MycN promotes TRPM7 expression and cell migration in neuroblastoma through a process that involves polyamines

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ABSTRACT

Neuroblastoma is an extra-cranial solid cancer in children. MYCN gene amplification is a prognostic indicator of poor outcome in neuroblastoma. Recent studies have shown that the multiple steps involved in cell migration are dependent on the availability of intracellular calcium (Ca$^{2+}$). Although significant advances have been made in understanding the role of Ca$^{2+}$ during migration, little has been achieved towards understanding its impact on the progression of diseases such as cancer. Interestingly, previous studies showed that cancer cell migration is regulated by TRPM7, a calcium-permeable ion channel. The objective of the current study was to elucidate the mechanism by which MycN promotes NB cell migration and the mechanism regulating TRPM7 expression. The results showed that MycN increased TRPM7 expression, induced TRPM7 channel activity, increased intracellular Ca$^{2+}$ signaling, and promoted cell migration in NB cells. The results also showed that inhibition or down-regulation of ornithine decarboxylase (ODC) inhibited TRPM7 expression, a process that was reversed by spermidine. Overall, this study provides evidence that MycN promotes TRPM7 expression and cell migration through a mechanism that involves ODC synthesis of polyamines.

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1. Introduction

Neuroblastoma (NB) is the most common extra-cranial solid tumor that occurs in children [26,32,45]. There is clinical heterogeneity observed between different NB tumors. The majority of localized NBs can be treated with surgical resection alone, and a subset of these tumors may regress spontaneously [34]. However, most patients present with metastatic tumors that have unfavorable biological features and an aggressive phenotype [7,9,45]. The long-term survival rate for such patients remains poor compared to patients with early stages of the disease, despite the availability of intensive multi-modal therapies. Thus, metastatic forms of the disease, with poor prognosis, remain a clinical concern [5,8,32]. MYCN gene amplification has been shown to drive tumor progression and is a negative prognostic indicator for NB [7,32]. MycN, the protein product of the MYCN gene, is a member of the myc family of transcription factors. MycN contains a C-terminal domain with a basic helix-loop-helix motif (bHLHz), and a N-terminal transactivation domain that binds to the promoter region of genes containing E-box sequences, and regulates the expression of those genes [35,55]. MycN was previously shown to transcriptionally regulate ornithine decarboxylase (ODC), a rate limiting enzyme in polyamine biosynthesis [24,28]. MYCN amplification and MycN over-expression has been shown to promote NB cell migration, invasion and metastasis [21,32,41,56,57]. Interestingly, a recent study showed that polyamines regulate NB cell migration [27]. Previous studies have identified genes that are transcriptionally regulated by Myc, whose protein products play a role in modulating these processes, including calcium permeable ion channels [18]. However, the roles of MycN and ODC in the regulation of TRPM7 have not yet been examined.

Calcium is a ubiquitous second messenger that plays an important role in many fundamental physiological processes, such as regulating cell cycle, survival, apoptosis, gene expression, cell migration, invasion and metastasis [1,4,10,16,18,19,30,33–40,43,44,48,50,54]. Recently, calcium-permeable ion channels (e.g., Orai1, Orai3 and TRPM7) and channel regulators (e.g., Stim1 and Stim2) have been shown to play a role in the malignant progression of a variety of cancers (e.g., breast cancer, lung cancer, prostate cancer, glioblastoma, melanoma and cervical cancer).
TRPM7 is a member of the transient receptor potential melastatin family. It is a six transmembrane protein that forms tetramers to create an outwardly rectifying non-selective cation channel that is permeable to calcium, magnesium, sodium and trace levels of zinc. TRPM7 is a channel protein that also contains an intrinsically ser/thr α-kinase domain at the intracellular C-terminal end [2,11–13,17,25,42–46]. Recent studies have elucidated a role for calcium channels and channel regulators in the progression of several cancers. In particular, TRPM7-mediated calcium signaling events have been linked to cell migration, and shown to regulate actin cytoskeletal rearrangement, focal adhesion turnover, actin and myosin contraction, protrusion of lamellipodia at the leading edge of cells and retraction at the trailing edge [10,19,23,37,48]. However, to date, there have been few studies that examine the mechanism regulating the expression of TRPM7. The present study examines whether a relationship exists between MycN, ODC, TRPM7 and cell migration in NB cells. Gaining a better understanding of the mechanisms that regulate NB malignant progression could lead to identification of novel targets for the development of more efficient treatment strategies for NB, in particular advanced stage NB with MYCN gene amplification. The results from the present study suggest that MycN promotes TRPM7 expression and NB migration through a mechanism that involves ODC synthesis of polyamines.

