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TRPM7 regulates the migration of human nasopharyngeal carcinoma cell by mediating Ca^{2+} influx

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ABSTRACT

Ion channels are involved in various physiologic and pathologic processes, including the migration of tumor cells that is required for metastasis. To determine whether transient receptor potential melastatin 7 (TRPM7) Ca^{2+} channels play an important role in the migration of tumor cells, we examined the potential function of TRPM7 channels in the migration of 5-8F and 6-10B human nasopharyngeal carcinoma cells. The migratory potential of 5-8F cells was significantly decreased by extracellular Ca^{2+} chelator (EGTA), TRPM7 inhibitors (La^{3+}, 2-APB), or TRPM7 knockdown. Conversely, the addition of TRPM7 activator Bradykinin and overexpression of TRPM7 promoted the migration of 5-8F and 6-10B cells. Furthermore, the sustained Ca^{2+} influx regulated by TRPM7 activated release of Ca^{2+} stores via ryanodine receptors by a calcium-induced calcium release (CICR) mechanism. This study suggests, first, that Ca^{2+} influx is required for the migration of human nasopharyngeal carcinoma 5-8F cells. Second, and more importantly, it identifies TRPM7 as a novel potential-regulator of the Ca^{2+} influx that allows migration of 5-8F cells. TRPM7, therefore, might have potential as a prognostic indicator and as a therapeutic target in nasopharyngeal carcinoma.

1. Introduction

Although nasopharyngeal carcinoma (NPC) is rare in Western countries, it is extremely common in southern regions of China. In Guangdong, for example, NPC accounts for 18% of all cancers [1]. Because NPC occurs close to the intracranial organs, the cancer cells invade the cranial cavity and metastasize. This leads to a poor prognosis for many NPC patients. Therefore, an improved understanding of the molecular mechanisms involved in the intracranial invasion and metastasis of NPC will lead to improved treatments and prognosis factors for NPC patients.

Cell migration is required during the invasion and metastasis of tumor cells [2,3], and the ubiquitous second messenger Ca^{2+} is a critical regulator of cell migration [4]. Recently, many studies have shown that Ca^{2+} influx and Ca^{2+} channels are essential for the migration of various cell types, including tumor cells such as breast cancer cells [5–7]. It is possible, therefore, that Ca^{2+} entry pathways also exist in NPC cells. Tumor cells often lack the voltage-operated Ca^{2+} channels and receptor-operated Ca^{2+} channels that play a pivotal role in Ca^{2+} entry in excitable cells. However, recent studies of tumor cells have revealed two other potential pathways for Ca^{2+} entry: store-operated Ca^{2+} channels and transient receptor potential (TRP) channels [8]. Three TRP channels, TRPM1, TRPM8 and TRPV6, have been shown to control Ca^{2+} influx, and to regulate the migration of murine melanoma cells (B16-F1/10), human glioblastoma cells (DBTRG) and hepatoblastoma (HepG2) cells, respectively [9–11].

TRP channels were first identified in Drosophila species. On the bases of homology and channel function, the TRP family is divided into three main subfamilies: classic (TRPC), vanilloid (TRPV), and melastatin (TRPM) [12]. TRPM7, a member of the TRPM subfamily, is a non-selective cation channel with predominant permeability for Ca^{2+} and Mg^{2+}. It regulates the calcium concentration in cells, which is imperative for many processes [4]. Recent studies have suggested more and more importance for TRPM7 in the processes of cell migration. Wei et al. showed that calcium flickers, which arose from TRPM7 at the migrating fibroblast front, had a crucial role in guiding directional movement—after TRPM7 knockdown and inhibition of calcium signalling, all migratory, turning, and chemotactic abilities were impaired [13]. Clark et al. demonstrated that activating TRPM7 could promote cytoskeletal relaxation and the conversion of focal adhesions to podosomes in mouse tumor cells (N1E-115) [14]. Despite these findings, the potential function of TRPM7 channels in the migration of tumor cells is not known. We hypothesized that TRPM7 Ca^{2+} channels are important in the migration of human NPC cells.

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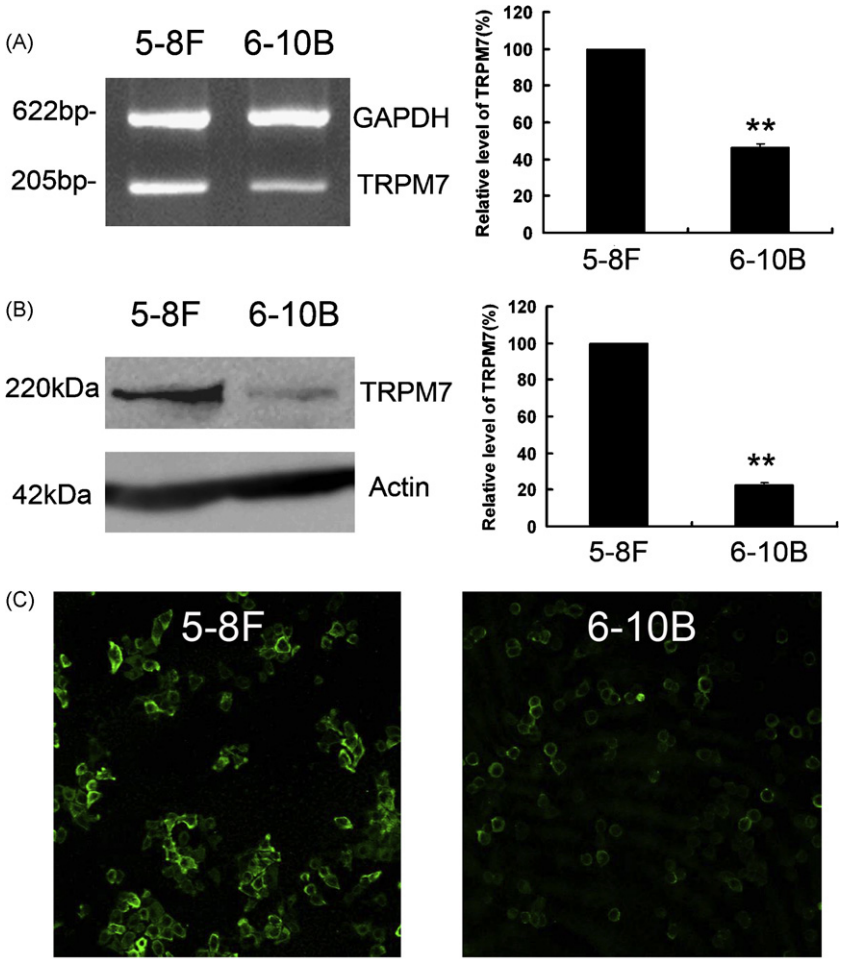

