The Mg\textsuperscript{2+} transporter MagT1 partially rescues cell growth and Mg\textsuperscript{2+} uptake in cells lacking the channel-kinase TRPM7

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**Abstract**

Magnesium (Mg\textsuperscript{2+}) transport across membranes plays an essential role in cellular growth and survival. TRPM7 is the unique fusion of a Mg\textsuperscript{2+} permeable pore with an active cytosolic kinase domain, and is considered a master regulator of cellular Mg\textsuperscript{2+} homeostasis. We previously found that the genetic deletion of TRPM7 in DT40 B cells results in Mg\textsuperscript{2+} deficiency and severe growth impairment, which can be rescued by supplementation with excess extracellular Mg\textsuperscript{2+}. Here, we show that gene expression of the Mg\textsuperscript{2+} selective transporter MagT1 is upregulated in TRPM7\textsuperscript{-/} cells. Furthermore, overexpression of MagT1 in TRPM7\textsuperscript{-/} cells augments their capacity to uptake Mg\textsuperscript{2+}, and improves their growth behavior in the absence of excess Mg\textsuperscript{2+}.

**1. Introduction**

As the most abundant intracellular divalent cation, Mg\textsuperscript{2+} is essential for a multitude of physiological processes, including protein synthesis and cell proliferation [1,2]. Transport of Mg\textsuperscript{2+} across membranes plays an important role in maintaining cellular equilibrium [3], yet the molecular mechanisms underlying vertebrate Mg\textsuperscript{2+} homeostasis have just begun to be characterized [4,5]. In this study we focus on the ability of one Mg\textsuperscript{2+} transporter, MagT1 (Magnesium transporter subtype 1), to complement the deletion of TRPM7 (Transient Receptor Potential Melastatin 7), an ion channel shown to play a major role in regulating cellular Mg\textsuperscript{2+} homeostasis [5,6].

TRPM7 and its closest relative, TRPM6, are the only examples of ion channels covalently linked to a kinase domain. Biophysical studies have shown that TRPM7 is a channel selective for divalent cations and can be inhibited by intracellular Mg\textsuperscript{2+} or MgATP [7]. The genetic disruption of TRPM7 in DT40 B cells results in reduced intracellular Mg\textsuperscript{2+} levels and cell growth arrest under physiological concentrations of Mg\textsuperscript{2+} (0.5–1 mM). Importantly, this phenotype can be reversed by supplementation with high (10 mM) extracellular Mg\textsuperscript{2+}, but not by adding Ca\textsuperscript{2+}, Mn\textsuperscript{2+}, Zn\textsuperscript{2+}, or Ni\textsuperscript{2+} to the culture media [8]. Similarly, patients deficient in TRPM6 suffer from lethal seizures, but can live normally if nutritionally supplemented with Mg\textsuperscript{2+} [9,10]. Others have demonstrated that in *C. elegans* and zebrafish, TRPM7 homologues are also involved in Mg\textsuperscript{2+} regulation [11,12]. TRPM7 deficiency in mice is embryonic lethal, though rescue by supplemental Mg\textsuperscript{2+} was not tested in this study [13]. Recently, a different group published that embryonic stem cells lacking TRPM7 kinase show minimal TRPM7 channel activity and a proliferation arrest phenotype that, similar to the TRPM7-deficient DT40 cells, can be rescued by Mg\textsuperscript{2+} supplementation [14].

Currently, the only known selective plasma membrane Mg\textsuperscript{2+} transporter is MagT1. It has been demonstrated to be essential for vertebrate Mg\textsuperscript{2+} influx [3], and is broadly expressed in human tissues [15]. It was originally discovered as an upregulated transcript in mouse distal convoluted tubule (MDCT) cells grown under hypomagnesic conditions [16]. MagT1 was also recently captured in a screen designed to identify human molecules that can complement for the deficiency in the Alr1p Mg\textsuperscript{2+}-transporter in yeast [15]. Knockdown of MagT1 leads to early developmental arrest in zebrafish embryos, and excess supplemental Mg\textsuperscript{2+} was shown to rescue this developmental block [15]. These studies and others also revealed that varying concentrations of extracellular Mg\textsuperscript{2+} can regulate expression of MagT1 in cell lines and murine tissues [17,18].

Given these reports, we asked whether MagT1 levels are increased in TRPM7\textsuperscript{-/} DT40 cells, possibly to help compensate for the negative effect of TRPM7 deficiency on intracellular [Mg\textsuperscript{2+}], and show here that this is indeed the case. Furthermore, we find that overexpression of recombinant MagT1 in TRPM7\textsuperscript{-/} cells par-
tially rescues the ability of these cells to uptake Mg²⁺, as well as their growth defect in the absence of Mg²⁺ supplementation. Our results lend further credentials to the notion that MagT1 is a potent Mg²⁺ transporter pathway in vertebrates.

