT, GAPDH (5'-3') were, GAAGGTGAAGGTCGGAGTC and GAAGATGGTGATGGGATTTC.

RT² profiler PCR array

Total RNA extraction was performed using the RNeasy Mini Kit as described above. cDNA was processed according to the manufacturer's protocol. Briefly, the first-strand cDNA synthesis was performed using a RT² First-Strand cDNA Synthesis kit (Qiagen, Valencia, CA) by 1000ng of total RNA. The cDNA template was then mixed with RT² Real-Time SYBR Green Master Mix to a reaction volume of 25 μ L for each well of the 96-well-plate format of the human stem cell signaling pathway PCR array (SABiosciences, PAHS-405ZD). Housekeeping genes as well as reverse transcription and positive controls were included in this format. Difference at mRNA transcript levels between A172 cells and A172 cells transfected with siTRPM7 were initially analyzed using SABiosciences web

portal software (<http://www.sabiosciences.com/pcrarraydataanalysis.php>). Fold changes and P values were calculated using Student's t-test. A p value < 0.05 with a fold change greater than 2.0 were considered to be a significant dysregulation.

Luciferase reporter gene assay

A172 cells were transfected using lipofectamine 2000 (Invitrogen, Carlsbad, CA) with 0.75µg of ALDH1 promoter-luciferase construct together with 100ng of pRL-TK, a cytomegalovirus-Renilla vector to control transfection efficiency. The amount of total DNA transfected was equalized with the appropriate amounts of control vectors. After transfection at different indicated conditions, cells were harvested and lysed in reporter lysis buffer (Promega, Madison, WI). Luciferase activity was determined by using the Dual Luciferase Kit (Promega, Sunnyvale, CA) and a luminometer (Turner Design, Sunnyvale, CA) according to the manufacturer's recommendation. All luciferase results were normalized to Renilla activity from the co-transfected pRL-TK plasmid. The data for luciferase activity were expressed as fold induction with respect to control cells and were the mean ± standard error of triplicate samples.

ChIP assay

The chromatin immunoprecipitation (ChIP) protocol used in this study was adapted from Guo et al ¹⁹ and from the protocol recommended by Upstate Biotechnologies. After cross-linking reaction, the cells lysates were made and sonicated to shear the DNA into 0.3~3-kb fragments, sheared chromatin was incubated with anti-STAT3, anti-pSTAT3 or rabbit serum (negative control) overnight at 4°C. Then, protein G beads were added and the chromatin was incubated for 2 hours in rotation. An aliquot of chromatin that was not incubated with an antibody was used as the input control sample. Antibody-bound protein/DNA complexes were eluted and subjected to PCR analysis. The primer sets used to amplify ALDH1 promoter with putative STAT3 binding sites were as follows: set 1, ALDH1 SBE1 (-79/-71), F: 5'-AAGTCAAAGGCTTCCTGCCCT-3' and R: 5'-TTGGCTTTATTTGTTCCCTTTT-3', which generated a 141-bp product; set 2, ALDH1 SBE3 (-236/-229), F: 5'-CCAGAGCAGCTGCTGCATACA-3', and R: 5'-AGGGCAGGAAGCCTTTGACTT-3, which generated a 148-bp product. PCR products were resolved on 1.8% agarose gels.

Migration and invasion assay

The migration and invasion potential were assessed as previously described²¹.

Statistical analysis

The results obtained in this work were expressed as mean ± SEM of at least 2 independent experiments done in triplicate. Paired t-test or ANOVA tests were performed for data analysis, and significant difference was defined as p<0.05.