Fig. 1. TRPM7 mRNA and protein are expressed at higher levels in 5-8F cells than in 6-10B cells. (A) RT-PCR showed higher expression of TRPM7 mRNA in 5-8F cells than in 6-10B cells. Western blotting (B) and immunofluorescence staining (C) also showed higher expression levels of TRPM7 protein in 5-8F cells, which have metastatic potential, than in 6-10B cells, which have the same parentage as 5-8F cells but no metastatic potential. ($^{*}P < 0.05$, $^{**}P < 0.01$.)

We used colony lines of the NPC SUNE1 cell line to investigate the presence of TRPM7 channels in 5-8F cells (highly tumorigenic with metastatic ability) and 6-10B cells (tumorigenic without metastatic ability). We found high expression of the TRPM7 gene and protein in 5-8F cells, but low expression in 6-10B cells. By activation, blockade, and knockdown of these ion channels, we showed that TRPM7 affected the migratory potential of 5-8F cells. Contrary to TRPM7 RNAi results in 5-8F cells, we found that overexpression of TRPM7 increased the migration of 6-10B cells. Moreover, we found that TRPM7 regulated the migration of 5-8F cells by controlling Ca^{2+} influx. Finally, pharmacological data showed that TRPM7 channels were not stores-operated channels but, instead, controlled the release of intracellular Ca^{2+} stores via ryanodine receptors by a calcium-induced calcium release (CICR) mechanism. Overall, our data suggest that TRPM7 might be critical for the migration of NPC cells.

2. Materials and methods

2.1. Cell and cell culture

Two colony lines of human NPC SUNE1 cells—5-8F cells, which are highly tumorigenic and have metastatic ability, and 6-10B cells, which are tumorigenic, but lack metastatic ability [15]—were stored in our laboratory (established by Oncology Institute of Sun Yat-sen University Cancer Center). Both cell lines originated from a poorly differentiated squamous cell carcinoma of the nasopharynx. The cells were grown in RPMI 1640 supplemented with 10% FBS (Hyclone, USA).

2.2. Materials and protocols

For the cell stimulations, bradykinin (BK), 2-aminoethoxydiphenyl borate (2-APB), lanthanum (La^{3+}), thapsigargin (TG), SKF96365, nimodipine, DL-2-amino-5-phosphonopentanoic acid (2-AP), CNQX, EGTA, ryanodine and xestospongin C were purchased from Sigma Chemical Co. (Shanghai China). Then, 5-8F cells were treated as follows: BK (TRPM7 activator: 100 nM for 16 h); 2-APB (TRPM7 non-specific inhibitor: 200 μM for 2 and 16 h); La^{3+} (TRPM7 non-specific inhibitor: 200 μM for 16 h); SKF96365 (stores-operated Ca^{2+} channels inhibitor: 30 μM for 16 h); nimodipine (voltage-operated Ca^{2+} channels inhibitor: 20 μM for 16 h); 2-AP (NMDA receptors inhibitor: 1 mM for 16 h); CNQX (AMPA receptors inhibitor: 10 μM for 16 h); EGTA (Ca^{2+} chelator: 2 mM for 16 h) ryanodine (RyR inhibitor 100 μM) and xestospongin C (IP_3R inhibitor: 5 mM).

2.3. Reverse transcription-PCR

Total RNA from 5-8F and 6-10B cells was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. First-strand DNA was generated from 0.5 μg of total RNA

