INTRODUCTION

TRPM7 (transient receptor potential melastatin 7) is one of only two vertebrate ion channels to contain both ion channel and kinase domains [1,2]. The other bifunctional channel is TRPM6, mutations in which cause HSH (familial hypomagnesaemia with secondary hypocalcaemia), an autosomal-recessive disease characterized by low serum Mg\(^{2+}\) accompanied by hypocalcaemia [3,4]. Whereas TRPM6 is expressed mainly in the kidney and the intestine [3,5], TRPM7 is expressed in nearly all cell types [1,2]. Evidence points to a pivotal role for TRPM7 in cellular Mg\(^{2+}\) homoeostasis. Scharenberg and colleagues revealed that TRPM7-deficient DT40 B-cells exhibited Mg\(^{2+}\) deficiency and growth arrest [6,7]. Supplementation of growth medium with extracellular Mg\(^{2+}\) or expression of the Mg\(^{2+}\)-transporter SLC41A2 (solute carrier family 41 member 2) in TRPM7-deficient cells restored the ability of TRPM7-deficient DT40 cells to proliferate [8]. In mice, homozygous deletion of TRPM7 resulted in early embryonic lethality [9]. Early developmental arrest caused by loss of the channel kinase in mice appears to be related to the channel’s ability to permeate Mg\(^{2+}\), as depletion of TRPM7 in Xenopus laevis embryos produced a disruption in convergent-extension cell movements during gastrulation that could be prevented by Mg\(^{2+}\) supplementation as well as by expression of the Mg\(^{2+}\)-transporter SLC41A2 [10]. Later in development, other physiological functions have been ascribed to the channel kinase, including skeletogenesis and melanophore maturation, kidney and pancreatic development, synaptic vesicle fusion and thymopoiesis [9,11–15]. The pleiotropic phenotypes caused by loss of the channel kinase is probably due to TRPM7’s bifunctional nature as well as to the channel’s ability to permeate multiple species of divalent cations [16]. The best illustration of this comes from studies of the channel’s role in cell death.

TRPM7 appears to play a major role in a cell’s response to cell stress. The first and perhaps most striking example of the channel’s influence on this process comes from the collective work by Tymianski, MacDonald and colleagues [17–19]. Their studies revealed that TRPM7 constitutes a Ca\(^{2+}\)-permeable non-selective cation conductance (I\(_{\text{Ca}}\)) that becomes activated by ROS (reactive oxygen species)/RNS (reactive nitrogen species) to promote Ca\(^{2+}\) overload and anoxic death in cultured cortical neurons subjected to OGD (oxygen glucose deprivation) [17]. Suppressing TRPM7 expression using siRNA (small interfering RNA) reduced the ischaemia-induced current, decreased Ca\(^{2+}\) uptake and increased cell viability [17]. Using intrahippocampal injections of adeno-associated viral vectors packaged with shRNA (small hairpin RNA) specific for TRPM7, a subsequent study by Tymianski and colleagues provided in vivo evidence that regional TRPM7 suppression provides a comparable level of protection against brain ischaemia [18]. Importantly, depletion of the channel had no negative effect on animal survival, dendritic morphology, neuronal excitability or synaptic plasticity [18]. In addition to its contribution to Ca\(^{2+}\) overload during OGD, TRPM7 is also required for Zn\(^{2+}\)-induced neuronal cell death, indicating that permeation of Ca\(^{2+}\) and Zn\(^{2+}\) both contribute to the TRPM7 channel’s ability to mediate cell death in neurons [20]. More recently, knockdown of TRPM7 in hippocampal neurons has been shown to reduce the increase in intracellular Mg\(^{2+}\) levels detected following OGD, suggesting that conduction of Mg\(^{2+}\) by
the channel during ischaemia may also be contributing to neuronal cell death [21]. Consistent with the notion that conduction of multiple ions are involved in TRPM7’s ability to mediate cell death, overexpression of TRPM7 in HEK (human embryonic kidney) cells increased Mg\(^{2+}\) and Ca\(^{2+}\) influx, which led to increased production of ROS and NO production [22]. The resultant oxidative stress caused by overexpression of the channel in turn activated the stress-activated protein kinases p38 MAPK (mitogen-activated protein kinase) and JNK (c-Jun N-terminal kinase), which caused loss of cell adhesion and increased cell death [22,23]. Conversely, depletion of TRPM7 in HEK-293 cells was protective against many forms of cell stress, including the apoptosis inducer DOX (doxorubicin), the translation inhibitor CHX (cycloheximide) and the broad kinase inhibitor STS (staurosporine) [23]. To further uncover how TRPM7 affects the cellular response to stress, we have employed a stable TRPM7-knockdown Swiss 3T3 fibroblast line (M7shRNA6 cells), which we previously used to investigate the mechanisms by which TRPM7 controls cell motility [24]. M7shRNA6 cells exhibit defects in the ability to form lamellipodia and migrate directionally, which can be rescued by re-expression of TRPM7 as well as by expression of the Mg\(^{2+}\) transporter SLC41A2 [24]. In the present study, we show that depletion of TRPM7 from fibroblasts lowered intracellular Mg\(^{2+}\), rendered cells more resistant to apoptotic stimuli and lowered the concentration of cellular ROS, which can all be reversed by re-expression of TRPM7, as well as by expression of the Mg\(^{2+}\) transporter SLC41A2. These results indicate that Mg\(^{2+}\) is not only playing a key role in TRPM7’s ability to control cell survival, but is also critical to the regulation of cellular ROS levels, both in the absence and presence of cell stress.