Results

(1) Analysis of human stem cell signaling pathways using real time RT² profile PCR arrays

GSCs harbour significantly increased tumor-initiating activity when compared to the glioma monolayers from which they were derived. We first assess the targeting genes of the stem cell signaling pathways induced by TRPM7 using real time RT² Profile PCR arrays (SABioscience, PAHS-405ZD). A172 cells were transfected with 50 nM TRPM7 specific siRNA to knock down TRPM7 or control siRNA. After six hours of transfection, A172 cells were grown in regular growth medium for another 48h. Total RNA was extracted and subjected to cDNA synthesis and then underwent RT² profile PCR array assay. Fig. 1A shows a list of down regulated and upregulated genes by knocking down TRPM7 gene with fold-change greater than 2 ($P < 0.05$ was considered to be significantly different). The down regulated genes included: 1) Notch pathway: Notch1, Jagged 1 (JAG1) and Delta-like 1, Drosophila (DLL1); 2) Wnt pathway: Wingless-type MMTV integration site family, member 1 (WNT1), adenosine deaminase, RNA-specific (ADAR) and cyclin D1 (CCND1); 3) Aldehyde dehydrogenase 2 family, mitochondrial (ALDH1). The upregulated gene included peroxisome proliferator-activated receptor λ (PPAR λ), which can inhibit growth and increase apoptosis of cancer cell lines ²². The heat-map and scatter plot are shown in Fig. 1B and 1C.

(2) STAT3 and Notch1 are involved in the regulation of glioma cells by TRPM7

The differential expression of the signaling pathways identified in RT² profile PCR assay was validated further. To this end, we detected Notch pathway activation, ALDH1 and CD133 expressions upon TRPM7 gene silencing by quantitative PCR assay (qPCR), Western blot assay, and immunofluorescence images. Consistent with the RT² profile PCR results, we found that silencing of TRPM7 was associated with down regulation of Notch1 mRNA and protein (Fig. 2A and 2B); Notch ligand Jagged-1 and other downstream genes such as Hey2 and Survivin (Fig. 2A) as well as ALDH1 (Fig. 2A and 2B) and WNT1 expressions (data not shown) were also reduced accordingly by siRNA-TRPM7 knock down. Since high persistent activation of transcriptional factor STAT3 is found in many types of human tumors, including glioma, and found constitutively activated in GSC ¹⁷, we therefore tested whether STAT3 is involved in regulation of glioma by TRPM7. As shown in Fig. 2B, down regulation of TRPM7 by siRNA markedly reduced the activation of STAT3 without changes in total STAT3 levels in A172 cells, suggesting that STAT3 as assessed by its tyrosine phosphorylation lies downstream of TRPM7 pathway activation. Taken together, these preliminary data illustrate that Notch1, STAT3, ALDH1 pathways are involved in the TRPM7 regulation of human glioma cells. CD133, a transmembrane glycoprotein, is an important cell surface marker for both stem cells and CSC in various tissues ²³ including GSC ^{15, 24}. A retrospective study showed that expression of CD133 predicted the clinical course of disease better than the histological grading ²⁵. The presence or absence of CD133 (+) in CSC may explain the crucial biological difference between individual cases. Although the importance of CD133 in glioma and GSC is largely investigated, the relationship between CD133 and TRPM7 is unclear. Our results indicated that CD133 was positively regulated by TRPM7 (Fig. 2A and 2B). TRPM7 was demonstrated to be required for sustained PI3K/AKT signaling activation and may act as

central modulator in lymphocyte growth and proliferation^{26–27}. However we did not detect any changes in gliomas when TRPM7 expression was reduced (Fig. 2B). Together, these results indicate that silencing TRPM7 is associated with down regulation of multiple signaling pathways regulating stem cell maintenance.

The immunofluorescence staining was used to determine the subcellular localization of diverse proteins described above (Fig. 3). A172 cells were treated with siTRPM7 for 48 h and then stained for antibodies as indicated. Notch1 was stained in Green (FITC, Fig3B, left), CD133, pSTAT3, pJAK2 in red (Cy3, Fig3C, 3D, and 3E, left) and nucleus in blue (DAPI, center), and the right panel gives the merged picture. The merged picture gives a clear co-localization. As shown in the panels of Fig. 3, it is apparent that TRPM7 localized predominately in the membrane and in the surrounding cytoplasm, while Notch1, pSTAT3 and pJAK2 are almost exclusively localized in the nucleus. CD133 is widely distributed and is visualized in the membrane, cytoplasm and nucleus. Notch1, CD133, pSTAT3 and pJAK2 expression are significantly decreased by TRPM7 silencing when compared with expression of corresponding proteins achieved with control siRNA (Fig. 3).