2. Materials and methods

2.1. Molecular biology

The coding sequence of chicken MagT1 was extracted from DT40 B cell cDNA using oligos designed from the gallus gallus MagT1 published sequence (GenBank ID: NM_001006435) 5'-AC GTGGTACCCATTAGGAAGTCTATGATGATATCC-3' and 5'-ACGTA AGCTATGGCGCCGCTGACTTTTG-3'. Chicken MagT1 was then cloned into the pcDNA4/TO-construct encoding C-terminally FLAG-tagged transporter.

2.2. DT40 cell line construction, cell culture, and growth curves

The generation of DT40 TRPM7⁻/⁻ cells, the cloning of human TRPM7 WT, and the establishment of stable inducible expression of hTRPM7 in TRPM7⁻/⁻ DT40 cells have been previously described [8]. TRPM7⁻/⁻ DT40 cells expressing the tet-repressor were transfected with a pcDNA4/TO-construct encoding C-terminally FLAG-tagged chicken MagT1. Cells were maintained in chemically defined HyQ CCM1 media supplemented with 10 mM MgCl₂, P/S, Glu, 1% chicken serum, and 1 mg/ml Zeocin (Invitrogen), 50 μg/ml Blasticidin (Invivogen). Growth curves were generated using customized Mg²⁺ free CCM1 media supplemented with 0.5 mM MgCl₂ and 1% chicken serum.

2.3. Real-time quantitative PCR analyses

DT40 WT and TRPM7⁻/⁻ cells were cultured in media containing 0 mM or 10 mM MgCl₂ for 24 h. Total RNA was isolated from cells using RNeasy (Qiagen) and converted to cDNA using Superscript III RT (Invitrogen). Quantitative RT-PCR was carried out using Sybr-Green Master Mix and DNA Engine Opticon 2 (BioRad formerly MJ Research). Primers were used for detection of MagT1 (For 5'–GTTGACATATGCGGAGTGAAC-3' and Rev 5'–TCCTAAAGTAA CACCACCTG-3') and GAPDH control (For 5'–TGTTTTCTGGTAT GCAATGACTT-3', Rev 5'–CTCACTCGTGATGCATGCT-3').

2.4. Immunoblotting

Stable cell lines expressing MagT1 were lysed in 0.5% Triton X lysis buffer, and proteins immunoprecipitated using anti-Flag M2 agarose beads at 4°C overnight under constant rotation. Beads were washed 3× with lysis buffer and proteins were eluted by boiling in 2x sample buffer. Proteins were separated by SDS-PAGE using 12.5% polyacrylamide gels, transferred to a polyvinylidene difluoride membrane, and analyzed by anti-FLAG (Sigma) immunoblotting.

2.5. Mg²⁺ measurements

Intracellular concentrations of Mg²⁺ were measured using a QM-6/2003 spectrophotometer (Photon Technology International). As previously described [19], cells were grown in chemically defined media supplemented with 10 mM MgCl₂ and when required for induction of gene expression, treated with doxycycline 48 h before being placed in 0.5 mM MgCl₂. At 14–18 h, 4×10⁶ cells per sample were loaded with 1 μg/ml Mag-Flura-2 (Invitrogen) for 30 min at room temperature in 0.5 mM MgCl₂ buffer (135 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 5.6 mM Glucose, 10 mM Hepes, adjusted pH to 7.5, then add 0.1% bovine serum albumin). The cells were washed, placed in a cuvette with a stirbar, and measured every 2 s at 340/380 nm excitation accompanied by monitoring 510 nm emission light for 10 min, with 9.5 mM or 19.5 mM of MgCl₂ added directly into the cuvette at t = 40 s. The area under the curve was calculated by averaging the fluorescence-ratio (F340/F380) of the first 39 s (before MgCl₂ addition) to obtain baseline average. The baseline was subtracted from each timepoint in the indicated response (40–600 s) and these values were summed and normalized to TRPM7⁻/⁻. The SEM was calculated for each group and a two-tailed t-test performed on four separate experiments.

3. Results

3.1. TRPM7⁻/⁻ DT40 cells show higher MagT1 gene expression levels than wildtype cells

TRPM7⁻/⁻ DT40 cells become Mg²⁺ deficient and die unless their culture medium is supplemented with 10–20 fold higher [Mg²⁺], than the normal 0.5–1 mM physiologic levels provided to DT40 wildtype (WT) cells. Since it has been previously demonstrated that MagT1 is upregulated under hypomagnesic conditions, we hypothesized that TRPM7 deficiency might also elicit an increase in MagT1 gene expression. To test this idea, using quantitative RT-PCR, we analyzed chicken MagT1 specific transcript levels in WT vs. TRPM7⁻/⁻ DT40 cells under varying Mg²⁺ levels.

