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## TRPM Channels and Magnesium in Early Embryonic Development

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### Abstract

Magnesium ( $Mg^{2+}$ ) is the second most abundant cellular cation and is essential for all stages of life, from the early embryo to adult.  $Mg^{2+}$  deficiency causes or contributes to many human diseases, including migraine headaches, Parkinson's disease, Alzheimer's disease, hypotension, type 2 diabetes mellitus and cardiac arrhythmias. Although the concentration of  $Mg^{2+}$  in the extracellular environment can vary significantly, the total intracellular  $Mg^{2+}$  concentration is actively maintained within a relatively narrow range (14 – 20 mM) via tight, yet poorly understood, regulation of intracellular  $Mg^{2+}$  by  $Mg^{2+}$  transporters and  $Mg^{2+}$ -permeant ion channels. Recent studies have continued to add to the growing number of  $Mg^{2+}$  transporters and ion channels involved in  $Mg^{2+}$  homeostasis, including TRPM6 and TRPM7, members of the transient receptor potential (TRP) ion channel family. Mutations in TRPM6, including amino acid substitutions that prevent its heterooligomerization with TRPM7, occur in the rare autosomal-recessive disease hypomagnesemia with secondary hypocalcemia (HSH). However, is the fact that genetic ablation of either gene in mice results in early embryonic lethality that has raised the question of whether these channels' capacity to mediate  $Mg^{2+}$  influx plays an important role in embryonic development. Here we review what is known of the function of  $Mg^{2+}$  in early development and summarize recent findings regarding the function of the TRPM6 and TRPM7 ion channels during embryogenesis.

### Keywords

magnesium; TRPM7; TRPM6; ion channel; gastrulation; and embryonic development

### Introduction

Unlike its flashy divalent cousin  $Ca^{2+}$ ,  $Mg^{2+}$  faithfully labors without fanfare in numerous vital biological processes, ranging from metabolic regulation, to DNA and RNA synthesis, neurotransmission, and cell stress reactions (Romani, 2007).  $Mg^{2+}$  homeostasis regulation in mammals occurs in the intestine, kidney and bone, where the divalent cation is initially absorbed, excreted and stored in its hydroxylapatite form, respectively. The kidney is believed to be the primary site for regulation of whole body  $Mg^{2+}$  homeostasis, where the

balance of  $Mg^{2+}$  reabsorption and excretion is controlled. It is well known that disruption of  $Mg^{2+}$  homeostasis is associated with numerous human diseases (for an excellent comprehensive review see (de Baaij *et al.*, 2015)). Low level of  $Mg^{2+}$  intake has been linked to the etiology of preeclampsia, stroke, diabetes, and cardiovascular disease. Beyond these pathologies, which primarily affects adults, it has been reported that insufficient  $Mg^{2+}$  intake during pregnancy may increase the risk of preterm birth and low birth weight (Conradt *et al.*, 1984, Doyle *et al.*, 1989, Makrides and Crowther, 2001).

Given the cation's association with fetal development and human diseases, it is very important to understand the molecular mechanisms that govern  $Mg^{2+}$  homeostasis. In this review, we summarize data illustrating the importance of  $Mg^{2+}$  homeostasis during embryonic development. We focus primarily on data from *Xenopus laevis*, where the most extensive and detailed analysis of the impact of  $Mg^{2+}$  on embryonic development has been conducted; this is presumably because *Xenopus* embryos can be developed *ex-vivo* and the concentration of ions bathing the embryo can be easily manipulated. We also discuss recent studies regarding the function of TRPM7 and TRPM6 ion channels in  $Mg^{2+}$  homeostasis and embryogenesis. Finally, we end our focused review by sharing our own unpublished results on the impact of  $Mg^{2+}$  on *Xenopus* development, including new observations that may be relevant to a role for this understudied cation in Parkinson's disease. For more detailed information, we refer the reader to more comprehensive reviews on TRPM6 and TRPM7 channels as well as  $Mg^{2+}$  homeostasis (Komiya *et al.*, 2014, Romani, 2011, Runnels, 2011).

## Cellular roles for $Mg^{2+}$ and its regulation by ion channels and transporters

Magnesium is involved in a wide variety of biological functions, including the cell cycle, ion channel regulation, ATPase activity, protein and nucleic acid synthesis, metabolism reactions, maintenance of body temperature and neurotransmission in nervous system (Romani, 2007).  $Mg^{2+}$  is a cofactor for many enzymes' activities. It has been estimated that at least 600 enzymatic reactions are directly or indirectly regulated by  $Mg^{2+}$  (de Baaij *et al.*, 2015). Total intracellular  $Mg^{2+}$  is 14 – 20 mM, making it the second most abundant cation (Grubbs and Maguire, 1987, Romani, 2011). However, unlike  $Ca^{2+}$ , whose cytosolic concentration can vary 10–100 fold, the free concentration of  $Mg^{2+}$  is comparably steady, hovering between 0.4 – 1 mM in concentration. This has lead some to question whether  $Mg^{2+}$  can function as a second messenger like  $Ca^{2+}$ . Nevertheless, rapid fluxes of  $Mg^{2+}$  into and out of cells have been detected in a number of cell types (Matsuura *et al.*, 1993, Romani and Scarpa, 1990a, Romani and Scarpa, 1990b). Disruption one of these processes was recently shown to be responsible for a novel X-linked human immunodeficiency characterized by CD4 lymphopenia, severe chronic viral infections, and defective T-lymphocyte activation (Li *et al.*, 2011). Mutations in the  $Mg^{2+}$  transporter MAGT1 abolished  $Mg^{2+}$  influx required for activation of phospholipase C- $\gamma$ 1 (PLC- $\gamma$ 1) and  $Ca^{2+}$  influx in T cells. Thus the impact of  $Mg^{2+}$  on cell signaling may be underappreciated. Indeed, low  $Mg^{2+}$  availability disrupts cell proliferation and cell differentiation in cell culture systems. Under low  $Mg^{2+}$  conditions, HL-60 leukemic cells accumulate in the  $G_0/G_1$  phase of cell cycle and have altered granulocytic differentiation (Covacci *et al.*, 1998). Low  $Mg^{2+}$  availability also inhibits cell proliferation of mammary epithelial HC11 cells by