(3) TRPM7-mediated activation of STAT3, upregulation of Notch1 and ALDH1 signaling pathways in GSC

A172-derived GSCs were generated using serum-free medium supplemented with EGF and bFGF as described previously (http://www.webmedcentral.com/article_view/557) (Fig. 4A). Compared to A172 glioma cells grown in monolayer (GC), mRNA expression of Notch1 and its ligand Jagged1 (JAG1), or target genes Hey2 and Survivin as well as ALDH1 (Fig. 4B), WNT1 (data not shown) were upregulated by qPCR assay. At protein level, active form of Notch1, cleaved Notch1, the Notch1 intracellular domain (NICD) protein, its target genes Hey2 and Survivin, as well as ALDH1 (Fig. 4B and 4C) and CD133 (Fig. 4B–4D) were also upregulated. Interestingly, the molecules under Notch pathway including Notch1, Hey2, JAG1, and Survivin as well as ALDH1 expressed significantly higher in spheroid GSCs when compared with monolayer cultures (Fig. 4B and 4C). Although these Notch-regulated genes and ALDH1, known positive regulators of pathways maintaining stem cell-like state, were up-regulated in GSCs, the negative regulators of these pathways, Notch2 and Sox11, were down-regulated (Fig. 4B). In addition, we provided strong evidence that the STAT3 signaling pathway as measured by pSTAT3 is activated in glioma cells and that activation is markedly greater in GSC (Fig. 4C). JAK2 (Tyr1007/1008) activation exhibited comparable kinetics in response to TRPM7 silencing (Fig. 4C) as pSTAT3. These data suggest that Notch, STAT3, ALDH1 and CD133 signaling pathways are involved in the regulation of GSC by TRPM7. The underlying mechanisms are however unknown and will be investigated next.

(4) ALDH1 promoter is activated by STAT3 in A172 cells

Phosphorylated STAT3 usually binds to the γ -interferon activation sequence (GAS)-like element in the promoter region of targeted genes²⁸. Sequence analysis revealed that the ALDH1 promoter harbors GAS-like elements TT(N4-6)AA (<http://www.cbrc.jp/research/db/TFSEARCH.html>), we therefore determined whether STAT3 directly binds to the ALDH1 promoter and how STAT3 transcribes ALDH1 gene and induces ALDH1

transcription in A172 cells. To this end, we cloned a human ALDH1 promoter (886bp) into a luciferase reporter plasmid, which was called ALDH1luc. The location of the 5' region of the ALDH1 promoter construct is indicated in Fig 5A, and the primers used to generate it are shown in blue and described in Methods. The construct was transfected into A172 cells, and the activity was assessed after TRPM7 gene knockdown as indicated in Fig 5. To determine if the expression levels of STAT3 would have any effect on the transcriptional activity of ALDH1, A172 cells were cotransfected with a ALDH1 luciferase reporter construct, a caSTAT3, dnSTAT3, and a siRNA of STAT3 (siSTAT3) and a control siRNA respectively as indicated. The cellular samples were lysed and assayed for luciferase activity. As shown in Fig 5B, dnSTAT3 and siSTAT3 down regulated ALDH1 luciferase activity by approximately 20% and 60%, whereas caSTAT3 upregulated ALDH1 luciferase activity by approximately 2.5 fold.

Tyrosine-phosphorylated STAT3 binds the ALDH1 promoters in A172 cells

When STAT3 is activated by a variety of ligands, STAT3 is phosphorylated on tyrosine 705 residues, translocates to the nucleus and subsequently activates the transcription of different types of target genes ²⁹. In order to determine whether activated nuclear protein STAT3 binds to the ALDH1 promoter, we conducted a ChIP analysis using STAT3-transfected A172 cells. We generated two specific primer sets for ChIP-PCR analysis. Both sets were designed to amplify promoter regions containing STAT3 putative binding sites, amplifying a region harboring GAS-like elements (Fig 5A). Briefly, we transfected A172 with a plasmid constitutively expressing active STAT3 (caSTAT3), dnSTAT3 and siSTAT3, followed by a ChIP assay. We found that pSTAT3 co-immunoprecipitated more fragments of the promoters of ALDH1 (Fig 5C) than the empty vector, dnSTAT3 and a siSTAT3 did (Fig 5C). As shown in Fig 5C, amplification was detected with both primer sets designed to amplify different regions having the STAT3 binding sites in ALDH1 promoter. These results, together with the data above shows that STAT3 binds to the ALDH1 promoter region and activates ALDH1 when STAT3 is activated in A172 cells.