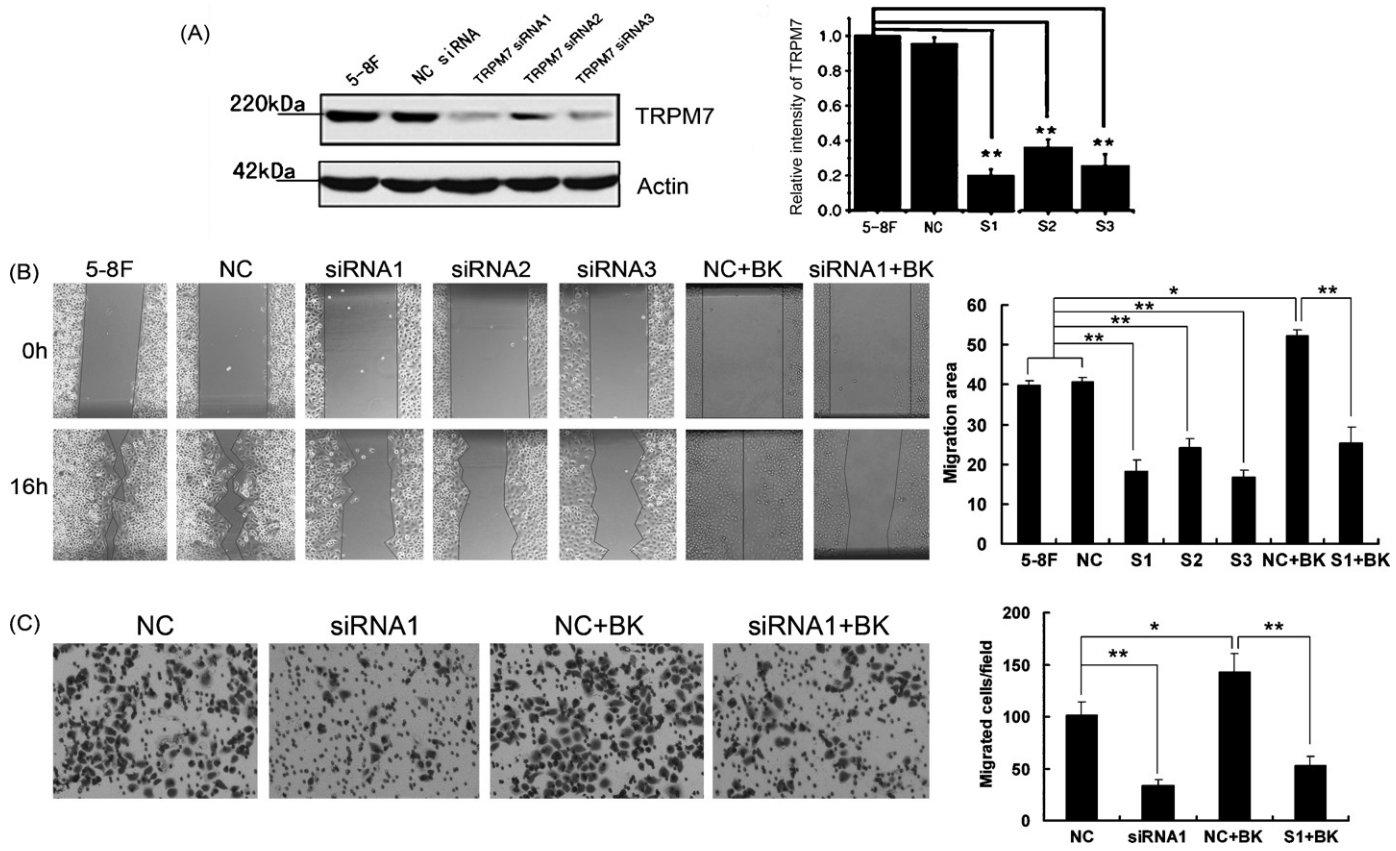

Fig. 2. TRPM7 potentially required for migration of 5-8F cells. (A) Immunoblot analyses showed that silencing TRPM7 (siRNA1, 2, or 3) decreased expression of the TRPM7 protein. (B) Wound-healing assays showed that silencing of TRPM7 (bars S1, S2, and S3) inhibited the migration of 5-8F cells in both the absence or presence of BK. (C) Transwell chamber assays confirmed that silencing TRPM7 (siRNA1) inhibited the migration of 5-8F cells in either absence or presence of BK. Data are either representative of three independent experiments or are shown as mean ± SEM of five experiments. (*$P < 0.05$, **$P < 0.01$.)

using oligo(dT)15 and reverse transcriptase SuperScript II (Invitrogen, USA) at a reaction volume of 20 μl. An oligonucleotide primer pair was synthesized over regions specific for human TRPM7 cDNA (GenBank accession no. NM_017672). PCR amplifications of TRPM7 with upstream primers (5′-GCACCATCTTGGACTCTT-3′) and downstream primers (5′-GAAATTGCCTTCACTTGTA-3′) were performed for 28 cycles under the following conditions: denaturing at 94 °C for 30 s, annealing at 56.6 °C for 30 s, and polymerization at 72 °C for 1 min. A primer pair for the detection of human GAPDH was used as the internal control. Reverse transcription-PCR (RT-PCR) amplification was done with Taq polymerase (Takara Japan) using a thermal cycler (96-Well GeneAmp® PCR System 9700). PCR products were separated by 1.0% agarose gel electrophoresis and visualized by ethidium bromide staining.

2.4. Western blot analysis

5-8F and 6-10B cells were washed twice with cold PBS and lysed on ice in RIPA buffer (1× PBS, 1% NP40, 0.1% SDS, 5 mM EDTA, 0.5% sodium deoxycholate, and 1 mM sodium orthovanadate) with protease inhibitors. The protein lysates were resolved on 10% SDS–polyacrylamide gel, electrotransferred to polyvinylidene fluoride (PVDF; Pall Life Sciences, NY, USA) membranes, and blocked in 5% non-fat dry milk in Tris-buffered saline (TBST) (pH 7.5; 100 mM NaCl, 50 mM Tris, and 0.1% Tween-20). Membranes were immunoblotted with anti-TRPM7 goat-polyclonal antibody (Abcam, USA) and anti-Actin antibody (Santa Cruz Biotechnology, CA, USA) overnight at 4 °C, and their respective secondary antibodies with conjugated to horseradish peroxidase (HRP). Immunoblots were detected by enhanced chemiluminescence (ECL; BeyoECL Plus, China).

2.5. Immunofluorescence confocal microscopy

5-8F and 6-10B cells were seeded on coverslips or in coverslip bottom dishes, precoated with poly-L-lysine (Sigma) for 24 h, fixed with 4% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 solution and blocked in 2% bovine serum albumin-0.1% Triton X-100. After extensive and repeated washing in PBS, cells were incubated with 1:100 dilution of TRPM7 primary antibody (Abcam, USA) for 2 h at room temperature, washed again in PBS, and incubated with fluorescein isothiocyanate (FITC)-labeled anti-goat donkey antiserum (Proteintech, USA) for 1 h. The coverslips were sealed with mounting medium containing 50% glycerol and 50% PBS to prevent quench. Immunostained cells were observed under a confocal laser scanning microscope (FV1000; Olympus) equipped with an argon/krypton laser source.