increasing oxidative stress (Wolf *et al.*, 2009).  $Mg^{2+}$  deficiency has been etiologically linked to many human diseases as well (de Baaij *et al.*, 2015, Volpe, 2013). For example, it has been reported that low  $Mg^{2+}$  and  $Ca^{2+}$  intake is associated with Parkinson's disease (PD) and higher dietary intake of  $Mg^{2+}$  as well as iron and zinc may have a protective effect against PD (Miyake *et al.*, 2011, Oyanagi *et al.*, 2006). In addition, the highly selective  $Mg^{2+}$  transporter NIPA2 is mutated in patients with childhood absence epilepsy (CAE). The NIPA2 protein mutants were expressed in the cytosol, whereas the wild type protein properly localized to the cell border (Xie *et al.*, 2014). Indeed, it is estimated that up to 60 % of all critically ill patients are  $Mg^{2+}$  deficient (de Baaij *et al.*, 2015). Therefore, regulation of cellular as well as whole body  $Mg^{2+}$  homeostasis is critical for human health. Consequently, normal cells work aggressively to maintain a relatively narrow range of intracellular free  $Mg^{2+}$  concentration, regardless of the extracellular  $Mg^{2+}$  concentration. This responsibility largely falls to  $Mg^{2+}$  transporters and ion channels.

Magnesium transporters were originally identified in prokaryotes and protozoan (Quamme, 2010). In 1976, CorA was identified as a  $Mg^{2+}$  transporter in bacteria. CorA has two transmembrane domains and function as a  $Mg^{2+}$  transporter when oligomerized (Park *et al.*, 1976). CorA can permeate  $Co^{2+}$ ,  $Ni^{2+}$  as well as  $Mg^{2+}$  and CorA is the primary transporter responsible for  $Mg^{2+}$  intake in most prokaryotes. In an effort to search for new CorA family members and homologues, many other  $Mg^{2+}$  transporters such as MgtE, Alr1, and Mrs2, have been identified in bacteria, fungi and yeast systems (Bui *et al.*, 1999, Graschopf *et al.*, 2001). These discoveries led to the identification of mammalian's  $Mg^{2+}$  transporters, which were identified in part based on homologies to their ancestral counterparts. While some of the identified transporters exhibit relatively high specificity for  $Mg^{2+}$  transport, other molecules shown to play a role in  $Mg^{2+}$  homeostasis were surprisingly found to be relatively non-selective (see (de Baaij *et al.*, 2015) for a review). The TRPM7 ion channel, which has been shown to play a vital role in both cellular and whole animal  $Mg^{2+}$  homeostasis, is a good example (Ryazanova *et al.*, 2010, Schmitz *et al.*, 2003). TRPM7 exhibits high permeation of  $Mg^{2+}$ , but is also permeable to other essential divalent cations, including  $Ca^{2+}$  and  $Zn^{2+}$  (Monteilh-Zoller *et al.*, 2003). Likewise, TRPM6, a homologue of TRPM7 that is mutated in the rare autosomal disorder familial hypomagnesemia with secondary hypocalcemia (HSH), is permeable to a similar range of divalent cations as TRPM7 (Li *et al.*, 2006, Voets *et al.*, 2004). TRPM7 and TRPM6 are members of transient receptor potential (TRP) superfamily, which comprises a large number of cation-permeable ion channels, many of which execute critical functions in sensory systems (Clapham, 2003, Nadler *et al.*, 2001, Runnels *et al.*, 2001). Among ion channels, TRPM7 and TRPM6 are quite unique because they are bifunctional molecules ("chanzymes") with functional kinases domains attached to COOH-termini (Figure 1). TRPM7 appears to be ubiquitously expressed, whereas the expression of TRPM6 is more restricted, showing highest expression in the intestine and the distal convoluted tubule (DCT) of the kidney (Schlingmann *et al.*, 2002, Walder *et al.*, 2002). The DCT is the final site for  $Mg^{2+}$  reabsorption before urinary excretion and functions to balance the body's  $Mg^{2+}$  content. TRPM6 is posited to play a critical role in this process. A positional candidate gene approach identified TRPM6 as the gene mutated in familial HSH (Schlingmann *et al.*, 2002, Walder *et al.*, 2002). Individuals affected by HSH are characterized by low serum  $Mg^{2+}$  and  $Ca^{2+}$ . Patients exhibit neurologic

