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Deletion of *Trpm7* Disrupts Embryonic Development and Thymopoiesis Without Altering Mg^{2+} Homeostasis

Jie Jin^{1,2,*}, Bimal N. Desai^{1,*}, Betsy Navarro¹, Adriana Donovan², Nancy C. Andrews^{2,3}, and David E. Clapham^{1,†}

¹ Department of Cardiology, Howard Hughes Medical Institute, Children's Hospital Boston, and Department of Neurobiology, Harvard Medical School, Enders Building 1309, 320 Longwood Avenue, Boston, MA 02115, USA

² Division of Hematology and Oncology, Children's Hospital Boston, Karp Family Building 8-125A, Boston, MA 02115, USA

³ Department of Pediatrics and Department of Pharmacology and Cancer Biology, Duke University School of Medicine, Durham, NC 27702, USA

Abstract

The gene *transient receptor potential-melastatin-like 7* (*Trpm7*) encodes a protein that functions as an ion channel and a kinase. TRPM7 has been proposed to be required for cellular Mg^{2+} homeostasis in vertebrates. Deletion of mouse *Trpm7* revealed that it is essential for embryonic development. Tissue-specific deletion of *Trpm7* in the T cell lineage disrupted thymopoiesis, which led to a developmental block of thymocytes at the double-negative stage and a progressive depletion of thymic medullary cells. However, deletion of *Trpm7* in T cells did not affect acute uptake of Mg^{2+} or the maintenance of total cellular Mg^{2+} . *Trpm7*-deficient thymocytes exhibited dysregulated synthesis of many growth factors that are necessary for the differentiation and maintenance of thymic epithelial cells. The thymic medullary cells lost signal transducer and activator of transcription 3 activity, which accounts for their depletion when *Trpm7* is disrupted in thymocytes.

The transient receptor potential (TRP) superfamily comprises cation-permeant ion channels that have diverse functions (1–3). TRPM7 (1,2) and TRPM6 (4,5) proteins also contain a C-terminal kinase domain (6). TRPM7 is expressed in all examined cell types (3) and mediates the outwardly rectifying Mg^{2+} -inhibitable current (MIC) (7). TRPM6 and TRPM7 exhibit nearly identical current-voltage (*I-V*) relations, conducting only a few pA of inward current at physiological pH levels (1,2,8,9).

A chicken DT-40 B cell line targeted for *Trpm7* gene disruption was reported to require high concentrations of extracellular Mg^{2+} (10 mM) for survival (10). Given the permeability of TRPM7 to Mg^{2+} , the results have been interpreted to indicate that TRPM7 was critical for cellular Mg^{2+} homeostasis in vertebrates. A role for TRPM7 in vertebrate development was suggested by a *Danio rerio* *Trpm7* mutant that exhibited abnormal skeletogenesis and melanophore development, but whether this developmental defect is related to Mg^{2+} homeostasis remains unclear (11).

We generated multiple mouse lines with a targeted deletion of the *Trpm7* gene (fig. S1A) (12). Mouse lines with disruption of *Trpm7* in all tissues (global deletion), generated using

[†]To whom correspondence should be addressed. E-mail: dclapham@enders.tch.harvard.edu.

*These authors contributed equally to this work.