2.6. Calcium imaging

Calcium-imaging experiments were performed as described previously [16]. Briefly, cells were plated onto glass coverslips and loaded with 4 μM Fura-2 AM (a ratiometric fluorescent Ca^{2+} indicator) at room temperature for 45 min in HBSS containing (in mM): 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 0.3 Na_2HPO_3, 0.4 KH_2PO_4, 4 $NaHCO_3$, 5 glucose, and 10 HEPES adjusted to pH 7.4 with NaOH. Cells were then placed in a perfusion chamber on the stage of the microscope (Olympus Xl 71) with a 40× objec-

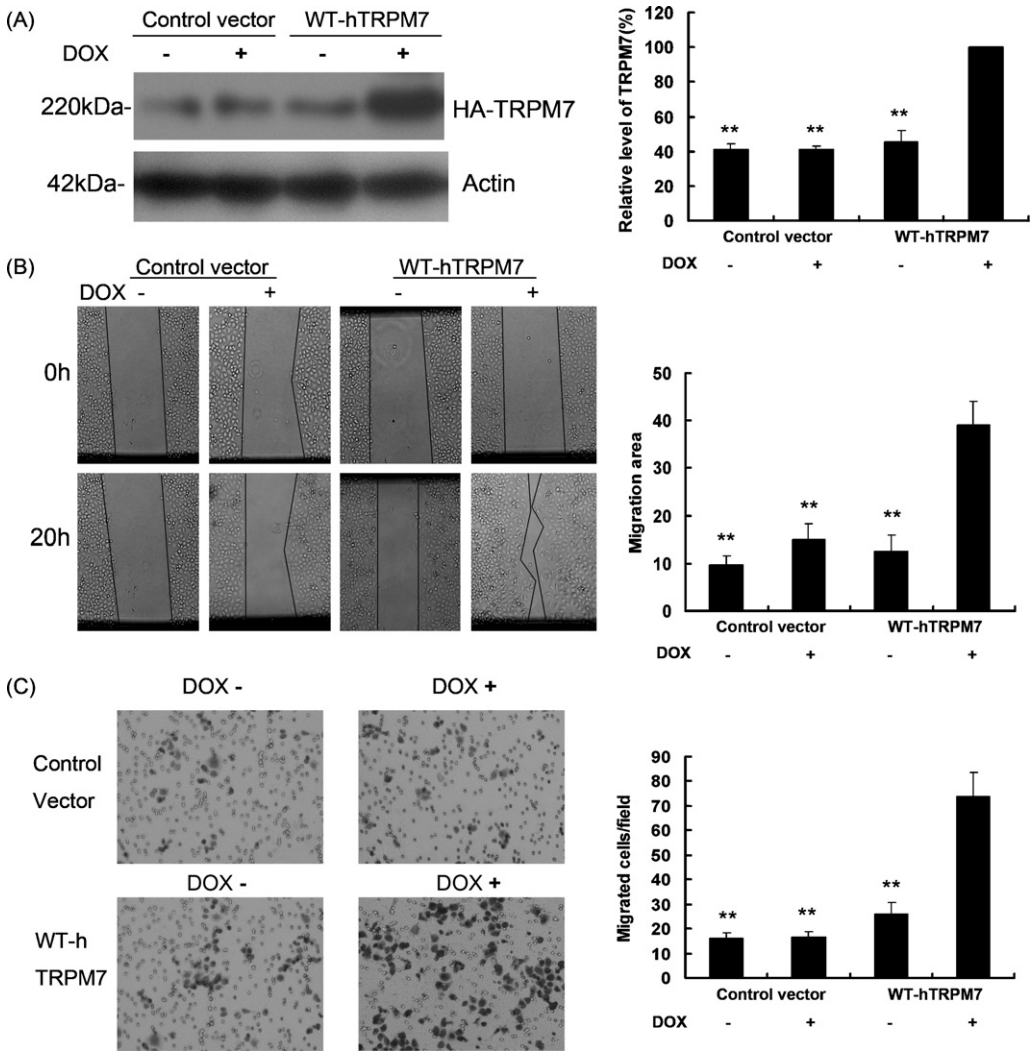

Fig. 3. Overexpression of TRPM7 increases migration of 6-10B cells. (A) Overexpression of TRPM7 in 6-10B cells was detected by Western blot using antibodies against the HA tag. Cells transfected the empty vector were used as control in all experiments. Control cells or TRPM7 cells were cultured in the absence or presence of doxycycline (DOX 1 μg/ml) for 1 day. Cells were lysed and TRPM7 and actin were determined by Western blotting. (B) Wound-healing assays and (C) transwell chamber assays showed that overexpression of TRPM7 increased the migration of 6-10B cells. Data are either representative of three independent experiments or are shown as mean ± SEM of five experiments. ($^{*}P < 0.05$, $^{**}P < 0.01$.)

tive (NA 0.8). Fluorescence images of the cells were recorded by a Ratio Vision digital fluorescence microscopy system (TILL Photonics GmbH, Germany) and analysed by TILL vision 4.0 software. The Fura-2 fluorescence, at an emission wavelength of 510 nm, was recorded by exciting the probe alternatively at 340 and 380 nm. The signal ratio at 340/380 nm was then converted into $[Ca^{2+}]_i$ level according to established methods [17]. The data are shown by one mean curve from average responses of 10–20 cells.