2. Materials and methods

2.1. Cell lines and treatment of cultured cells

All cells were authenticated by the Cell Line Authentication Testing Services at Genetica DNA Laboratories (USA) using STR DNA typing to verify each cell line and verify pure cells (no contamination). The human NB cell lines SK-N-SH (ATCC, USA), IMR-32 (ATCC, USA) h-TERT retinal pigment epithelial cells (provided by Michael D. Hogarty, PA, USA) and MYCN2 (provided by Jason Shohet, TX, USA) were maintained in RPMI-1640 (Mediatech, Inc., Manassas, VA, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA, USA). The MYCN2 cells are SHEP-1 cells with doxycycline inducible MycN over-expression [49]. The hTERT rat pigment epithelial cells (RPE-1) were also used. RPE-1 are non-cancerous cells with tamoxifen-inducible MycN over-expression. Cells in early log-phase were seeded and for MYCN2 cells, doxycycline (100 ng/ml) was added 3 h after seeding. For pharmacological inhibition of ODC1, NB cells were treated with 5 mM α-difluoromethylornithine (DFMO) for 72 h. NB cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

2.2. Western blot analysis

Cell lysates were prepared in RIPA buffer [20 mM Tris–HCl, pH 7.5, 0.1% (w/v) sodium lauryl sulfate, 0.5% (w/v) sodium deoxycholate, 135 mM NaCl, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 2 mM EDTA], supplemented with Complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA), and phosphatase inhibitors sodium fluoride (NaF) (20 mM) and sodium vanadate (Na3VO4) (0.27 mM). Western blot analysis was performed as previously described [28]. The total protein concentration was determined using the protein assay dye reagent from Bio-Rad Laboratories (Hercules, CA, USA). Cell lysates in SDS-sample buffer were boiled for 5 min and equal amounts of total protein analyzed by 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting. The antibodies used in this study are: rabbit monoclonal TRPM7 (at a 1:1000 dilution from Epitomics, Inc. (Burlingame, CA, USA), rabbit polyclonal MycN (at a 1:500 dilution), goat polyclonal ODC (at a 1:500 dilution) and mouse monoclonal GADPH (at a 1:1000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary anti-mouse (at a 1:10,000 dilution) IR-680 or IR-800 (LI-COR Biosciences, Lincoln, NB, USA). Proteins were detected using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NB, USA) and analyzed with Licor Image Studio 2.0 acquisition and analysis software.

2.3. Reverse transcription polymerase chain reaction

RNA was isolated using TRIzol/chloroform (Invitrogen Life Technologies, Grand Island, NY, USA). Template cDNA was generated using GoScript Reverse Transcriptase (Promega, Madison, WI, USA). PCR reactions were performed using GoTaq DNA Polymerase (Promega, Madison, WI, USA). Primer sequences for GADPH were:

- Sense: TTGGTCCACTGGGGGTGCTC
- Antisense: ATGGCCACATGGGGAAGCC

Primer sequences for TRPM7 were:

- Sense: TGAGAAGTGTGGCCTTGA
- Antisense: TGGACAGTGCTTGCTTGA

Primer sequences for Orai3 were:

- Sense: TCCCTGATGTACATCAGTC
- Antisense: AGAAAGATGGTGTGTGTTGA

Primer sequences for Stim1 were:

- Sense: GATGCCGGCCCTGTACCCG
- Antisense: AGAAACCTGGAAATGCCGAGG

Primer sequences for Stim2 were:

- Sense: GTGCTGTGGCCGCTTACCCG
- Antisense: CCAAGGGCAGGTGACCCG

Primer sequences for TRPC1 were:

- Sense: AGTCCGGCCCTGTACCCG
- Antisense: AGAAACCTGGAAATGCCGAGG

Primer sequences for KCa3.1 were:

- Sense: GTGCTGTGGCCGCTTACCCG
- Antisense: CCAAGGGCAGGTGACCCG

Primer sequences for p27 were:

- Sense: AGTCCGGCCCTGTACCCG
- Antisense: AGAAACCTGGAAATGCCGAGG

2.4. Knockdown experiments with siRNA

Cells were transfected with siRNA from Santa Cruz Biotechnology, Inc. The TRPM7-specific siRNA (sc-42662) used in this study were:

sc-42662A:

- Sense: GAGAUGUUGGUGUCCGUAATT
- Antisense: UAAGGAGCAACCACCGUGA

sc-42662B:

- Sense: CCAAUUUGGGCGACGUAAAT
- Antisense: UUCAUCUGACCCAAUUGGTT
sc–42662C:
  - Sense: GCAUAUGUUGCCUGUUAAGAtt
  - Antisense: UCUUACACGGAUCAACUUGCtt

The MycN specific siRNA (sc–36003) used in this study were:
sc–36003A:
  - Sense: CAGCAGCGAUGCUAAAGATT
  - Antisense: UCUUAGCAACUGCGUGUUGTT
sc–36003B:
  - Sense: GGAUGGUCAUACGUAAUATT
  - Antisense: UCUUACGGAUCAACUUGCtt

sc–36003C:
  - Sense: CCUGUAACUUAUGAUGATT
  - Antisense: UCUUACGAACUGCGUGUUGTT

The ODC-specific siRNA (sc–43982) used in this study were:
sc–43982A:
  - Sense: CUUUCAGCCUGCUUAAAtt
  - Antisense: UCUUACGGAUCAACUUGCtt

sc–43982B:
  - Sense: GUGAUUGGACUUCUUGAtt
  - Antisense: UCAAAAGCAAGGGAAGCAtt

sc–43982C:
  - Sense: GGAUUGGGUCAACUUAAtt
  - Antisense: UCUUACGGAUCAACUUGCtt

Note: all sequences are provided in 5′→3′ orientation.
The scrambled control siRNA (sc–36869) was from Santa Cruz.
Briefly, siRNA (40–80 pmol) and Lipofectamine 2000 reagent (10 µl) were diluted, in separate vials, in serum-free RPMI1640. After 5-min incubation at room temperature, the siRNA and Lipofectamine 2000 were mixed together and incubated at room temperature for an additional 30 min, then added to the cells. The medium was exchanged with RPMI1640 supplemented with 10% FBS after overnight incubation. The cells were analyzed at 48 h post-transfection.

2.5. Transwell migration assay

Migration and invasion assays were performed as outlined in the manufacturer’s protocol (Treivant, Gaithersburg, MD, USA). Briefly, transwell plates were either coated with basement membrane extract (BME) solution and allowed to incubate for 4 h at 37 °C in a CO2 incubator, or left uncoated. Serum-starved cells (5×10⁴ cells) were seeded into the top chamber in medium without FBS, while medium with FBS was present in the bottom chamber. The cells were incubated for 24 h. The media and remaining cells were aspirated from the top chamber and washed 2 times with 1× wash buffer. The bottom chamber was aspirated and washed 2 times with 1× wash buffer. Calcein-AM in cell dissociation buffer was added to the bottom chamber, and incubated for 1 h. The top chamber was removed and the fluorescence intensity (calcein-AM labeled cells) was measured at 485 nm excitation (520 nm emission) using a plate reader.

2.6. Immunofluorescent calcium measurements

For immunofluorescence microscopy, cells were grown in 96 well format incubated with Fluo–4 AM (Invitrogen Life Technologies, Grand Island, NY, USA) at 2 µM for 45 min and washed once in PBS containing calcium. Epifluorescence measurements were performed using an Operetta High Content Imaging System (PerkinElmer, Santa Clara, CA, USA). Fluorescence intensity was quantified using Harmony (PerkinElmer, Santa Clara, CA, USA).

2.7. Patch-clamp measurements

Patch-clamp experiments were performed under whole-cell configuration at 21–25 °C using a HEKA EPC10 amplifier. Voltage ramps of 100 ms duration spanning from −100 to +100 mV were delivered at a rate of 0.5 Hz from a holding potential of 0 mV. External solution contained (mM) 140 NaCl, 2.8 KCl, 1 MgCl2, 1 CaCl2, 10 HEPES and 11 mM glucose. The standard internal solution contained (mM) 140 Cs-Glutamate, 8 NaCl, 10 HEPES and 10 BAPTA. 1 mM ATP was added to the internal solution for IMR32 and SK-N-SH experiments. Currents were normalized to cell size in pf. Data points for inward and outward current amplitudes were obtained at −80 and +80 mV respectively. Currents were normalized to the current obtained before development of TRPM7 mediated currents.

