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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, $Ca^{2+}$ and $Mg^{2+}$ 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 TRMP7-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.



## 1. 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 temozolomide [1]. 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 chemoresistance [2–4]. 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 $Ca^{2+}$ and $Mg^{2+}$ [5]. In addition to its channel activity, TRPM7 contains a functional α-kinase domain within its C terminus [6]. 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 lines [7]. 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 cancer [8], gastric cancer [9], head and neck cancer [7], nasopharyngeal carcinoma [10], pancreatic cancer [11], prostate cancer [12], retinoblastoma [13], and leukaemia [14]. Increased TRPM7 expression is found to be correlated with clinicopathological parameters, such as tumor grade, Ki67 proliferation index and patient survival [11].

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 invasion [15]. 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 GBM [16]. 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 cells [17]. Signal

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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 glioma [17]. In our pilot study, we found a strong evidence to support an involvement of TRPM7 channels in mediating the $Ca^{2+}$-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.

## 2. Material and methods

### 2.1. 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.

### 2.2. 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, 20 ng/ml EGF and 20 ng/ml FGF-2 (Invitrogen, Carlsbad, CA), and 5 μg/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% $CO_2$, 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.

### 2.3. 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 48 h or 72 h 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 described [18].

### 2.4. 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 previously [19]. 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 GCTCG AGAA 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.

### 2.5. MTT assay

A172 cells were seeded at $1\times10^4$ cells in 100 μl of medium per well into 96-well plates and were transfected with 50 nM specific siRNA or control using Lipofectamine™ for indicated times. 10 μl 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 570 nm was measured using 690 nm as reference filter using a CytoFluorTM 2300 plate reader.

### 2.6. 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 immunodetectable bands were performed as previously described [20]. 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, ati-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: CCCTTTGGTGGATTCAAGAT and TTGAAGAGCTTC TCTCCACTCTT, CD133 (5′-3′) were, CAGAAGGCATATGAATCCAAAA and ATAAACAGCAGCCCCAGGAC, Notch1 (5′-3′) were, CAC TGT GGGC GGGTCC and GTTGTATTGGTTCGGCACCAT, Survivin (5′-3′) were, GCCC AGTGTTTCTTCTGCTT and CCTCCCAAAGTGCTGGTATT, Hey2 (5′-3′) were, AAAAAGCTGAAATATTGCAAATGA and GTACCGCGCAACTTCTGTT, Jagged1(5′-3′) were, GACTCATCAGCCGTGTCTCA and TGGGGAACACTC ACACTCAA, WNT1(5′-3′) were GCGCTTCCTCATGAACCTT and GTGCAT GAGCCGACATC, Notch2(5′-3′) were, AATCCCTGACTCCAGAACG and TGGTAGACCAAGTCTGTGATG AT, Sox11(5′-3′) were, GACCCAGACTGG TGCAAGAC and GCCCAGCCTCTTGGAGAT, GAPDH (5′-3′) were, GAAG GTGAAGGTCGGAGTC and GAAGATGGTGATGGGATTTC.

### 2.7. $RT^2$ 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 $RT^2$ First-Strand cDNA Synthesis kit (Qiagen, Valencia, CA) by 1000 ng of total RNA. The cDNA template was then mixed with $RT^2$ Real-Time SYBR Green Master Mix to a reaction volume of 25 μL 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.