2.7. Small interfering RNA silencing

5-8F cells were seeded onto six-well plates 24 h before transfection. RNAi sequences for TRPM7 were designed using RNAi Designer (http://www.invitrogen.com/rnai), and all siRNA duplexes were synthesized by Shanghai GenePharma Co. In the wells, 50 nM of TRPM7 siRNA1 (5′-CCUCAUGAAGCACCAUCUU-3′), TRPM7 siRNA2 (5′-GCAUGGUGUUGUAAAUACA-3′), TRPM7 siRNA3 (5′-AGGAGAAGAUGCAAUUAAA-3′), or negative control siRNA (5′-UUCUCCGAACGUGUCACG-3′), and 10 μl of Lipofect AMINE 2000 (Invitrogen) was added to Opti-MEM and mixed gently. The wells were added to plates and incubated for 48 h until ready for further assay. All Western blotting and functional studies were carried out after a 48–72 h transfection.

2.8. Construct and 6-10B cells transfection

The expression constructs WT human TRPM7/pCDNA4/TO were gifts from A. Scharenberg, A. Perraud, and C. Schmitz [18]. This recombinant hTRPM7 is tagged with the hemagglutinin (HA) epitope. 6-10B cells were transfected with the hTRPM7/pCDNA4/TO construct and empty construct by using Lipofectamine 2000 (Invitrogen, USA) and selected for stable transfectants by zeocin treatment (400 μg/ml). TRPM7 expression was induced 1 day before use by adding 1 μg/ml doxycycline (DOX) to the culture medium. 6-10B cells transfected with the empty vector served as a control for all experiments. Western blot analysis, wound healing assay and transwell chamber migration assay were performed 16–24 h after induction.

2.9. Wound healing assay

5-8F cells (2.0×10^5) were cultured in 12-well tissue culture plates to confluence without vacant space. A pipette tip (10 μl)

was used to scratch a wound midline of the culture well then cells were pretreated with 10 μg/ml mitomycin C (Sigma–Aldrich, Shanghai, China) for 1 h at 37 °C. After 16 h of culture in RPMI 1640 supplemented with 2% serum, migration of cells was evaluated by measuring the difference in width of the wounds at 0 h and at 16 h.

2.10. Transwell chamber migration assay

The migration activities of 5-8F cells, siNC-transfected 5-8F cells, and siTRPM7-transfected 5-8F cells were assayed using transwell cell culture chambers. Cells were added to the transwell chamber (8-μm pore size; Corning, NY, USA). The motilities of NC and TRPM7 knockdown cells in the transwell chamber assay were stimulated by the serum alone (as a negative control) or the serum in combination with BK. The serum in bottom wells acts as chemotatic factors and BK in both top and bottom wells acts as expected motility boosting factors. After 48 h the cells that migrated through the membrane were counted. Cells were counted in 5 random fields and expressed as the average number of cells per field under a light microscope.

2.11. Statistical analysis

Statistical data are expressed as mean ± SEM. When appropriate, Student's *t*-test and one-way ANOVA were applied. In all cases, $p < 0.05$ was considered statistically significant. All experiments were repeated at least three times.

3. Results

3.1. Expression of TRPM7 higher in 5-8F cells than in 6-10B cells

From colony lines of the NPC SUNE1 cell line, two cell clones with differing metastatic potentials were established: 5-8F cells are highly tumorigenic and have metastatic ability, and 6-10B cells are tumorigenic but lack metastatic ability [15]. The expression of TRPM7 in 5-8F and 6-10B cells was examined by Western blot, immunofluorescence staining, and RT-PCR. Levels of TRPM7—both mRNA and protein—were significantly higher in 5-8F cells than in 6-10B cells (Fig. 1).

3.2. Silencing of TRPM7 inhibits migration of 5-8F cells

To investigate whether TRPM7 plays an important role in the migration of 5-8F cells, RNA interference (RNAi) was used to suppress TRPM7 expression selectively in 5-8F NPC cells. Immunoblotting analyses showed that, within 48 h after transfection of 5-8F cells with TRPM7 siRNA Nos. 1–3, the expression of TRPM7 protein was suppressed 65–80% (Fig. 2A). Moreover, wound-healing assays showed that serum-induced migration of 5-8F cells was inhibited 50–70% by TRPM7 siRNA (Fig. 2B). Finally, the motility of TRPM7 knockdown cells was analyzed using the transwell chamber assay. Like the down-regulated expression of TRPM7 and the decreased influx of Ca^{2+}, motility was also reduced in cells transfected with TRPM7 siRNA. Compared with negative control siRNA cells, TRPM7 knockdown (siRNA1) cells showed a 68% reduction in the number of cells that crossed the membrane (Fig. 2C), indicating that TRPM7 channels were required for migration.

3.3. Overexpression of TRPM7 enhances migration of 6-10 cells

To examine whether overexpression of TRPM7 alters migratory ability of 6-10B cells, 6-10B cells were transfected with the hTRPM7/pCDNA4/TO construct and empty construct and selected for stable transfectants by zeocin treatment. Then the cells were verified inducible hTRPM7/empty vector expression by Western blot after grown in the absence or presence of the inducer, Doxycycline (DOX), for 24 h (Fig. 3A). Two faint bands (left lanes) representing low-level expression of native TRPM7 can be detected in 6-10B in the absence or presence of DOX. Interestingly, wound-healing assays and transwell chamber assays (Fig. 3B and C) showed that BK and serum in combination or serum-induced migratory abilities increased 260–460% in 6-10B cells transfected with WT-hTRPM7 construct when compared with control 6-10B cells.