Since TRPM7⁻/⁻ cells need to be maintained in 10 mM MgCl₂ to ensure their survival, we cultured the WT cells under the same conditions for better comparison. As shown in Fig. 1, we detect MagT1 gene expression in WT DT40 cells at 10 mM MgCl₂, and its level is elevated when the cells are cultured in Mg²⁺ free medium for 24 h (P < 0.05). In the same experiment, we also found that MagT1 gene expression is further increased in TRPM7⁻/⁻ cells, even in 10 mM MgCl₂, but most substantially when TRPM7⁻/⁻ cells were deprived of Mg²⁺ (P < 0.01 and P < 0.001 respectively, as compared to WT DT40s under the same Mg²⁺ conditions). We therefore conclude from these results that in an effort to compensate for the Mg²⁺ deficiency caused by the absence of TRPM7, TRPM7⁻/⁻ cells have been previously described [8].

3.2. Complementation with MagT1 partially restores growth in TRPM7⁻/⁻ cells under physiologic extracellular [Mg²⁺]

Based on the observations that TRPM7⁻/⁻ cells exhibit higher levels of MagT1-transcript, we asked the question whether overex-
expression of recombinant MagT1 would allow TRPM7−/− cells to grow under physiologic concentrations of Mg2+ (0.5–1 mM). To this aim, chicken MagT1 cDNA was isolated from WT DT40 cells and cloned into an expression vector that adds a C-terminal Flag-tag for easy detection of the overexpressed protein by immuno-Western blot. We subsequently transfected TRPM7−/− DT40 cells cultured in 10 mM MgCl2 with the MagT1 construct, selected for zeocin-resistant clones, and screened for those with stable expression of flag-tagged MagT1. Two MagT1-expressing clones are shown in Fig. 2A, and clone #2 was used for subsequent experiments.

In order to monitor the growth of the MagT1-overexpressing TRPM7−/− DT40 cells in comparison to TRPM7−/− and WT DT40s, all cell lines were grown in parallel for 6 days in chemically defined media containing either 10 mM or 0.5 mM MgCl2. As anticipated, these cell lines show similar growth behavior under 10 mM MgCl2 (not shown to improve graph clarity). However, at 0.5 mM MgCl2, as previously reported, TRPM7−/− cells do not grow [8], whereas growth is partially restored in TRPM7−/− cells overexpressing MagT1, albeit not to WT levels. Thus, overexpression of the MagT1 Mg2+ transporter is beneficial to DT40 cells lacking TRPM7, although given the different biological properties of these two molecules, it is perhaps not surprising that the complementation is only partial.

3.3. Overexpression of MagT1 increases intracellular Mg2+ recovery in TRPM7−/− cells

To further characterize the functional impact of MagT1 overexpression in TRPM7−/− cells, we wanted to investigate whether MagT1 alters the capacity of TRPM7−/− cells to uptake Mg2+. We therefore performed Mg2+ add back experiments in our DT40 cell lines cultured under hypomagnesic conditions, and monitored changes in cytosolic free Mg2+ levels by loading the cells with the Mg2+ sensitive fluorescent dye Mag-Fura-2, as previously described [19]. All cells were preincubated in 0.5 mM MgCl2 for 14–18 h, and cytosolic Mg2+ was measured after the addition of either

![Fig. 2. MagT1 overexpression partially reverses growth defect of TRPM7−/− DT40 cells. (A) Western Blot showing stable expression of Flag-tagged MagT1 in TRPM7−/− cell clones. Flag-MagT1 was immunoprecipitated using anti-Flag M2-agarose beads, and blot was probed with anti-Flag antibody. TRPM7−/− parental cell line was used as the negative control. Positive clone #2 was selected for all subsequent experiments. (B) Growth curves recorded in parallel of the following DT40 cell lines: Wildtype, TRPM7−/−, or TRPM7−/− overexpressing MagT1, all grown in the presence of 0.5 mM MgCl2 for 6 days. Dotted line indicates cell death.](image1)