symptoms such as seizures and muscle spasms (Shalev *et al.*, 1998). Life-long supplementation of  $Mg^{2+}$  can be used to treat HSH. Thus far, however, there has been no report of a mutation in the *TRPM7* gene for a HSH patient. While a majority of the mutations in individuals affected with HSH are either nonsense or frameshift mutations in *TRPM6* that are easily compatible with a loss-of-function phenotype, one missense mutation entails the exchange of a highly conserved serine for a leucine at amino acid position 141 (S141L), which disrupts the ability of TRPM6 to form heterooligomers with TRPM7 (Schlingmann *et al.*, 2002). In two sets of studies, TRPM6 was reported to be dependent upon TRPM7 for cell surface expression (Chubanov *et al.*, 2004, Schmitz *et al.*, 2005). Full-length variants of TRPM6 failed to form functional channel complexes when heterologously expressed in HEK-293 cells and *Xenopus* oocytes (Chubanov *et al.*, 2004). This data suggests that TRPM7 may also participate in whole body  $Mg^{2+}$  homeostasis, either by itself, or by forming heterooligomeric channels with TRPM6. In support of such a role, heterozygous mice engineered to lack TRPM7's kinase domain (TRPM7<sup>KINASE</sup>) exhibited a mild hypomanaemia phenotype, which was most likely due to diminished TRPM7 channel activity resulting from deletion of the kinase domain. The heterozygous TRPM7<sup>KINASE</sup> mutant mice exhibited high  $Mg^{2+}$  excretion, altered  $Mg^{2+}$  homeostasis and high sensitivity to low-magnesium diet (Ryazanova *et al.*, 2010). A more severe phenotype was observed for homozygous TRPM7<sup>KINASE</sup> null embryos, which were able to initiate gastrulation and mesoderm formation, but died between day 7.5 and 8.5 of embryogenesis. Thus, studies in mice have elaborated these channels role in whole body magnesium homeostasis, but have also pointed to additional critical functions during early embryogenesis.

## TRPM7, TRPM6 and embryogenesis

The requirement of TRPM7 and TRPM6 for embryogenesis has been demonstrated by ablation of their genes in mouse models. In the mouse embryo, expression of TRPM7 is dramatically increased from embryonic day 10.5 (E10.5) to E11.5 and global expression was observed through E14.5 (Jin *et al.*, 2008). No homozygous TRPM7 mutants were born, dying at E7.5, the period when gastrulation cell movements commence. During mouse embryogenesis, the primitive streak is formed around E6.5, after which cells start moving dynamically to establish the anterior-posterior axis. From E8.5 to E15 the neural plate is formed and the neural tube closes. Jin and colleagues also generated conditional knockout mice of TRPM7 using a tamoxifen-inducible and multiple tissue specific Cre recombinase lines (Jin *et al.*, 2012). Tamoxifen-dependent deletion of TRPM7 at E7.5 to E8.5 caused embryonic lethality within 48–72 hours, while the depletion at E14.5 did not cause embryonic lethality, with mutant mice developing normally. Taken together, results from TRPM7 mutant mice demonstrate a temporal requirement of TRPM7 during early gastrulation in mouse. In addition, Jin *et al.* demonstrated that disruption of TRPM7 in induced pluripotent stem (iPS) cells induced apoptosis, indicating a contribution of TRPM7 to stem cell survival (Jin *et al.*, 2012). A similar dependence on TRPM7 was observed for the survival of bone marrow derived mesenchymal stem cells and human dental pulp stem cells (Cheng *et al.*, 2010, Cui *et al.*, 2014), but not neural stem cells (Jin *et al.*, 2012). This may be because not all cells solely depend upon TRPM7 for cellular magnesium

homeostasis. Nevertheless, an indispensable function for TRPM7 in certain stem cell populations may impact developmental processes dependent upon cell differentiation. Later in development, TRPM7 has also been demonstrated in mice to have critical functions during nephrogenesis, the development of neural-crest-derived pigment cells, and myocardial proliferation during early cardiogenesis (Jin *et al.*, 2012, Sah *et al.*, 2013). Additional studies in zebrafish have also highlighted roles for TRPM7 in pigmentation, but have uncovered additional developmental roles in other organs' development, including the pancreas (McNeill *et al.*, 2007, Yee *et al.*, 2011). Magnesium's contribution to TRPM7 function later in development remains poorly understood.