three different approaches, did not yield any live *Trpm7^{null/null}* animals. Mendelian ratios of littermate genotypes indicated that *Trpm7^{null/null}* mice died prenatally (194 pups analyzed) (fig. S1, D and E). To rule out the possibility that embryonic viability was compromised by the disruption of maternoembryonic transport, we deleted *Trpm7* by using paternal *Sox-2 Cre*, which deletes the gene in the embryonic cells but not in extra-embryonic visceral endoderm (13). Eight litters from this mating scheme failed to produce *Trpm7^{-fl}* (*Sox-2 Cre*) mice, indicating that embryonic lethality resulted from a requirement for TRPM7 in the developing embryo rather than a compromise of materno-embryonic nutrient transport. For further analysis, we used mice in which a β -geo cassette (coding for β -galactosidase) was inserted in the first intron of *Trpm7* (*Trpm7^{geo}*) (12). The β -geo transcript has a splice acceptor site but not a splice donor site; thus, barring alternative splicing, *Trpm7^{geo}* generates a null allele of *Trpm7*. We isolated and analyzed embryos from *Trpm7^{geo/+}* intercrosses at various times after fertilization and determined that *Trpm7^{null/null}* embryos died before day 7.5 of embryogenesis (E7.5) (fig. S1F). LacZ staining of *Trpm7^{geo/+}* embryos revealed a predominant expression in the fetal heart at E9.5, followed by a gradual and intense expansion of the expression across the ventral region at E10.5, peaking throughout the embryo at E11.5 and E12.5. The broad expression pattern was maintained through E14.5 (fig. S1G). Thus, TRPM7 is expressed in embryonic stem cells (fig. S1B), expression is increased in the early embryo, and the expressed TRPM7 has a nonredundant and vital role in the embryonic development of the mouse.

Using *lck-Cre* mice, we selectively deleted *Trpm7* in developing thymocytes. Deletions of *Trpm7*-exon 17, in thymocytes and mature T-lymphocytes isolated from *Trpm7^{-fl}* (*lck-Cre*) mice, were confirmed by reverse transcription polymerase chain reaction (RT-PCR) (fig. S2A) and quantified using quantitative RT-PCR directed against exon 17. Unmodified transcripts in thymocytes ($8.9 \pm 0.28\%$ of normal) were lower than in T lymphocytes ($16.8 \pm 0.96\%$ of normal) when compared with those in wild-type (*wt*) cells, which probably reflected the presence of contaminating cells of non-T cell lineage in Thy-1.2-directed immunoaffinity preparations from mouse spleens.

Whole-cell patch-clamp recordings revealed that MIC current (I_{MIC}) in thymocytes was potentiated by extracellular application of 10 mM NH_4Cl (Fig. 1A) and reversibly inhibited by 10 mM MgCl_2 (Fig. 1A) or 100 μM 2-aminoethoxydiphenylborate (2-APB) (fig. S3A) (7, 14). I_{MIC} was abrogated in T cells derived from *Trpm7^{-fl}* (*lck-Cre*) mice (Fig. 1, B and C), whereas K^+ currents ($\text{K}_v1.3$, $\text{K}_v3.1$, and $\text{K}_{Ca}3.1$) (15–18) were unaffected (fig. S3B). I_{MIC} was still present in a small population of T cells, probably because of incomplete *Cre* expression and inadvertent patch clamping of Thy-1.2+ splenocytes other than T lymphocytes.

We used a cell-permeable fluorescent indicator [KMG104AM (19)] to evaluate Mg^{2+} influx in freshly isolated T cells from *wt* and *Trpm7^{-fl}* (*lck-Cre*) mice. T lymphocytes incubated with KMG104AM and maintained in a Mg^{2+} -free medium responded with an increased fluorescence intensity to extracellular perfusion with solutions containing 10 mM MgCl_2 but not to solutions containing 10 mM CaCl_2 (fig. S3C). Mg^{2+} influx in *wt* T lymphocytes was insensitive to 0.5 mM 2-APB (Fig. 1D). Similarly, intracellular alkalization induced by extracellular 50 mM NH_4Cl , which potentiates I_{TRPM7} (14), did not result in higher Mg^{2+} influx (fig. S3D). Mg^{2+} influx in thymocytes freshly isolated from *Trpm7^{-fl}* (*lck-Cre*) mice was insensitive to deletion of TRPM7 (Fig. 1F), which indicates that TRPM7 does not mediate the observed Mg^{2+} influx in T cells. To test the tissue Mg^{2+} -dependence on TRPM7, we used inductively coupled plasma mass spectrometry (ICP-MS) to measure total Mg^{2+} in freshly isolated T cells. The total $[\text{Mg}^{2+}]$ in T cells obtained from *wt* and *Trpm7^{-fl}* (*lck-Cre*) mice were not statistically different (Fig. 1E). These data indicate that TRPM7 is not essential for cellular Mg^{2+} homeostasis in mice.