![Fig. 3. Overexpression of MagT1 increases intracellular Mg2+ recovery in TRPM7−/− cells. (A) Changes in free cytosolic Mg2+ in DT40 wildtype cells in comparison to TRPM7−/− DT40 cells complemented with TRPM7 or MagT1. All cell lines were cultured under hypomagnesic conditions (0.5 mM MgCl2) for 14–18 h, and then loaded with the Mg2+ sensitive fluorescent dye Mag-Fura-2 for 30 min in a 0.5 mM MgCl2 buffer, and samples analyzed using a fluorometer. 9.5 mM MgCl2 were added to the samples as indicated by the black filling in the bar above the graph. Shown trace is representative of four separate experiments. (B) Experimental design as in A, except that 19.5 mM MgCl2 were added. Data is representative of six separate experiments. (C) Quantification of the results presented under A and B where bars represent mean area under the curve normalized to TRPM7−/− cells at 10 mM final concentration. For statistical analysis, results were compared to values obtained from TRPM7−/− cells at the corresponding MgCl2 concentrations. Data shown are from 4 individual experiments, the mean ± SEM; **P < 0.01.](image2)
9.5 mM (Fig. 3A), or 19.5 mM MgCl₂ (Fig. 3B). Notably, baseline fluorescence ratio in TRPM7/C0 cells already appear to reflect lower levels of intracellular free Mg²⁺ than in cells complemented with MagT1 or TRPM7. Following 14–18 h at 0.5 mM MgCl₂, TRPM7/C0 cells show the least cytosolic Mg²⁺ recovery when MgCl₂ is added, as previously published [8]. When complemented with MagT1, Mg²⁺ uptake is increased in TRPM7/C0 cells, and as would be expected, Mg²⁺ uptake is strongest when TRPM7 is re-introduced into these cells. The addition of 19.5 mM instead of 9.5 mM MgCl₂ results in similar effects, but more pronounced, indicating that the amplitude of the Mg²⁺ uptake correlates with the amount of added extracellular MgCl₂ (Fig. 3B and C). In sum, these results show that the improved growth behavior of MagT1 complemented TRPM7/C0 DT40 cells is accompanied by an amelioration of the Mg²⁺ uptake capacity of these cells, suggesting that this is the mechanism by which MagT1 compensates for the absence of TRPM7.

4. Discussion

In this study, we demonstrate that cells lacking the regulator of Mg²⁺ homeostasis, TRPM7, increase gene expression of the Mg²⁺ transporter MagT1, indicating that MagT1 function might be beneficial in situations of TRPM7 deficiency. This notion is further supported by our results showing that overexpression of MagT1 enables TRPM7/C0 cells to regain viability and some growth under normal levels of Mg²⁺. This is in contrast to TRPM7/C0 cells that require extracellular Mg²⁺ levels an order of magnitude higher than physiologic concentrations.

Our findings about MagT1 gene expression regulation are reminiscent of work done in HEK and MDCT cells where MagT1 expression was also upregulated in response to hypomagnesia [15,16]. Together with our data, these studies suggest that when cells are sensing suboptimal extracellular Mg²⁺, they respond by transcriptional upregulation of MagT1, probably in an effort to avoid intracellular Mg²⁺ deficiency by increasing Mg²⁺ uptake. Importantly, TRPM7 itself is not required to mediate this hypomagnesia-induced MagT1 upregulation given that this takes place in DT40 cells deprived of TRPM7. Since TRPM7 is described as a master regulator of Mg²⁺ regulation, it could be required to sense environmental Mg²⁺ availability and elicit upregulation of cellular Mg²⁺ transport capacity, but this appears not to be the case for MagT1. From our review of the literature, MagT1 gene expression does not appear to be uniformly regulated in all tissues. Other studies have shown that in low concentrations of Mg²⁺, mammary epithelial MagT1 expression remains unchanged [20], while rumen epithelial cells actually downregulate MagT1 expression [17,18]. These results imply that there may be differential regulation of Mg²⁺ and its transport machinery in various cell types.

We observed that overexpression of MagT1 in TRPM7/C0 cells is capable of augmenting growth and increasing cytosolic Mg²⁺ recovery in the absence of supplemental Mg²⁺. This suggests that MagT1 enhances Mg²⁺ accumulation in TRPM7/C0 cells, and supports the role of MagT1 as a Mg²⁺ influx system. These results further establish TRPM7/C0 DT40 cells as a valid model system to test functionality of Mg²⁺ transport pathways. This experimental approach has been used previously to demonstrate that SLC41A2, another putative Mg²⁺ transporter, can indeed function as such. Similarly to MagT1, SLC41A2 restores partial growth of the TRPM7/C0 cells under normomagnesic conditions [21]. In the past, we have also used this system to demonstrate that the closest homologue of TRPM7 closest homologue and only other known channel-kinase fusion, TRPM6, cannot functionally compensate for TRPM7 in DT40s [19]. Together, the only partial TRPM7-complementations reported with either SLC41A2 or MagT1 suggest that TRPM7 remains essential for DT40s to proliferate and accrue Mg²⁺ when grown in physiological concentrations of Mg²⁺. Future studies will need to determine whether overexpression of multiple Mg²⁺ transporters in the absence of TRPM7 can lead to a complete rescue of the growth phenotype without supplemental Mg²⁺. In summary, our data suggest that MagT1 is an integral component of Mg²⁺ uptake in TRPM7/C0 DT40s.

Acknowledgements

This work was supported by National Institutes of Health Pre-doctoral Traineeship (NIH T32 AI07405), and by grants R01GM068801 (ALP), K08AI060926 and R01GM09123 (CS).

References