Surprisingly, knockout of TRPM6 from mice is also embryonically lethal (Walder *et al.*, 2002, Woudenberg-Vrenken *et al.*, 2011). In experiments reported by Walder and colleagues, homozygous TRPM6 knockout embryos died by E12.5 and exhibited neural tube closure defects (Walder *et al.*, 2002). The expression pattern of TRPM6 during embryogenesis demonstrated a significant increase at E10, suggesting TRPM6 is also temporally required for early development. Heterozygous TRPM6 knockout mice exhibited mild hypomagnesemia (Walder *et al.*, 2002, Woudenberg-Vrenken *et al.*, 2011). Interestingly, in one of the studies dams fed with a high  $Mg^{2+}$  diet slightly suppressed the embryonic lethality caused by knockout of TRPM6, suggesting that embryonic lethality may in part be due to  $Mg^{2+}$  deficiency. However, in the second study by Woudenberg-Vreken *et al.*, a high  $Mg^{2+}$  diet failed to rescue the lethality caused by homozygous deletion of *TRPM6* (Woudenberg-Vrenken *et al.*, 2011). Studies from zebrafish also support a role for  $Mg^{2+}$  during early embryonic development. Zhou and Clapham have demonstrated that knockdown of the MagT1 and TUSC3  $Mg^{2+}$  transporters in zebrafish embryos causes early developmental arrest, with embryos exhibiting an apparent defect in brain and eye development (Zhou and Clapham, 2009). Supplementation of  $Mg^{2+}$  in the growth media partially rescued the embryonic arrest caused by depletion of MagT1, demonstrating the importance of  $Mg^{2+}$  transporters and  $Mg^{2+}$  during embryogenesis. In a more recent study, mutations in the gene encoding for cyclin M2 (CNNM2) were demonstrated to be causative for mental retardation and seizures in patients with hypomagnesemia (Arjona *et al.*, 2014). In patients with a recessive mode of inheritance, the intellectual disability is more severe and is accompanied by motor defects and brain malformations. In zebrafish, CNNM2 has two orthologues, *cnnm2a* and *cnnm2b*. Consistent with the human pathology, knockdown of *cnnm2a* in zebrafish resulted in reduced total magnesium content and in morphological phenotypes characterized by enlarged pericardial cavities and notochord defects. At 25 hour post-fertilization (hpf) maldevelopment of the midbrain hindbrain boundary (MHB) was observed. *Cnnm2b* morphants also had lower total magnesium and were characterized by enlarged pericardial cavities, kidney cysts, accumulation of cerebrospinal fluid in the cerebrum, and at 25 hpf, also exhibited defects in development of the MHB. These studies highlight a particularly important role for CNNM2, and potentially for magnesium, in heart, kidney and especially brain development.

## TRPM7 function and magnesium deficiency in the *Xenopus* embryo

*Xenopus laevis* embryos constitute a classic animal model to investigate early developmental processes. Many of the signaling molecules that regulate early embryogenesis were

originally identified in *Xenopus*. It is well known that key factors of early development, such as BMP, Wnt and FGF signaling molecules, are functionally conserved between *Xenopus* and mammals. Since *Xenopus* eggs can be externally fertilized, it is easy to observe each step of embryogenesis. This model also generates a relatively large embryo, which allows for targeted microinjection and microsurgery. Microinjection of morpholino antisense oligonucleotides (MOs), which inhibit protein translation from their mRNA targets, produces phenotypes that are typically milder than those produced by homozygous gene deletion. Because of the early embryonic lethality of TRPM7 and TRPM6 knockout mice, a moderate knockdown of the ion channels facilitates investigation of the developmental processes they are affecting. In addition, *Xenopus* embryos as well as dissected explants can survive in a simple buffer, allowing us to easily manipulate the ionic composition of the culture buffer and to examine the significance of different ions during early embryogenesis.

Previously, we have demonstrated a crucial role of TRPM7 in gastrulation cell movements during *Xenopus* embryogenesis (Liu *et al.*, 2011). While *Xenopus* TRPM7 expression is very consistent throughout embryogenesis, *in situ* hybridization analysis showed specific TRPM7 expression in dorsal mesoderm and ectoderm during the gastrula and in neural plate during neurulation. Downregulation of TRPM7 by MO injection induced severe gastrulation defects, which were characterized by a short and curved axis and widely opened neural tube. Interestingly, co-injection of TRPM6 or the SLC41A2 Mg<sup>2+</sup> transporter with the TRPM7 MO suppressed the MO-induced gastrulation defects. Supplementation of Mg<sup>2+</sup>, but not Ca<sup>2+</sup>, in the culture buffer was similarly effective. These results indicate Mg<sup>2+</sup> intake through TRPM7 is required for gastrulation in the *Xenopus* embryo. During gastrulation, neuroectoderm and dorsal mesoderm undergo dynamic morphological changes. Polarized cells move toward the midline and intercalate mediolaterally. Afterwards the ball-shape embryo narrows and lengthens its head-tail axis. This coordinated movement of cells establishes anterior-posterior axis and is referred to as “convergent extension movements” (Wallingford *et al.*, 2002). It is well known that non-canonical Wnt pathway plays a pivotal role in convergent extension movements (Wallingford and Habas, 2005). We have demonstrated that knockdown of TRPM7 strongly inhibits convergent extension movements and disrupts cell polarization (Liu *et al.*, 2011). Importantly, TRPM7 MO-induced suppression of cell movements can be rescued by the supplementation of Mg<sup>2+</sup> in culturing buffer, indicating maintaining Mg<sup>2+</sup> concentration in embryo is critical for cell movements during early embryogenesis. Although molecular mechanism is not fully understood, our co-injection studies suggest that the TRPM7 signaling merges with non-canonical Wnt pathway. Overall, our experiments with *Xenopus* embryos uncovered the importance of TRPM7 as well as Mg<sup>2+</sup> for cell movements during gastrulation. Since expression of a Mg<sup>2+</sup> transporter and Mg<sup>2+</sup> supplementation could rescue the defects induced by the TRPM7 MO, maintenance of cellular Mg<sup>2+</sup> homeostasis is likely crucial for gastrulation.