In the intestine of adult *Trpm7^{-fl} (lck-Cre)* mice, T lymphocytes were readily detected at a density comparable to that of *wt* mice (fig. S7), whereas a small reduction in T cell density was evident in the lymph nodes (fig. S8) and spleen (fig. S4B) of *Trpm7^{-fl} (lck-Cre)* mice. Flow cytometry of splenocytes isolated from *wt* and *Trpm7^{-fl} (lck-Cre)* mice revealed a small reduction in the percentage and numbers of T cells but not of B cells (fig. S4, C and D). Despite the decrease in the splenic T cell numbers, the results show that mature T lymphocytes in *Trpm7^{-fl} (lck-Cre)* mice are able to survive and populate the periphery.

The thymi in *Trpm7^{-fl} (lck-Cre)* mice developed morphological abnormalities with an 85% phenotypic penetrance ($n = 27$ mice), which suggests defective thymopoiesis. Histology of thymic sections derived from 12-week-old *Trpm7^{-fl} (lck-Cre)* and *Trpm7^{+/fl}* littermate controls showed abnormal thymic architecture in *Trpm7^{-fl} (lck-Cre)* mice (Fig. 2A). The boundary between cortical and medullary areas was easily visible in *wt* thymi but not in *Trpm7*-deficient thymi (Fig. 2A and fig. S6A). In contrast to *wt* thymi, where the CD3+ T cells remained confined to the medulla (outlined and marked as T), the CD3+ cells in the thymi of *Trpm7^{-fl} (lck-Cre)* mice were uniformly distributed across the thymic stroma (Fig. 2B and fig. S6B). Evaluation of thymic cellularity indicated a substantial reduction in the number of thymocytes in *Trpm7^{-fl} (lck-Cre)* mice (Fig. 2C).

Thymocytes from *Trpm7^{-fl} (lck-Cre)* mice contained a higher percentage (Fig. 2D) and number (Fig. 2E) of CD4⁻ CD8⁻ [double negative, (DN)] cells than did thymocytes from *wt* controls, which indicates a partial developmental block in transition from the DN to double-positive (DP) stage. This developmental defect may account for the reduced number of T cells in *Trpm7^{-fl} (lck-Cre)* mice. Analysis of the DN population based on the cell-surface expression of CD44 and CD25 revealed a significantly higher percentage of DN thymocytes in the DN3 (CD44⁻ CD25⁺) stage (Fig. 3A), which indicates a failure to down-regulate CD25 expression during T cell development. Because of the block at the DN stage, the cell number is significantly higher in *Trpm7*-deficient thymi. Overlays of CD25 expression of the total thymocyte population and of DN thymocytes show that the proportion of cells expressing CD25 was significantly higher in *Trpm7*-deficient thymi (Fig. 3B). We calculated the changes in percentages (Fig. 3C) and number (Fig. 3D) of DN cells in DN1 (CD44⁺ CD25⁻), DN2 (CD44⁺ CD25⁺), DN3 (CD44⁻ CD25⁺), and DN4 (CD44⁻ CD25⁻) stages. These data indicated that a portion of *Trpm7*-deficient thymocytes fails to down-regulate high-affinity interleukin-2 receptors (CD25), exhibiting a block during the transition from the DN3 to DN4 stage. Cell-surface expression of T cell receptor β (TCR- β) chain (fig. S5C) was not substantially altered, which suggests that the developmental defect was not due to a failure in TCR- β locus rearrangement.