3.4. Ca^{2+} influx mediated by TRPM7 potentially critical for migration of 5-8F cells

Pharmacological approaches were used to verify that influx of extracellular Ca^{2+} affected the migration of 5-8F cells. In the wound-healing assay, removal of extracellular Ca^{2+} by EGTA (2 mM) significantly inhibited migration of 5-8F cells, indicating that influx of extracellular Ca^{2+} is critical for the migration of 5-8F cells. Next, wound-healing assays were employed to investigate the effect on cell migration of pharmacological inhibitors for various ion channels that mediated Ca^{2+} influx. Fig. 4 showed that four inhibitors of three different Ca^{2+} channels (nimodipine for voltage-operated channels, 2-AP for NMDA receptor-operated channels, CNQX for AMPA receptor-operated channels, and SKF96365 for store-operated Ca^{2+} channels) had no effect on migration of 5-8F cells. However, the addition of a non-specific TRPM7 channel inhibitor (2-APB, 200 μM) reduced the migration of 5-8F cells by 69.7%. Likewise, another non-specific TRPM7 channel inhibitor (La^{3+}) inhibited the migration of 5-8F cells by 95.8%. Conversely, the addition of BK, a peptide agonist that activates and promotes expression of TRPM7 in N1E-115 cells [14], promoted the migration of 5-8F cells (Fig. 4). Transwell chamber assays also revealed that both 2-APB and La^{3+} inhibited the migration of 5-8F cells (data not shown).

3.5. TRPM7 channel potentially a major regulator of intracellular Ca^{2+} response

In pharmacological approaches to further confirm that TRPM7 mediated Ca^{2+} influx in 5-8F cells, Fura-2-based Ca^{2+} imaging was performed. Addition of BK triggered a rapid increase in cytosolic Ca^{2+} from internal stores in 5-8F cells. The initial increase in Ca^{2+} was transient, but was followed by a sustained phase of elevated Ca^{2+} that lasted for several minutes before Ca^{2+} returned to basal levels (Fig. 5A). Ratiometric analysis showed that compared with control (NC), silencing of TRPM7 (siRNA1) significantly decreased the transient phase of the response (720 ± 70 nM versus 550 ± 50 nM, $^{*}P < 0.05$), but it decreased the sustained phase of the response much greater (400 ± 50 nM versus 190 ± 50 nM, $^{**}P < 0.01$).

Fura-2-based Ca^{2+} imaging was also used to examine the mechanism by which TRPM7 inhibitors influenced influx of extracellular Ca^{2+} and release of intracellular Ca^{2+} stores in 5-8F cells. Both of TRPM7-mediated Ca^{2+} influx (plateau phase) and Ca^{2+} store Ca^{2+} release (peak phase) could be induced consecutively two times by BK (TRPM7 specific activator) without any desensitization in the control group (Fig. 5B, comparing BK1 and BK2; $P < 0.001$). However, compared with the control group, both La^{3+} (Fig. 5D) and 2-APB (Fig. 5E) completely inhibited the amplitude of the peak and plateau phases of the second BK-induced Ca^{2+} response ($P < 0.001$). Interestingly, similar results were observed with EGTA, which chelated extracellular Ca^{2+} (Fig. 5C, $P < 0.001$). These data indicated that Ca^{2+} response was not induced by BK without extracellular Ca^{2+}.

Moreover, ryanodine receptor (RyR) inhibitor ryanodine (100 μM) and inositol-1,4,5-trisphosphate receptor (IP3R) inhibitor xestospongin C (5 mM) were used to investigate whether

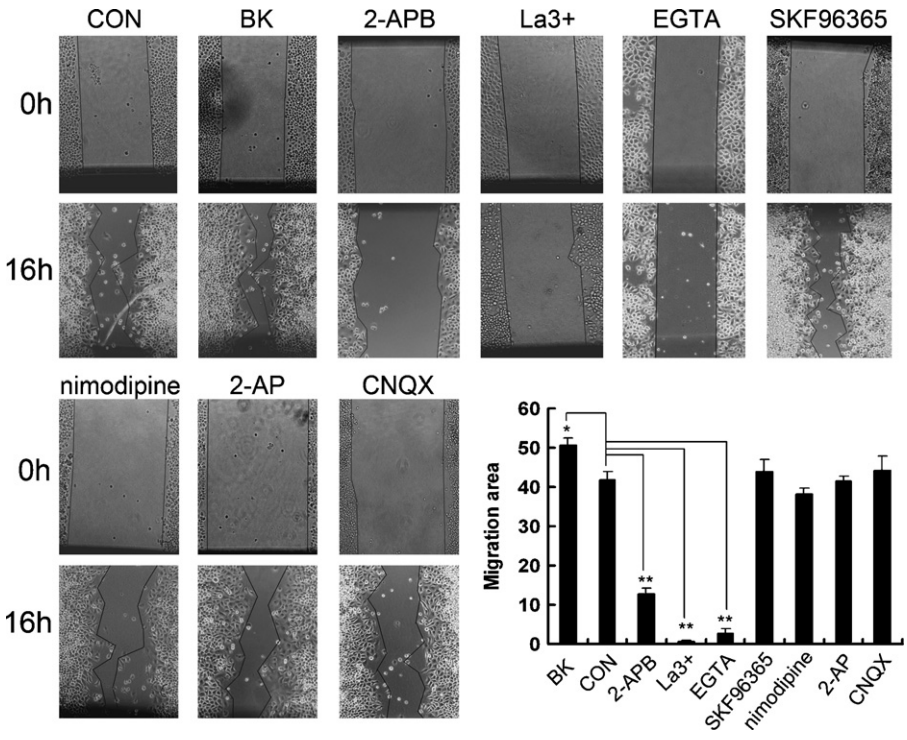

Fig. 4. Ca^{2+} influx mediated by TRPM7 potentially required for migration of 5-8F cells. Wound-healing assays showed inhibition of 5-8F cell migration by EGTA (2 mM), 2-APB (200 μM) and La^{3+} (200 μM); no such effect by nimodipine (20 mM), 2-AP (1 mM), CNQX (10 mM), or SKF96365 (10 mM); and promotion of cell migration by BK (1 μM). ($^{*}P < 0.05$, $^{**}P < 0.01$.)

calcium-induced calcium release (CICR) occurred via the RyR or IP_3R in 5-8F cells. We compared the first response to BK in the absence of ryanodine with the corresponding second BK response after ryanodine pre-incubation. These results showed that ryanodine significantly reduced BK-induced Ca^{2+} peak responses (Fig. 5F, comparing BK1 and BK2; $P < 0.001$) while having no significant effect on BK-induced Ca^{2+} plateau phases. However, different results were obtained by inhibiting the IP_3R with xestospongin C. The corresponding second BK response after xestospongin C pre-incubation was similar with the first BK response (Fig. 5G, $P > 0.05$).