In 1977 Miller and Landesman performed the first comprehensive investigation of the significance of Mg<sup>2+</sup> during *Xenopus* embryogenesis (Miller and Landesman, 1977). Typically, *Xenopus* embryos are cultured in a simple buffer such as 0.1 × MMR or 0.1 × MBS, solutions that contains 0.1 μM MgSO<sub>4</sub>. 0.1 × MMR contains (in mM) 0.5 HEPES (pH 7.8), 10 NaCl, 0.2 KCl, 0.2 CaCl<sub>2</sub> and 0.1 MgSO<sub>4</sub>. 0.1 × MBS contains (in mM) 0.5 HEPES

(pH 7.8), 8.8 NaCl, 0.1 KCl, 0.07 CaCl<sub>2</sub>, 0.1 MgSO<sub>4</sub>, 0.25 NaHCO<sub>3</sub>. These buffers also contain sodium, potassium, and calcium ions, which are also essential for embryonic development since *Xenopus* embryos cannot survive in distilled water alone. In their experiments, the authors determined the impact of Mg<sup>2+</sup> on embryogenesis by varying the concentration of Mg<sup>2+</sup> in the culture buffer from 100 nM to 10 mM. When the Mg<sup>2+</sup> concentration was lower than 10 μM, embryos exhibited reduced melanophores at stage 35–36. By stage 40 the embryos showed shorter tail expansion, abnormal coiling of the gut, slow head enlargement, restricted heart growth, and edema. In addition, abnormal somite development was observed, which may be related to paralysis of the Mg<sup>2+</sup> deficient embryos. In control embryos, the total Mg<sup>2+</sup> content began to increase after hatching. By comparison, embryos cultured in Mg<sup>2+</sup> deficient buffer maintained a very low level of total Mg<sup>2+</sup> throughout development. Their results suggested that proper Mg<sup>2+</sup> uptake is a critical factor during early developmental processes in *Xenopus*.

The Miller and Landesman article has very detailed drawings of the embryonic phenotypes caused by Mg<sup>2+</sup> deficiency, but to the best of our knowledge there are no photographs of the embryos from their experiments. To update the information on Mg<sup>2+</sup> deficiency during *Xenopus* embryogenesis, we conducted similar experiments to those of Miller and Landesman by examining the effect of Mg<sup>2+</sup> depletion from culture medium on the developmental processes of *Xenopus laevis* (Figure 2A). We observed the morphology of *Xenopus* embryos cultured in regular MMR solution or Holtfreter's buffer with or without 0.1 mM MgSO<sub>4</sub>. It should be noted that our buffer does not contain the chelating agent, sodium pyrophosphate, since Miller and Landesman reported that sodium pyrophosphate induces cell dissociation and inhibits gastrulation.

Consistent with their earlier findings, early developmental processes such as cleavage, early gastrulation, and neurulation were not significantly affected by Mg<sup>2+</sup> depletion in our experiments (data not shown). At the tadpole stage, embryos cultured in Mg<sup>2+</sup> deficient media exhibited lighter pigmentation in eyes and body than control embryos reared in normal buffer (Figure 2A). All embryos cultured in Mg<sup>2+</sup> deficient media did not elongate their axis properly: the length of anterior-posterior axis was significantly shorter than control embryos. In addition, embryos cultured in Mg<sup>2+</sup> deficient media had spinal cords that were not straight, with some of embryos exhibiting a mildly kinked axis. Many of the embryos cultured in Mg<sup>2+</sup> deficient media had a slightly smaller head size. Although we did not perform histological exams, in some of embryos the gut appeared abnormal as reported by Miller and Landesman. We did not observe severe defects during early gastrulation and the reason for this is not clear. The concentration of Mg<sup>2+</sup> from egg to the stage 9 embryo, just before gastrulation begins, does not vary much from 14 mM (Slack *et al.*, 1973). The rate at which the embryos lose Mg<sup>2+</sup> in Mg<sup>2+</sup> deficient media is not known and could be too slow to produce a severe phenotype at earlier stages in development. More research on the regulation of Mg<sup>2+</sup> homeostasis in the developing embryo is required to address this question.

Nevertheless, given the fact that Mg<sup>2+</sup> supplementation can rescue the convergent extension defect induced by TRPM7 depletion suggests that Mg<sup>2+</sup> influences cell movements during early embryogenesis. In addition to the result from *Xenopus* embryos, Su *et al.* have

demonstrated the essential role of TRPM7 and  $Mg^{2+}$  in cytoskeletal arrangements and polarized cell movements in fibroblasts (Su *et al.*, 2011). Knockdown of TRPM7 by RNAi inhibited the activity of Rho, Rac, and Cdc42, which interfered with the establishment of cell polarity and lamellipodia formation in a cellular wound assay. Similar to what was observed in *Xenopus* experiments, these cellular defects could be rescued by expression of the  $Mg^{2+}$  transporter SLC41A2. These results support a role for  $Mg^{2+}$  as well as for TRPM7 in controlling polarized cell movements. To directly determine whether  $Mg^{2+}$  influences gastrulation cell movements we investigated the effect of  $Mg^{2+}$  depletion on convergent extension using a Keller explant assay. After fertilization, embryos were grown in regular MMR or  $Mg^{2+}$ -free MMR until the early gastrula stage, after which the dorsal marginal zone was dissected. The isolated explants were then cultured in buffer containing  $Mg^{2+}$  or  $Mg^{2+}$ -free buffer. For the control condition (with  $Mg^{2+}$ ), dorsal tissue explants underwent convergent extension movements and became well elongated. The explants that were pre-cultured with  $Mg^{2+}$ -free MMR also elongated, but the length was shorter than the controls, indicating that elongation was impaired (Figure 2B). These results suggest that the short axis phenotype in embryos cultured in  $Mg^{2+}$  deficient media could be explained by mild inhibition of convergent extension cell movements.