(5) Knockdown of TRPM7 inhibits migration and invasion of A172 cells

To examine the role of TRPM7 in glioma cell migration and invasion, transwell matrigel invasion assay ³⁰ was then performed. A172 cells were transfected with siRNA control or siTRPM7 for 48h followed by being cultured in the transwell chambers for another 24h, some cells migrated from the upper surface to the lower surface (Fig 6A). Compared with the siRNA control, siTRPM7 greatly decreased the number of migrated cells to 30.0% (Fig 6A-C). Migration potential was assessed by previously described monolayer cell wound healing ²¹. Both siTRPM7 and control cells migrated to the wound area within 24 hr. Although complete wound closure was not observed within 24 hr, siTRPM7 cells exhibited less wound closure than control cells. These results indicated that knockdown of TRPM7 significantly delayed wound closure (Fig 6D and 6E). Using the same procedures as described above, A172 cells were treated with either the control or siTRPM7 from 24 to 72 h followed by evaluation of cell proliferation using MTT assay. siTRPM7 induced 30.4% to 46.7% of cell proliferation (Fig 6F). Based on our findings, a schematic model for the regulation of TRPM7 in glioma tumorigenesis and GSC was shown in Fig 6G.

Discussion

The A172 glioblastoma cell line is a representative and widely used cell line in regulation of GSC^{31–32}. In this study, we utilized an *in vitro* system consisting of human glioma cancer (GC) cells and human glioma cancer stem cells (GSC) derived from A172 to facilitate an expanded range of inquiry that can be rapidly explored to test the significant role of TRPM7 in differentiation and self-renewal of GSC. This study aimed to investigate the effects of TRPM7 pathway activation on glioma cells and stem-like cells derived from them. In particular, we found that TRPM7 activates JAK2/STAT3 and/or Notch signaling pathways and leads to increased cell proliferation and migration. In addition, we found that TRPM7-induced upregulation of ALDH1 expression is associated with increases in ALDH1 activity and is detectable in stem-like cells when expanded as spheroid CSCs. These findings for the first time demonstrated that TRPM7 (1) activates a previously unrecognized STAT3→ALDH1 pathway, and (2) promotes the induction of ALDH activity in glioma cells.

The features that TRPM7 is essential for cancer cell growth, proliferation and metastasis are the basis for the increasing interest in the channel as a target for cancer therapeutics. We previously found that lowering extracellular Ca^{2+} induced a large nondesensitizing current reminiscent of Ca^{2+} -sensing cation current or TRPM7 current previously described in other cells. This Ca^{2+} -sensing current can be inhibited by Gd^{3+} , 2-aminoethoxydiphenyl borate (2-APB), or intracellular Mg^{2+} , consistent with the TRPM7 current being activated. Interestingly, we identified that Ca^{2+} is critical for the growth and proliferation of FaDu cells and blockade of TRPM7 channels by Gd^{3+} and 2-APB or suppression of TRPM7 expression by siRNA inhibited the growth and proliferation of these cells. Similar to FaDu cells, SCC25 cells also express TRPM7 channels and suppression of the function of TRPM7 channels inhibited the proliferation of SCC25 cells⁷. In addition, we identified the potential functions of TRPM7 channels in human umbilical vein endothelial cells (HUVECs)³³. Silencing TRPM7 with small interference RNA (siRNA) decreased the level of TRPM7 mRNA, the TRPM7-like current, as well as phosphorylation of extracellular signal-regulated kinase (ERK), indicating that TRPM7 channels also play an important role in the function of vascular endothelial cells and angiogenesis³³. Our recent observation further demonstrates that TRPM7 channels contribute hyperglycemia-induced injury of vascular endothelial cells³⁴. It remains a high priority for researchers and clinicians to discover new targets and therapeutic strategies to increase the survival rate and improve the clinical outcomes of GBM. Our electrophysiological and pharmacological data strongly indicated an involvement of TRPM7 channels in mediating the Ca^{2+} -sensing current in A172 glioma cells and glioma patients' brain tissues. Importantly, suppression of TRPM7 expression also significantly inhibited the growth, proliferation, migration and invasion of A172 cells indicating that TRPM7 channels may represent a novel and promising target for therapeutic intervention in malignant glioma (Leng et al. manuscript in preparation).