2.8. Affymetrix DNA micro-array hybridization and analysis

There are expression profiles for 88 NB tumors in the Affymetrix “NB88” NB tumor dataset, and 649 NB tumors in the “Kocak-649” tumor dataset. Both tumor sets have documented genetic and clinical features. Briefly, total RNA was extracted from frozen NB tumors which contained >95% NB cells, and labeled cRNA was hybridized to Affymetrix Human genome U133 Plus 2.0 microarray (Affymetrix, Santa Clara, CA, USA) as previously described [47]. The NB tumor sets were accessed through the Gene Expression Omnibus (GEO) database at the NCBI website (GEO: GSE16476, GSE45547). The mRNA levels were determined from data image files using MAS5.0 from Affymetrix. Affymetrix probe-sets were selected using the R2 bio-informatic platform (http://r2.amc.nl). The Kocak-649 tumor set was analyzed for correlation between MycN and TRPM7, as the tumor set contained the largest number of non-amplified and MYCN gene amplified NB tumors. The NB88 tumor set was used for Kaplan–Meier graph to analyze the survival prognosis of patients based on high or low expression of TRPM7. Further analysis of the NB88 tumor set was performed in NB tumors to separate alive and not-alive patient samples. The tumors were separated into one group with surviving patients (right panel-alive category was highlighted in green) and those that are not alive (left panel-alive category was highlighted in red), and further divided into MycN amplified tumors and TRPM7 expression. All analyses were performed using R2, an Affymetrix analysis and visualization platform developed in the Department of Oncogenomics at the Academic Medical Center at the University of Amsterdam. R2 can be accessed at: http://r2.amc.nl. Anova, Student’s t-test or Kruskal–Wallis t-tests were used as appropriate. 2logPearson correlation was used to assess statistical significance of correlation of mRNA expression.

2.9. Statistical analysis

In those cases where statistical comparisons were made, paired Student’s t-test was used.
3. Results

3.1. TRPM7 expression correlates with MYCN amplification and lower survival probability

To examine whether MycN correlated with TRPM7 mRNA expression, the Kocak-649 tumor set and the R2 microarray analysis visualization platform were used (http://r2.amc.nl). The mRNA expression for MycN and TRPM7 was measured with Affymetrix profiling. The MYCN gene amplified tumors were separated from the MYCN non-amplified tumors and the mRNA expression of TRPM7 were examined. Fig. 1A shows that TRPM7 was expressed in all tumor samples. However, TRPM7 expression was significantly higher in MYCN amplified tumors, and there is a correlation between MycN expression and TRPM7 expression ($p < 2.3 \times 10^{-7}$). This suggests that MycN expression promotes TRPM7 expression.

Next, we examined the expression of TRPM7 and its clinical relevance in NB. Using a Kaplan–Meier scan analysis, a correlation between TRPM7 mRNA expression and NB patient survival was observed (Fig. 1B). The significant $p$-value was calculated when NB88 tumor set was divided into 31 tumors with low TRPM7 mRNA expression, and 57 tumors with high expression of TRPM7 mRNA. The survival of patients with low TRPM7 expression ($n = 31$) was ~80% for up to 216 months, while the survival of patients with high TRPM7 expression ($n = 57$) dropped to ~50% ($p = 0.02$). The NB88 tumor set was further analyzed for TRPM7 and MYCN expression and patient survival. Fig. 1C shows that 33 NB patients from the NB88 tumor set did not survive. 29 of the 33 patients that did not survive had stage 4 NB (Fig. 1C). 14 out of the 16 patients with MYCN amplified NB tumors did not survive, and had higher TRPM7 expression (Fig. 1C). These data suggest that TRPM7 expression correlates with MycN expression and decreased survival of NB patients.

3.2. MycN regulates TRPM7 expression

Previous studies have shown that MycN over-expression and MYCN gene amplification promotes NB cell migration, invasion and metastasis [41]. In order to identify the channel protein(s) involved in MycN-induced NB cell migration, semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) was performed on tetracycline-inducible MycN over-expressing NB cells, MYCN2 cells. Previous studies have optimized the induction of MYCN2 cells with doxycycline. Cells grown in culture containing doxycycline for 96 h consistently showed significant increases in MycN expression, and expression of down-stream MycN targets. Therefore, the same conditions were used in the current study. MYCN2 cells cultured in media containing doxycycline were designated as MycN-off and MYCN2 cells cultured in media with doxycycline were designated as MycN-on. The mRNA expression of TRPM7 was determined using total RNA extracted from MycN-on and MycN-off cells, and performing semi-quantitative