The results from Miller and Landesman as well as our own results indicate a vital contribution of  $Mg^{2+}$  to pigmentation development. Body pigmentation develops from the presence of melanin in melanocytes (the melanophore is the non-mammalian vertebrate counterpart of the mammalian melanocyte) and retinal pigment epithelium. Melanin is produced by specialized lysosomal-related organelles called melanosomes. Interestingly, it has been reported that the TRPM7, TRPM1 and TRPA1 ion channels are present in melanocytes and are involved in the regulation of melanin synthesis (Bellono and Oancea, 2014). As mentioned earlier, TRPM7 mutant zebrafish exhibit pigmentation defects as a result of having fewer melanophores (McNeill *et al.*, 2007). The loss of pigmentation appears to be caused by apoptotic and necrotic cell death of melanoblasts, precursor cells of melanophores, as well as melanophores. In melanophores of TRPM7 mutant zebrafish, many irregular shaped melanosomes were observed. In the surrounding tissue, melanosomes were also seen, suggesting that the plasma membrane of melanophore cells ruptured and caused necrotic cell death. Interestingly, inhibition of melanin synthesis by a tyrosinase inhibitor can rescue melanophore cell death in the TRPM7 mutant zebrafish (McNeill *et al.*, 2007). These results indicate that abnormal melanin synthesis is related to the death of melanophores of TRPM7 mutant fish. In addition,  $Mg^{2+}$  supplementation was also partially successful in suppressing the death of melanophores. Melanosomes contain a high concentration of metals, including iron, zinc, copper, calcium and magnesium, indicating that the organelle may also function as an intracellular storage site for these metals (Hong and Simon, 2007). Although the molecular mechanisms remain unclear, the data suggests that  $Mg^{2+}$  accumulation into melanosomes may contribute to melanogenesis.  $Mg^{2+}$  may be affecting melanin synthesis by binding to enzymes or intermediates of melanin synthesis that contribute to pigmentation development. Thus, loss of TRPM7 or embryonic development in a low  $Mg^{2+}$  environment causes abnormal melanin synthesis that somehow contributes to melanocyte cell death. This could explain for why melanin-positive cells may be more sensitive to loss of TRPM7 and to  $Mg^{2+}$  deficiency than other cell types.

## Magnesium homeostasis and neurodegenerative diseases

In addition to defects of cell movement and pigmentation, we observed that embryos grown in  $Mg^{2+}$ -free medium did not readily move at the tadpole stage. Control tadpoles swam actively in the culture dish, whereas  $Mg^{2+}$  deficient tadpoles exhibited a paralysis phenotype (Supplementary Movie 1). When touched with a paintbrush,  $Mg^{2+}$  deficient tadpoles were often unresponsive to touch stimulation. In comparison to control tadpoles that swam away immediately when touched, some  $Mg^{2+}$  deficient tadpoles twitched and swam away abnormally slow. This touch response defect was of our particular interest because TRPM7 mutant zebrafish lines have been shown to also exhibit movement defects (Decker *et al.*, 2014, Low *et al.*, 2011). In wild type zebrafish, a light-to-dark transition stimulates swimming behavior. Decker and colleagues demonstrated that the swimming distance of TRPM7 mutant fishes under these conditions was shorter than wild type. Interestingly, the paralysis phenotype of TRPM7 mutant zebrafish is reported to be due to abnormal differentiation of dopaminergic neurons. Decker *et al.* demonstrated that in the brain of TRPM7 mutant zebrafish the number of cells expressing tyrosine hydroxylase (an enzyme involved in dopamine synthesis) was significantly lower than in wild type fish. Interestingly, application of levodopa (L-dopa), a precursor of dopamine, could rescue the motility defect of TRPM7 mutants. Their results suggested that loss of function mutations in the *TRPM7* gene in zebrafish is caused by abnormal differentiation of dopaminergic neurons, which ultimately resulted in a motility defect. However, mutation in TRPM7 has also shown to be responsible for a touch-unresponsive zebrafish mutant. This phenotype could be alleviated by forcing the expression of TRPM7 in primary sensory neurons, suggesting that TRPM7 is required transiently for function or differentiation of embryonic primary sensory neurons (Low *et al.*, 2011). A defect in a touch-evoked escape behavior was also observed in *cnm2a* and *cnm2b* zebrafish morphants, which was speculated to be caused to lack of excitation of sensory neurons via TRPM7 and/or to defects in early brain development (Arjona *et al.*, 2014). Given the similarity of the phenotypes between  $Mg^{2+}$  deficient embryos and the *cnm2* and TRPM7 mutant fishes, the movement defect in  $Mg^{2+}$  deficient *Xenopus* tadpoles could be caused by a defect in sensory neurons and/or dopaminergic neurons. This hypothesis remains to be explored.