The initiation and progression of diverse tumors, including glioma, are driven by a population of cells with stem cell properties. In recent years, GSC has been successfully isolated from patient tumor specimens. Biochemical and biological characterization of these cancer initiating cells has implicated their critical roles in cancer growth, malignancy and

resistance to conventional treatments^{35–36}. The targeting of GSC may represent a powerful treatment approach. Notch signaling is central to the normal and neoplastic development of the central nervous system, playing important roles in proliferation, differentiation, apoptosis and CSC regulation. It is also involved in the regulation in response to hypoxia and angiogenesis, which are common features of tumorigenesis and, more specifically, for GBM^{37–38}. One of another TRP family member, TRPC6 has recently been recognized to cause sustained elevation of intracellular calcium, and linked to Notch signaling pathway in GBM growth and invasiveness³⁹. Previous studies in glial transformation illustrated that STAT3 has dichotomous roles in human glioblastoma tumorigenesis and is depending on the glioblastoma-associated genetic mutation. STAT3 is tumor suppressive in PTEN-deficient glioblastoma tumors whereas it functions in an oncogenic manner in EGFRvIII-expressing tumors^{40–41}. In human tumors harboring both PTEN loss and EGFRvIII expression, STAT3 is anticipated to play an oncogenic role based on genetic evidence seen in astrocyte transformation in which both mutations are introduced⁴⁰. Interestingly, constitutive activation of the STAT3/NFκB signaling pathway and upregulation of STAT3- and NFκB-dependent genes are found in GSC and regulate the Notch pathway¹⁷. Among GSC, CD133(+) cells are a well-defined population and that the presence of CD133(+) GSC correlates highly with patient survival, making these cells an ideal immunotherapy target⁴². ALDH1 is also recognized as a novel stem cell marker in human GBM¹⁶. Moreover, emerging evidence indicates that ALDH1 is not only a marker for stem cells, but may also play key functional roles related to self-protection, differentiation, expansion⁴³ and proliferation^{44–45}.

A few number of ion channels and transporters were identified to be involved in the function of GSC. The Na-K-Cl cotransporter1 (NKCC1) protein which transports 1 Na⁺, 1 K⁺ and 2 Cl⁻ ions, was expressed in both GC and GSC and played an essential role in regulatory volume increase (RVI) in response to hypertonic cell shrinkage⁴⁶. TRPV2, a calcium-permeable cation channel, promotes GSCs differentiation and inhibits their proliferation⁴⁷. Our results showed that TRPM7 activates JAK2/STAT3 and/or Notch signaling pathways and leads to increased cell proliferation and migration. In addition, we defined molecular cross-talk between the TRPM7, STAT3 and ALDH1 signaling pathways in glioma CSCs, TRPM7 activates a previously unrecognized STAT3→ALDH1 pathway, and promotes the induction of ALDH activity in glioma cells.

TRPM7 is unique in the sense that it encodes an α kinase domain fused to the ion channel moiety, the kinase and channel domain can be mutually regulated, but the presence of the kinase domain is not required for TRPM7 channel activity although it contributes partly to the modulation of the channel sensitivity to Mg²⁺⁴⁸ and cAMP^{49–50}. Future studies will be required to identify the separate effects of kinase activation and channel activity on STAT3, ALDH1 and Notch activation. Several compounds have been reported to irreversibly suppresses the TRPM7 channels, such as waixenicin A, an organic extract of the soft coral *S. edmondsoni*¹⁴, but they lack specificity and therefore are of limited use. The activation of STAT3 and Notch signaling pathway that leads to the regulation of ALDH1 pathway genes in GSC identify potential therapeutic targets for the treatment of glioma (Fig. 6 G).