Fig. 1. (A) TRPM7 gene expression is correlated to MycN expression and patient prognosis. TRPM7 gene expression correlates with MycN expression in NB tumor set, NB88. Illustration of TRPM7 and MycN expression in all 88 NB tumors. There is a direct correlation between TRPM7 and MycN expression with $p < 2.3 \times 10^{-7}$. (B) The Kaplan–Meier graph shows the survival prognosis of patients with tumors that are part of the NB88 tumor set. The survival prognosis is based on high or low expression of TRPM7. Survival probability is significantly higher in patients with low TRPM7 expression, compared to those with high TRPM7 expression ($p < 0.02$). (C) The tumors were separated into one group with surviving patients (right panel-alive category highlighted in green) and those that are not alive (left panel-alive category highlighted in red), and further divided into MycN amplified tumors and TRPM7 expression. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
reverse-transcriptase polymerase chain reaction (RT-PCR). The mRNA expression of Orai3, Stim1, TRPC1 and \( K_{\text{Ca}3.1} \) were higher in MycN-on compared to MycN-off cells. However, the mRNA expression of TRPM7 expression in MycN-on cells was consistently significantly higher than that of MycN-off cells (Fig. 2A). Therefore, in the current study, we focused on investigating the regulation of TRPM7 in NB. In order to confirm the increase in TRPM7 expression at the protein level, whole cell lysates from MycN-on and MycN-off NB cells were analyzed by western blot. In addition, western blot analysis was also performed using whole cell lysates from SK-N-SH and IMR-32 cells, MYCN non-amplified and MYCN gene amplified NB cells, respectively. The results showed that TRPM7 protein expression was significantly higher in MycN-on cells compared to MycN-off cells, and TRPM7 protein expression was significantly higher in IMR-32 cells compared to SK-N-SH cells (Fig. 2B). The band density in each lane of the western blot was quantified, and the level of TRPM7 protein in SK-N-SH, IMR-32, MycN-off, and MycN-on cells was normalized to GAPDH expression. MycN-on cells had \( \sim 4.5 \times \) higher TRPM7 expression than MycN-off cells (Fig. 2C). The TRPM7 expression in IMR-32 cells was \( \sim 9 \times \) higher than SK-N-SH cells (Fig. 2C). The data indicates that there is a correlation between MycN and TRPM7 expression in NB cells.

In order to confirm the regulation of TRPM7 expression by MycN, MycN specific and scrambled siRNA were transfected into IMR-32, NB cells with MYCN gene amplification. Whole cell lysates were prepared from IMR-32 cells transfected with MycN siRNA, scrambled siRNA, and mock transfected cells (transfection reagent only). TRPM7 protein expression was analyzed by western blot. MycN siRNA significantly decreased MycN and TRPM7 expression compared to scrambled siRNA and mock transfected cells (Fig. 2D). In order to provide further evidence that MycN regulates TRPM7 expression, MycN was over-expressed in hTERT-immortalized retinal pigment epithelial cells (RPE-1 cells), a non-cancerous cell line, using a tamoxifen-inducible system. Tamoxifen induced MycN over-expression in RPE-1 cells, and significantly increased TRPM7 expression in RPE-1 cells, compared to RPE-1 cells without tamoxifen-induced MycN over-expression (Fig. 2E). These results show that down- or up-regulation of MycN expression leads to a corresponding decrease or increase in TRPM7 expression, respectively, providing additional evidence that MycN regulates TRPM7 expression in NB cells.