Loss of dopaminergic neurons is a hallmark of Parkinson's disease (PD). Interestingly, these dopaminergic neurons are neuromelanin-positive. Given the fact that both TRPM7 and  $Mg^{2+}$  contribute to melanophore cell development as well as to the motility of zebrafish and *Xenopus* embryos, it is tempting to speculate that  $Mg^{2+}$  deficiency may be related to etiology of Parkinson's disease. In fact, epidemiological evidence and animal studies support a link between  $Mg^{2+}$  deficiency and loss of dopaminergic neurons in PD. In Guam and Kii peninsula in Japan, where extremely low concentrations of  $Mg^{2+}$  and  $Ca^{2+}$  in drinking water have been reported, individuals have a higher risk of developing parkinsonism-dementia complex (PDC) and amyotrophic lateral sclerosis (ALS). Parkinson's patients have also been found to have low  $Mg^{2+}$  concentrations in cortex, white matter, basal ganglia, and brain stem (Yasui *et al.*, 1992). Oyanagi *et al.* have attempted to replicate the condition of low  $Mg^{2+}$  and  $Ca^{2+}$  intake in rats. Rats were fed varying concentrations of  $Mg^{2+}$  and  $Ca^{2+}$  in their diet for over a generation; a significant loss of

dopaminergic neurons occurred in the substantia nigra of rats fed a low  $Mg^{2+}$  diet (Oyanagi *et al.*, 2006). Furthermore, it has been shown that  $Mg^{2+}$  supplementation of the culture medium for an *in vitro* PD model prevented loss of dopaminergic neurons induced by the parkinsonian agent (1-methyl-4-phenylpyridinium,  $MPP^+$ ) (Hashimoto *et al.*, 2008). Another group investigated the effect of low  $Mg^{2+}$  and  $Ca^{2+}$  diet on PD using mice. Mice fed low  $Mg^{2+}$  and  $Ca^{2+}$  for 3–6 weeks developed akinesia and rigidity concomitant with a decrease in the number of their dopaminergic neurons (Taniguchi *et al.*, 2013). Interestingly, a polymorphism in the *TRPM7* gene (T1482I) has been associated with a subset of patients with Guamanian amyotrophic lateral sclerosis (ALS-G) or parkinsonism dementia (PD-G). The T1482I mutation may render the TRPM7 channel more sensitive to inhibition by intracellular  $Mg^{2+}$  (Hermosura *et al.*, 2005), although this finding has been disputed (Demeuse *et al.*, 2006). In addition, a rare gain-of-function mutation in the  $Mg^{2+}$  transporter gene *SLC41A1*, which was shown to lower intracellular  $Mg^{2+}$  when expressed in HEK-293 cells, is putatively linked to PD (Kolisek *et al.*, 2013). Moreover, *SLC41A1* is located on the PARK16 locus that is associated with Parkinson's disease. Taken together these studies suggest that an appropriate amount of  $Mg^{2+}$  is required for survival, differentiation and/or function of dopaminergic neurons and that  $Mg^{2+}$  deficiency may be related to the etiology of PD. Since, TRPM7 appears to be involved in the function of dopaminergic neurons, magnesium's role in this devastating disease warrants further investigation.