Taken together, these results in 5-8F cells confirm and characterize the mediating effects of TRPM7 channels, both on influx of extracellular Ca^{2+} and release of intracellular Ca^{2+} stores via RyRs, but not IP_3Rs.

4. Discussion

It is well known that calcium ions are of great importance for various cellular processes because they activate or inhibit cellular signal pathways and Ca^{2+}-regulated proteins. These cellular processes include electrical signaling, gene expression, cell proliferation and cell migration. Some Ca^{2+}-mediated signal pathways are implicated in cellular motility [19,20], and the nature of intracellular Ca^{2+} signaling is implicated in the invasion and migration of cancer cells such as MDA-MB-231 human breast cancer cells, A549 lung carcinoma cells, and HT1080 tumor cells [5,21,22].

Four major classes of ion channels mediate the influx of extracellular Ca^{2+}: voltage-operated calcium channels, receptor-operated calcium channels, store-operated calcium channels, and transient receptor potential (TRP) channels [23]. We found that pharmacological inhibitors of the first three types of channels did not affect the migration of 5-8F cells, but migration was significantly inhibited by two inhibitors of TRP channels, TRPM7 RNA interference and extracellular Ca^{2+} chelator EGTA. Conversely, overexpression of TRPM7 significantly increased the migratory ability of 6-10B cells, which are tumorigenic, but lack metastatic ability (Fig. 3). Taking these findings together, we conclude that the TRPM7 channel and the Ca^{2+} influx mediated by TRPM7 might be critical for human nasopharyngeal carcinoma cells migration.

Earlier cellular studies have shown stimulus-induced Ca^{2+} responses that normally consist of two phases: a peak phase abetted by release of Ca^{2+} from intracellular stores, and a plateau phase abetted by influx of extracellular Ca^{2+}. The rapid elevation of Ca^{2+} in the peak phase is particularly relevant for rapid responses when components of the ion reactions and their downstream effectors are closely associated. In contrary, sustained elevation of Ca^{2+} is implicated in the control of many slow processes such as gene transcription, cell proliferation, and cell migration [20,24]. Our evidence showed that the TRPM7 promoter BK induced intracellular Ca^{2+} elevation but this response was totally abolished by the addition of EGTA (Fig. 5C), indicating that extracellular Ca^{2+} is required. Treatment with TRPM7 inhibitors (Fig. 5D and E) or TRPM7 knockdown (Fig. 5A) led to complete or significant inhibition both in the amplitude of the peak and plateau phases of the BK-induced Ca^{2+} response, indicating that an influx of extracellular Ca^{2+} through TRPM7 channel is involved. Pretreatment of 5-8 cells with the RyR inhibitor ryanodine (Fig. 5F) completely inhibited calcium transients but not the affected BK-induced plateau phase of Ca^{2+} response, indicating that this plateau phase was mediated by TRPM7 and these Ca^{2+} transients were mainly from intracellular Ca^{2+} store via RyRs by TRPM7-mediated CICR within the cell. These results are basically concordant of those of Wei et al. [13].

Moreover, these RNAi data showed that downregulated TRPM7 channels significantly decreased the sustained phase of the response much greater than the transient phase (Fig. 4A). We presume that Ca^{2+} influx mediated by 20–30% TRPM7 channels is enough to induce the Ca^{2+} release from ER and thus induce more Ca^{2+} store receptors (RyRs) opening through the CICR mechanism in 5-8F cells. However, this presumption needs future investigation.