We found a progressive loss of thymic medullary cells [cytokeratin 5⁺ (K5⁺); Fig. 4A, green] but not thymic cortical cells (K8⁺; Fig. 4A, red) in comparing 4- and 12-week-old *wt* and *Trpm7^{-fl} (lck-Cre)* mouse thymic sections. In *wt* mice, the CD3⁺ cells (Fig. 4B, green) were distributed preferentially within medullary regions of the thymus and showed minimal overlap with cortical thymic epithelial cells (TECs) (K8⁺; Fig. 4B, red). In contrast, the loss of medullary regions in *Trpm7*-deficient mice was accompanied by a uniform distribution of CD3⁺ thymocytes in the thymic cortex, as detected by an extensive overlap of K8 and CD3 staining (Fig. 4B).

We conducted a quantitative RT-PCR analysis of freshly isolated thymocytes for mRNA that encoded 82 growth factors with proposed roles in tissue growth and maintenance (fig. S9). We identified seven growth-factor mRNAs whose abundance increased by more than threefold and five growth-factor mRNAs present at <33% of normal levels in *Trpm7*-deficient thymocytes (Fig. 4C). Growth-factor mRNAs that were down-regulated included fibroblast growth factor 13 (FGF-13), FGF-7, and midkine. FGF-7 is an important growth factor for

thymic epithelial cells (20,21), and FGF receptors activate signal transducer and activator of transcription 3 (STAT3)– mediated transcriptional responses (22), a pathway crucial for the maintenance of thymic medullary cells (23). Midkine induces mesenchymal-epithelial transition through the activation of STAT3 (24,25).

Because STAT3 is autoregulated, the levels of STAT3 are a useful indicator of ongoing STAT3 activity (26). In *wt* mice, STAT3 was specifically expressed in medullary TECs (identified by expression of the K5 marker) and progressively lost in medullary TECs in *Trpm7*-deficient thymi (Fig. 4D). In 12-week-old *Trpm7*^{-fl} (*lck-Cre*) mice, STAT3 was not detectable in the remnants of the atrophic thymic medulla. Similarly, although activated phospho-STAT3 was readily detected immunohistochemically in the nucleus of *wt* medullary TECs, there was no evidence of activated phospho-STAT3 in *Trpm7*-deficient medullary TECs (Fig. 4E). These data show that deletion of *Trpm7* in thymocytes results in reduced STAT3 activity and abundance in thymic medullary cells, which is expected to lead to a progressive loss of thymic architecture.

TRPM7 is the first TRP channel to be identified with a nonredundant role in embryogenesis and the only ion channel known to be necessary for thymopoiesis. The most notable feature of TRPM7 is the permeation of Ca²⁺, Mg²⁺, and trace metals in the very same structure that contains a kinase. TRPM7 mediates exceedingly low inward conductance, which suggests that the actions of the permeant species are localized and do not substantially affect global Mg²⁺ levels. Our work is now concentrated on how this bifunctional protein mediates these effects on cell- differentiation processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. I_{MIC} and Mg^{2+} homeostasis in *Trpm7*-deficient cells

(A) I - V relationship of I_{MIC} in *wt* and *Trpm7*-deficient [knockout (KO)] thymocytes. (B) I_{MIC} densities in *wt* ($n = 13$ cells) and KO ($n = 18$ cells) T lymphocytes ($P < 0.0001$, two samples independent t test). Box charts are shown as a box (25 to 75 percentile), vertical bars (5 to 95 percentile), and data point (diamonds) overlap with the mean value (empty square) and median value (horizontal line in the box). (C) I_{MIC} densities in *wt* ($n = 9$ cells) and KO ($n = 10$ cells) thymocytes ($P < 0.0001$, two samples independent t test). (D) Mg^{2+} uptake in *wt* T lymphocytes loaded with KMG104AM, as indicated by the averaged ratio of fluorescence intensity F at indicated time (seconds) over the initial fluorescence F_0 at 0 s. Mg^{2+} uptake in the absence (blue, $n = 25$ cells) or presence (red, $n = 25$ cells) of 0.5 mM 2-APB is shown. (E) ICP-MS quantitation of total Ca^{2+} and Mg^{2+} in HNO_3 extracts. Average concentration of total cellular Mg^{2+} ($n = 3$ mice) as calculated by normalizing to a $[K^+]$ of 120 mM is shown. Error bars indicate $\pm$ SD. (F) Mg^{2+} uptake in *wt* (blue, $n = 117$ cells) and KO (red, $n = 102$ cells) thymocytes. $[Ca^{2+}]$ and $[Mg^{2+}]$ are in mM.