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**Fig. 2.** (A) Semi-quantitative RT-PCR were performed using total RNA extracted MycN-off and MycN-on cells, to assess levels of Orai3, STIM1, STIM2, TRPM7, TRPC1, and \( K_{\text{Ca}3.1} \) expression. P27, GAPDH and water were used as controls. (B) Western blot analysis was performed on whole cell lysates prepared from SK-N-SH, IMR32, MycN-off and MycN-on cells. The membranes were probed for TRPM7 and MycN expression. GAPDH was used as a loading control. TRPM7 (at a 1:1000 dilution from Epitomics, Inc. (Burlingame, CA, USA), rabbit polyclonal MycN (at a 1:500 dilution), and mouse monoclonal GADPH (at a 1:1000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary anti-mouse (at a 1:10,000 dilution) IR-680 or IR-800 (LI-COR Biosciences, Lincoln, NB, USA). The density of the TRPM7 band was quantified and data expressed as mean ± stdev, \( n = 3 \) independent experiments, \( p \leq 0.05 \). (D) Western blot analysis was performed on whole cell lysates from control (CTR) IMR-32 cells that were mock transfected (lipofectamine only), IMR-32 cells transfected with scrambled siRNA (Scr), and IMR-32 cells transfected with MycN specific siRNA (KD MycN). The PVDF membranes were probed for MycN and Full length TRPM7 expression, and GAPDH was used as a loading control. TRPM7 (at a 1:1000 dilution from Epitomics, Inc. (Burlingame, CA, USA)), rabbit polyclonal MycN (at a 1:500 dilution), and mouse monoclonal GADPH (at a 1:1000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary anti-mouse (at a 1:10,000 dilution) IR-680 or IR-800 (LI-COR Biosciences, Lincoln, NB, USA). Secondary anti-mouse (at a 1:10,000 dilution) IR-680 or IR-800 (LI-COR Biosciences, Lincoln, NB, USA). All the data presented were \( n = 3–5 \) independent experiments.
3.3. MycN regulation of TRPM7 expression results in a corresponding increase in TRPM7 channel activity

In order to determine whether MycN-induced TRPM7 expression leads to a corresponding increase in functional TRPM7 channels, electrophysiological measurements of TRPM7 channel activity were recorded using the whole-cell configuration. Characteristically, upon membrane rupture and initiation of current recordings, TRPM7 currents develop over time. TRPM7 current density was examined in MycN-on cells and MycN-off cells at +80 mV (Fig. 3A). TRPM7 mean current densities increased to a peak current of \( \sim 7 \) pA/pF (at \( \sim 500 \) s) in MycN-on cells, compared to \( \sim 4 \) pA/pF currents (at \( \sim 400 \) s) in MycN-off cells (Fig. 3A). The current/voltage relationship (IV curve) was characteristic of TRPM7 channels (Fig. 3B), displaying a large outwardly rectifying current, and a very small inward current. In addition, TRPM7 mean current densities developed to a peak current of \( \sim 15 \) pA/pF in IMR32 cells, compared to \( \sim 4 \) pA/pF in SK-N-SH cells (Fig. 3C). The IV curves were also characteristic of TRPM7, and displayed a larger outward current in IMR32 cells compared to SK-N-SH cells (Fig. 3D). The data suggest that MYCN gene amplification and MycN over-expression significantly increases TRPM7 channel activity under conditions where physiological MgCl\(_2\) (1 mM) was present in the external solution.

In order to confirm that MycN resulted in an increase in functional TRPM7 channels, and that the increased current was mediated by TRPM7, electrophysiological measurements were recorded on NB cells perfused with different concentrations of MgCl\(_2\). MgCl\(_2\) has been shown to be a potent inhibitor of TRPM7-mediated currents. Fig. 3E shows that TRPM7 currents developed in IMR-32 cells to greater extent when external MgCl\(_2\) was kept at 0 mM, and application of 3 mM MgCl\(_2\) effectively abolished TRPM7 currents. Removing the 3 mM MgCl\(_2\) restored TRPM7

![Fig. 3](image-url)

Fig. 3. Electrophysiological measurements were recorded, and TRPM7 current densities are shown in (A) MycN-off and MycN-on cells, (C) IMR-32 and SK-N-SH cells. (B) The red arrows indicate the current/voltage relationship characteristic of TRPM7 was observed in MycN-off and MycN-on cells, (D) IMR-32 and SK-N-SH cells. Data are expressed as mean ± SEM, \( n = 7–20 \). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
3.4. TRPM7 regulates NB cell migration

In order to confirm the role of TRPM7 in regulating NB cell migration, TRPM7 specific siRNA was used to down-regulate TRPM7 expression. TRPM7 specific or scrambled control siRNA was transfected into MYCN2 cells, or cells were mock transfected (transfection reagent only). In order to confirm down-regulation of TRPM7 protein expression, western blot analysis was performed on whole cell lysates from TRPM7 siRNA and scrambled siRNA transfected cells, as well as mock transfected control cells. Fig. 4A shows that TRPM7 expression in MycN-off (Fig. 4A) and MycN-on (Fig. 4B) cells is decreased by TRPM7 siRNA, compared to scrambled siRNA and mock transfection control. The band densities in each lane of the blots were quantified and normalized to GAPDH. TRPM7 siRNA decreased the protein expression of TRPM7 by 36% and 65%, respectively, compared to scrambled control cells (Fig. 4E and F). The results provide further evidence that TRPM7 plays a role in regulating NB cell migration.