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What's new?

Glioma cancer stem cells (GSC) initiate and maintain glioma tumorigenesis and being responsible for resistance to therapies. In studies of GSC and glioma monolayer cultures from which they were derived, we found that TRPM7 activates JAK2/STAT3 and/or Notch signaling pathways and leads to increased cell proliferation and migration. These findings for the first time demonstrates that TRPM7 (1) activates a previously unrecognized STAT3→ALDH1 pathway, and (2) promotes the induction of ALDH1 activity in glioma cells.

Figure 1. Analysis of human stem cell signaling pathways using real time RT² profile PCR arrays
Human stem cell signaling pathways were regulated by TRPM7 assessed by real time RT² Profile PCR arrays in A172 cells. The genes with fold change greater than 2 are listed as (A). Heat map (B) and scatter plot (C) are also shown.

Figure 2. STAT3 and Notch1 are involved in the regulation of glioma cells by TRPM7
Down regulation of TRPM7 reduced mRNA expression of Notch1, JAG1, Hey2, Survivin, ALDH1 and WNT1 by qPCR (A) as well as STAT3 activation, protein expression of Notch1 and target genes (Hey2, Survivin), ALDH1 and CD133 by Western blot analysis (B).

Figure 3. Determination of the subcellular localization of diverse proteins by immunofluorescence staining

TRPM7 localized predominately in the membrane and in the surrounding cytoplasm (A), while Notch1, pSTAT3 and pJAK2 (B, D and E) are almost exclusively localized in the nucleus. CD133 is widely distributed and is visualized in the membrane, cytoplasm and nucleus (C). Down regulation of TRPM7 (A) reduced Notch1 (B), CD133 expression (C), activation of STAT3 (D) and pJAK2 (E) by immunofluorescence study.

Figure 4. TRPM7-mediated activation of STAT3, upregulation of Notch1 and ALDH1 signaling pathways in Glioma CSC

A172-derived GSCs were generated using serum-free medium supplemented with EGF and bFGF (Fig. 4A). mRNA expression of Notch1 and its ligand or target genes Jagged1 (JAG1), Hey2 and Survivin as well as ALDH1, WNT1 were upregulated by qPCR assay (Fig. 4B). Compared to parental A172 cells (GC), active form of Notch1, cleaved Notch1, the Notch1 intracellular domain (NICD) protein, its ligand or target genes Hey2, Survivin, ALDH1 (Fig. 4C) as well as CD133 (Fig. 4C and 4D) were also upregulated.

Figure 5. Tyrosine-phosphorylated STAT3 binds the ALDH1 promoters in A172cells
The location of the 5' region of the ALDH1 promoter construct is indicated in Fig 5A, and the primers used to generate it are shown in blue. As shown in Fig 5B, dnSTAT3 and siSTAT3 down regulated ALDH1 luciferase activity by approximately 20% and 60%, whereas caSTAT3 upregulates ALDH1 luciferase activity by approximately 2.5 fold. pSTAT3 was found to co-immunoprecipitate more fragments of the promoters of ALDH1 than the empty vector, dnSTAT3 and siSTAT3 did (Fig 5C).

Figure 6. Knockdown of TRPM7 inhibits migration and invasion of A172 cells

A172 cells were transfected with siRNA control or siTRPM7 for 48h. After being cultured in the transwell chambers for 24h, some cells migrated from the upper surface to the lower surface (Fig 6A and B). Compared with the siRNA control, si TRPM7 greatly decreased the number of migrated cells to 30.0% (Fig 6A-C). Wound Healing was observed at the indicated time points along the scrape line and a representative field for each treatment condition was photographed. Assays were done in triplicate and two independent experiments were repeated. Panel D indicate the representative photomicrographs of each treatment in the wound-closure assay. Panel E indicates the quantification of the wound-closure assay (n=3). Panel G is a schematic figure to show that TRPM7 activity may mediate GBM progression through JAK2/STAT3-ALDH1 and Notch1 pathways and TRPM7 is a potential GBM drug target. Inhibition of TRPM7 channels at three steps is therefore expected to suppress protein synthesis and cell proliferation and invasion.