Fig. 2. Deletion of *Trpm7* in thymocytes leads to defective thymopoiesis

(A) Hematoxylin and eosin–staining of thymus sections from 12-week-old *wt* (top) and *KO* (bottom) mice at 4× (left) and 20× (right) magnification (red box). The boundary between medullary and cortical regions is highlighted with a solid white line where evident. (B) Thymocytes were immunolocalized by antibody-to-CD3 staining (brown) against a nuclear counterstain (blue) in the thymus sections obtained from *wt* (top) and *Trpm7*-deficient (bottom) mice.; (Left): 4× magnification. Red boxes indicate the areas that are shown at 20× magnification to the right. The CD3+ T cell–enriched medullary regions are highlighted in a *wt* thymus (see fig. S6 for larger images). (C) Box chart showing the reduced number of thymocytes in *Trpm7*-deficient mice (red, *n* = 9 mice) as compared with *wt* mice (black, *n* = 9 mice). Box charts shown as a box (±SD), vertical bars (maximum–minimum values), and data overlap. The *P* values in all of the box charts were calculated using the two-sample independent *t* test. (D) Flow cytometry of CD4 and CD8 on thymocytes from *wt* and *KO* mice. (E) Box charts comparing the total number of thymocytes in the DN, DP, CD4+, and CD8+ thymocytes are shown (*n* = 7 mice).

Fig. 3. *Trpm7*-deficient thymocytes are partially blocked at the DN3 stage

(A) Flow cytometry of CD44 and CD25 expression in DN (CD4[−] CD8[−]) thymocytes. Thymocytes were stained with antibody to CD4 [fluorescein isothiocyanate (FITC)], antibody to CD8 (FITC), antibody to CD44 [phycoerythrin (PE)] and antibody to CD25 [phosphatidylcholine 7 (PC7)]. FITC-negative cells (DN) were analyzed for CD44 and CD25 expression. (B) (Left) Overlay of cell-surface CD25 expression in *wt* (black) and *Trpm7*-deficient (red) thymocytes. (Right) Overlay of CD25 expression in DN thymocytes. (C) Box charts showing percentage of DN population found in the (left to right) DN1, DN2, DN3, and DN4 stages. (D) Box charts showing total number of thymocytes found in the (left to right) DN1, DN2, DN3, and DN4 stages.

Fig. 4. Deletion of *Trpm7* in thymocytes results in progressive loss of medullary epithelial cells (A) Immunofluorescence staining of thymus sections with antibodies to thymic epithelial markers K8 (red) and K5 (green). Letters indicate thymic cortex (C) and medullary (M) regions. Scale bar, 200 μ m. (B) Staining of thymus sections to CD3 (green) and K8 (red). (C) Dysregulated mRNA encoding growth factors in knockout thymocytes relative to *wt* thymocytes identified by quantitative RT-PCR. Growth factors with increased (blue) or decreased (red) mRNA abundance are presented as average $\Delta\Delta Ct$ values ($n = 3$ mice). Error bars indicate SD. A complete list of quantitative RT-PCR results is in fig. S9. (D) Immunofluorescence staining of thymus sections with antibody to STAT3 (red) and K5 (green). (E) Immunofluorescence staining of phospho-STAT3 (Tyr705) (red) and K5 (green) in thymus sections. Scale bar, 20 μ m.