3.5. Pharmacological inhibition of TRPM7 currents inhibit NB cell migration

In order to determine the role of TRPM7 in regulating NB cell migration, NB cells were treated with 100 μM 2-aminoethoxydi-phenyl borate (2-APB) or left untreated. 2-APB has been shown to inhibit TRPM7-mediated currents [22]. The TRPM7 channel activity was measured in IMR-32 cells (MYCN gene amplified cells). After TRPM7 currents developed, IMR-32 cells were perfused with 2-APB. Fig. 5A shows that 2-APB application significantly decreased TRPM7 channel activity. The current voltage relationship was characteristic of TRPM7, and indicates that the current was mediated by TRPM7 (Fig. 5B). In addition, 2-APB inhibited NB cell migration by 9.2% and 46.4% in MycN-off and MycN-on cells, respectively (Fig. 5C). These results suggest that TRPM7 channel activity is critical for NB cell migration.

3.6. Polyamines regulate TRPM7 expression

Previous studies showed that MycN regulates ODC expression and activity [20,24,28]. In addition, studies have shown that the irreversible ODC inhibitor, α-difluoromethylornithine (DFMO), significantly depleted intracellular polyamine levels which led to inhibition of NB cell migration, an effect that was attenuated by addition of exogenous polyamines in the culture media [27]. However, the mechanism by which MycN regulates TRPM7 and NB migration remains unknown. To examine the effect of intracellular polyamines on TRPM7 expression, ODC was pharmacologically inhibited by 5 mM DFMO using standard conditions and time-points to optimize the depletion of intracellular polyamines, as described in previous literature [24,25,28,53]. Whole cell lysates from untreated and DFMO-treated cells were analyzed by western blot for TRPM7 expression. DFMO significantly inhibited TRPM7 expression, compared to untreated cells (Fig. 6A). This effect was reversed by supplementing the culture media with 10 μM spermidine during DFMO treatment (Fig. 6A). PMA was used to stimulate ODC however TRPM7 expression was not significantly altered under these conditions. The band densities in each lane of the blots were quantified and normalized to GAPDH. Fig. 6C shows that DFMO increased TRPM7 expression by 27.8% and 65.7% in MycN-off and MycN-on cells, respectively. To further examine and confirm the role of intracellular polyamines in the regulation of TRPM7
ODC1-specific siRNA was used to down-regulate ODC expression. ODC1-specific siRNA effectively down-regulated ODC and TRPM7 expression, compared to scrambled siRNA control (Fig. 6B). The band densities in each lane of the blots were quantified and normalized to GAPDH. ODC siRNA decreased ODC protein expression by 38.4% and 58.7% in MycN-off and MycN-on cells, respectively (Fig. 6D). ODC siRNA also decreased TRPM7 protein expression by 49.1% and 65.1% in MycN-off and MycN-on cells, respectively (Fig. 6D). These results show that intracellular polyamines regulate TRPM7 expression.