## Conclusion

Magnesium is an abundant and essential divalent cation in the body. Not surprisingly, disruption of  $Mg^{2+}$  homeostasis has the potential to contribute to birth defects as well as to many other human diseases. Knockout mouse studies have uncovered indispensable roles for TRPM7 and TRPM6 during early development. However, experiments in zebrafish and *Xenopus* embryos have given insight into the role that  $Mg^{2+}$  may be playing not only in gastrulation, but in body pigmentation, brain development and body movement as well. Excitingly, these observations may be relevant to the etiology of many human diseases, including neural tube closure defects and neurological disorders such as epilepsy and Parkinson's disease. The mechanisms by which  $Mg^{2+}$  exerts its effects during development and disease are still poorly understood. This is in part due to the fact that  $Mg^{2+}$  functions as a cofactor in so many cellular processes. In addition,  $Mg^{2+}$  could be indirectly affecting the homeostasis of  $Ca^{2+}$  or other metal ions. Gain-of-function and loss-of-function studies, such as those that have been conducted in *Xenopus* and zebrafish, have the potential to uncover the mechanisms by which  $Mg^{2+}$  exerts its *in vivo* effects. Research into the function of  $Mg^{2+}$  will give much needed insight into approaches that may prevent or limit the devastating effects of the diseases caused or made worse by dysregulation of this vital divalent cation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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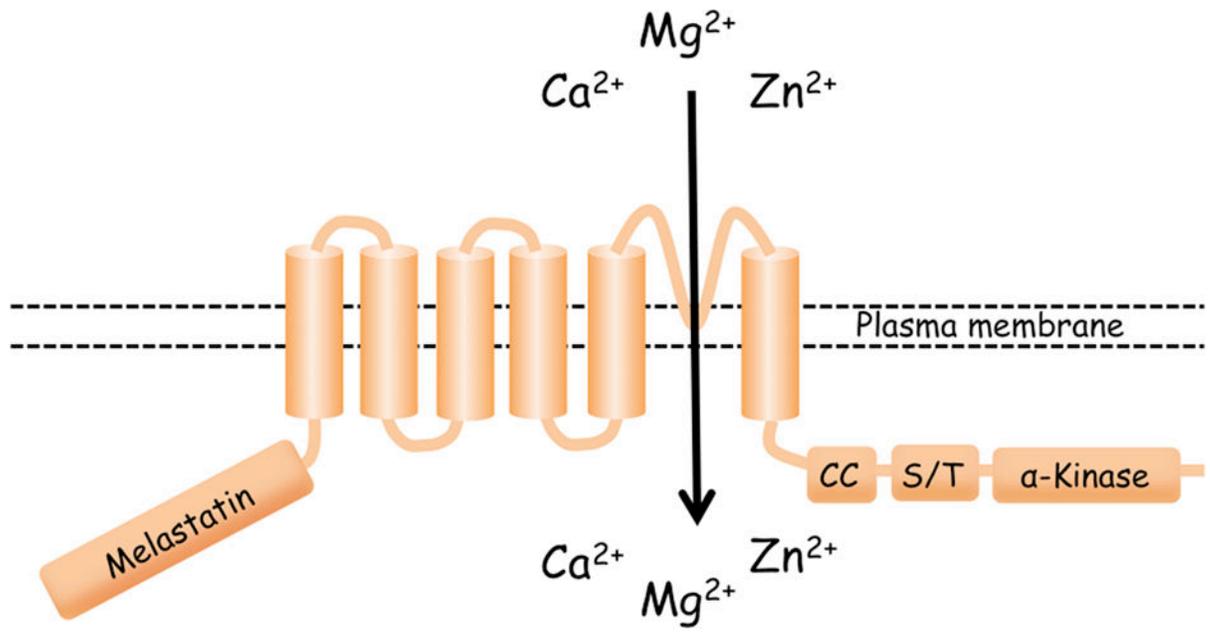
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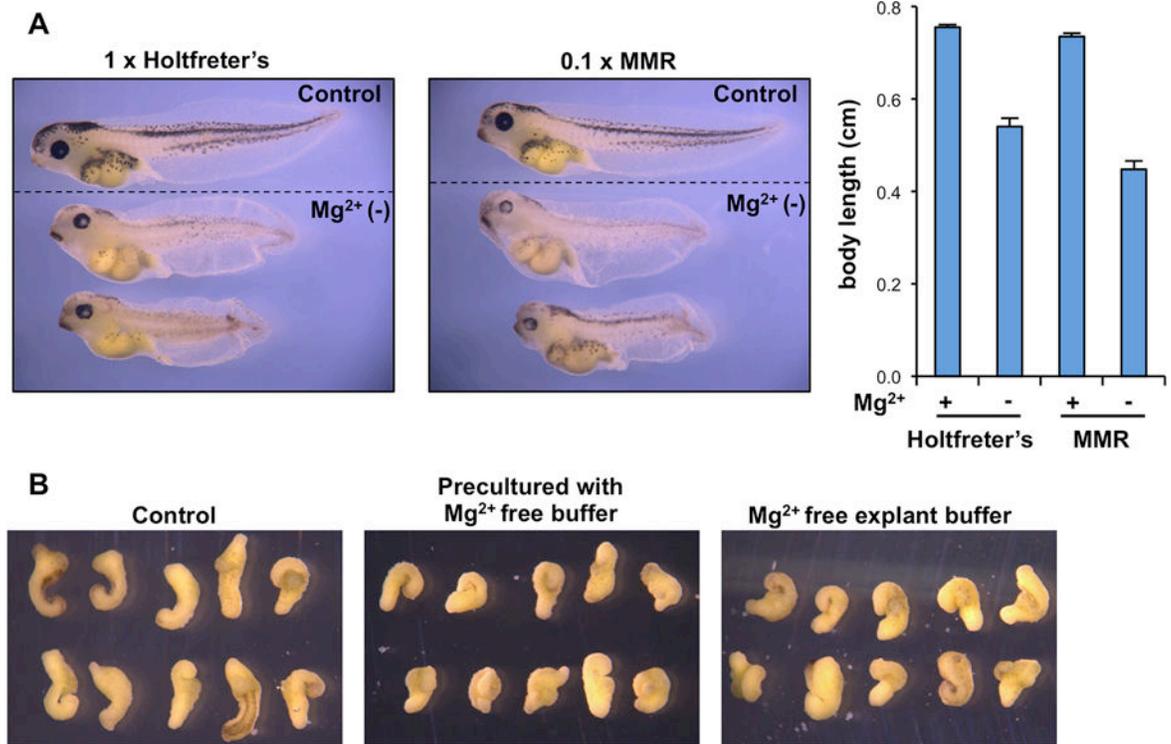
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**Fig. 1. Schematic model of the membrane topology of TRPM7 and TRPM6**

TRPM7 and TRPM6 have 6 transmembrane spanning domains and a pore-loop that is localized between fifth and sixth transmembrane domains. TRPM7 and TRPM6 possess coiled-coil (CC), Serine/Threonine-rich (S/T), and  $\alpha$ -kinase domains in the intracellular COOH-terminus region.



**Fig. 2. Magnesium depletion causes developmental abnormalities in *Xenopus laevis* embryos** (A) Mg<sup>2+</sup> deficient embryos showed pigmentation defects and a shortened axis. Top embryos in each panel are wild type embryos reared in normal buffer. Right panel shows average of body length at stage 42. (B) Mg<sup>2+</sup> depletion suppressed convergent extension movements. At stage 10.5, dorsal marginal zone was dissected and cultured in buffer with or without Mg<sup>2+</sup> until siblings reach stage 22–23.