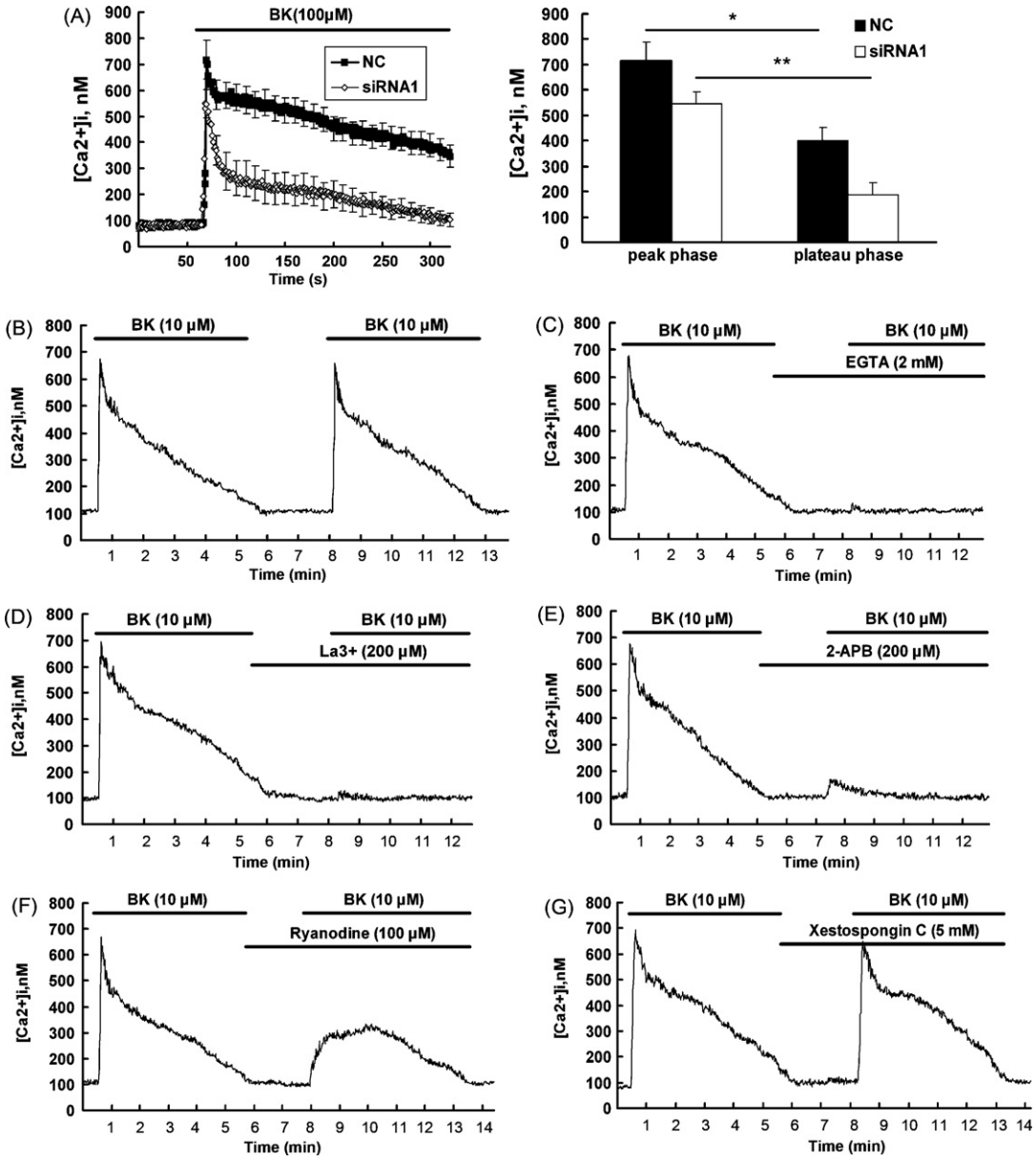

Fig. 5. Fura-2 AM Ca^{2+} imaging of 5-8F cell showed the TRPM7 channel as a potentially major regulator of intracellular Ca^{2+} response. (A) Addition of BK into 5-8F/NC cells triggered a Ca^{2+} increase that consisted of a peak phase and a plateau phase. In contrast, silencing of TRPM7 (siRNA1) significantly decreased the transient phase of the response ($P<0.05$) and decreased the sustained phase much greater ($P<0.01$). Each data-point represents mean value of the $[Ca^{2+}]_i$ signal in 10 cells. Note that the SEM values are shown for every 10th data-point for clarity. (B) The effect of double BK application in control condition. (C) Pretreatment with EGTA completely inhibited both transient and sustained phases. (D and E) Treatments with La^{3+} and 2-APB completely inhibited BK-induced Ca^{2+} response. (F) RyR inhibitor ryanodine completely inhibited BK-induced Ca^{2+} transient responses but not sustained phase. (G) IP_3R inhibitor xestospongin C (5 mM) had no effect on both BK-induced Ca^{2+} transient and sustained phases. The data are shown by one mean curve from average responses of 10–20 cells. Data are either representative of three independent experiments or are shown as mean ± SEM of five experiments. ($^*P<0.05$, $^{**}P<0.01$.)

Recently, Clark et al. [14] have shown that BK can induce a transient calcium signal in cells not expressing TRPM7, while BK activated a more sustained Ca^{2+} entry in TRPM7-expressing cells, indicated that BK, an IP3 agonist, might induce this transient calcium signal from Ca^{2+} store via IP_3R or both of two receptors in mouse N1E-115 cells. Contrary to these results, our RyR and IP_3R inhibitor data (Fig. 4E and F) provided the evidence that CICR could mainly be elicited from RyR but not IP_3R and thus could explain why BK could not induce any transient calcium signal in 5-8F cells without extracellular Ca^{2+}. These results indicated that most of the endoplasmic reticulum in 5-8F cell might behave mostly as single homogeneous caffeine-sensitive Ca^{2+} pools that could release Ca^{2+} mainly via RyRs.

Furthermore, our further pharmacological data (data not shown) from 5-8F and 6-10B cells showed that the emptying of intracellular Ca^{2+} stores by TG could not activate TRPM7 and other SOC channels, indicating that TRPM7 was not a SOC channel and SOC channels might be completely absent in these NPC cells. However, these data are preliminary and more concrete evidence is needed to confirm this presumption in further studies.

Recently, some TRP channels have been identified as prognostic indicators. For example, the TRPM1 channel protein has been used as a specific marker of malignant melanoma, and loss of TRPM1 in highly metastatic cells is considered a prognostic marker for metastasis [9,25]. Conversely, TRPM8 is up-regulated in prostate cancer cells, and assessment of TRPM8 mRNA has therefore been used as a sign of poor prognosis in patients with prostate cancer

[26,27]. Furthermore, recent studies have reported that activation of the TRPM7 channel is critical for growth and proliferation of cancer cells including retinoblastoma [28], breast cancer [29], and malignant head and neck tumor cells [30]. Therefore, increased expression in the TRPM7 channel of NPC cells might represent potential biomarkers of NPC, as well as future therapeutic targets.

In conclusion, this study supports the influx of Ca^{2+} as a requirement for the migration of human NPC 5-8F cells and identifies TRPM7 as a novel regulator not only of Ca^{2+} influx but also of 5-8F and 6-10B cells migration, thus showing TRPM7 as a potential prognostic indicator and therapeutic target for NPC patients.

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