4. Discussion

Neuroblastoma (NB) is an extra-cranial pediatric cancer. Metastatic forms of the disease, with poor prognosis, remain a clinical concern [6,7,9,35]. The current study examined the mechanism of...
by which MycN drives these processes in NB by examining the relationship between MycN, ODC, TRPM7 channel, and cell migration in NB cells with different MYCN status (e.g., non-amplified MYCN gene, MYCN gene amplified and MycN over-expression). TRPM7 expression has been shown to promote cell proliferation and metastasis in various cancers [10,16,19,22,36,43,48]. Previous studies have shown that MycN promotes NB migration and invasion by regulating the expression of genes involved in cell migration, invasion and metastasis, including integrin proteins, focal adhesion kinase [3,15,31], ODC and TRPM7 [27,29]. The results from the current study showed that TRPM7 expression correlated with MycN expression, and Kaplan–Meier survival data showed that TRPM7 expression correlated with decreased patient survival. In vitro data demonstrated that TRPM7 mRNA and protein expression was increased in NB cells with MYCN gene amplification (IMR-32), and MycN-on NB cells, compared to non-amplified (SK-N-SH) and MycN-off cells. Next, we showed that MycN specific siRNA effectively decreased MycN and TRPM7 expression. Interestingly, MycN over-expression in RPE-1 cells (a non-cancerous, retinal pigment epithelial cell line) also resulted in an increase in TRPM7 expression. Therefore, the expression of MycN promotes the expression of TRPM7 in non-cancerous and cancer cell lines. The data from the current study provide strong evidence that up- or down-regulation of MycN induces a corresponding increase or decrease in TRPM7 expression. In addition, electrophysiological measurements showed increased TRPM7 currents (I_\text{TRPM7}) in cells with MYCN gene amplification and over-expression of MycN, compared to cells with single copy of the MYCN gene. The current/voltage relationship (IV curve) was characteristic of TRPM7 channels (Fig. 2B), displaying a large outwardly rectifying current, when positive potentials promotes the transport of mainly monovalent ions out of the cell, and a very small inward current representing the transport of divalent cations (e.g., calcium and magnesium) from the extracellular space into the cells down their concentration gradients. This specific IV relationship is only observed with TRPM7 channels. The IV relationships of other TRP channels involve a more linear curve at both negative and positive potentials and/or less outward rectification. In addition, the TRPM7 current was completely abolished by 3 mM MgCl₂, which has been established as a potent blocker of the TRPM7 channel activity [2]. These data provide strong evidence that MycN increases TRPM7 expression and consequently induces an increase in TRPM7 channel activity. This is the first study that shows a relationship between MycN and TRPM7 expression and channel activity. However, it is not known whether MycN regulates TRPM7 directly, or through an indirect mechanism. Review of current literature and data from Myc target databases revealed that TRPM7 is not a transcriptional target of MycN. However, it has been shown that ODC is a transcriptional target of MycN in NB cells. A recent study demonstrated that the irreversible inhibitor of ODC, DFMO, depleted intracellular polyamine levels and significantly inhibited NB cell migration, effects that were attenuated by supplementing external media with exogenous polyamines. The results from the study suggests that polyamines regulate NB cell migration. However, the mechanism by which polyamines regulate NB cell migration remain unknown. Therefore, the current study investigated whether or not intracellular polyamines act down-stream of MycN to regulate TRPM7 expression. Previous studies have established that 5 mM DFMO effectively decreased ODC activity and depleted intracellular polyamine levels in NB cells [20,28,53]. The same conditions were employed in the current study. DFMO and ODC specific siRNA effectively decreased TRPM7 protein expression in NB cells. The results suggest that the mechanism by which MycN regulates TRPM7 expression involves intracellular polyamines. Polyamines, at physiological pH, are positively charged, and are able to bind to DNA, RNA and protein. Consequently, polyamines have been shown to regulate DNA replication, transcription, translation and protein function. Due to the fact that MYCN upregulates TRPM7 expression, at both the mRNA and protein level, suggests that the regulation may occur at the level of transcription. The mechanism by which MycN promotes NB migration may involve MycN-induced polyamine synthesis, polyamine-mediated up-regulation of TRPM7 expression and increased TRPM7 channel activity. These events appear to play a critical role in regulating NB cell migration.

TRPM7 is a bi-functional protein that contains an ion channel fused to an α-kinase domain at the C-terminal end. TRPM7 has been shown to modulate focal adhesion number, cell–cell adhesion, actomyosin contractility, invadopode formation and directional cell movement. In the current study, NB cell migration was inhibited by TRPM7 specific siRNA providing further evidence that MycN potentiation of NB cell migration may occur through a mechanism that involves TRPM7. Previous studies have shown that cleavage of the TRPM7 kinase domain plays a role in regulating NB migration. TRPM7 kinase mediates phosphorylation of myosin IIHA, regulating actomyosin contraction during cell migration [14,52]. A recent study showed that TRPM7 regulates the formation of invadopodes and focal adhesions independent of TRPM7-mediated calcium influx. The current study focused on the regulation of the TRPM7 channel expression by MYCN, events that do not involve the TRPM7-kinase but are also required for NB cell migration.

In conclusion, the results from the present study suggests that MYCN transcriptionally regulates ODC expression and increases polyamine biosynthesis, which leads to increased TRPM7 expression, increased TRPM7 channel activity, and increased intracellular calcium, all of which ultimately promotes NB cell migration. This is the first study demonstrating a link between the prognostic factor, MycN, and NB malignant progression with a calcium channel, in this case TRPM7. In addition, this study provides evidence that MycN regulates TRPM7 through a mechanism that involves intracellular polyamines and ODC.

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