**EXPERIMENTAL**

**Reagents**

All chemicals, unless otherwise stated, were obtained from Sigma. STS, a broad inhibitor of protein kinases, was obtained from Calbiochem. Mag-Indo-1, Fluo-4-AM (fluoro-4 acetoxyethyl ester) and CM-H\(_2\)DCFDA [5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate, acetyl ester] were obtained from Invitrogen.

**Cell lines**

The TRPM7-knockdown fibroblast cell line (3T3-M7shRNA6) and the non-silencing control line (3T3-shRNA-C) originated from Swiss 3T3 cells [A.T.C.C. (Manassas, VA, U.S.A.) number CCL-92] and were made by standard approaches using a previously characterized shRNA (shRNA6) that specifically targets mouse TRPM7 and using shRNA-C (non-silencing control shRNA) [24]. Cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium) with 10% (v/v) FBS (fetal bovine serum), unless otherwise indicated. Cells from frozen stocks were used 1–2 weeks after thawing to allow them to recover their normal morphology. The methods used to create the cell lines and a description of their full characterization were as described previously [24].

**Cell viability measurements**

Cell viability in response to treatment of cells with DOX, STS and CHX was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay or by manual cell counting using Trypan Blue exclusion. For the MTT assay, cells were incubated with 1 \(\mu\)g/ml MTT for 4 h and then dissolved in 150 \(\mu\)l dimethyl sulfoxide. The \(A_{570}\) was measured and normalized to untreated cells.

**Detection of PARP (poly(ADP-ribose) polymerase), caspase 3 and MAPKs**

Cells were lysed in ice-cold RIPA buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Igepal CA-630, 0.5% deoxycholate and 0.1% SDS] with protease inhibitors (Roche). Samples of the lysates were analysed by SDS/PAGE (8% gel for PARP and 12% gel for all others) and Western blotting using standard procedures. PARP and caspase 3 were detected with rabbit polyclonal antibodies obtained from Cell Signaling Technology. ERK1/2 (extracellular-signal-related kinase 1/2) and JNK were detected with monoclonal antibodies from Santa Cruz Biotechnology. Levels of p38 MAPK (phospho-p38 (Thr180/Tyr182)) were detected, with monoclonal antibodies from Santa Cruz Biotechnology. Activated p38 MAPK (phospho-p38 (Thr180/Tyr182)) and JNK (phospho-JNK (Thr183/Tyr185)) were detected with antibodies from Cell Signaling Technology. For immunochromatometric detection, the SuperSignal West Maximum Sensitive Substrates (Pierce) Pico and Dura were used, depending the strength of the signal.

**Measurement of ROS levels**

Swiss 3T3 fibroblasts were trypsinized, washed with PBS and resuspended in Phenol Red-Free culture medium. Cells were then labelled with 5 \(\mu\)M CM-H\(_2\)DCFDA for 20 min at 37°C under 5% CO\(_2\). After being washed with PBS, the cells were resuspended in Phenol Red-free DMEM and analysed using a Beckman Coulter Cytomics FC500 Flow Cytometer equipped with a 488-nm argon ion laser and a 525-nm bandpass emission filter.

**Measurement of intracellular Mg\(^{2+}\) and ATP**

For measurement of intracellular Mg\(^{2+}\). Swiss 3T3 fibroblasts were trypsinized, washed twice with PBS, and resuspended in Phenol Red-free DMEM. Cells were then labelled with 5 \(\mu\)M Mag-Indo-1 and incubated for 35 min at 37°C under 5% CO\(_2\). After being washed twice with PBS, cells were resuspended in Phenol Red-free DMEM, and blue and violet fluorescence emissions were collected using a Beckman Coulter MoFlo XDP cell sorter equipped with a 355-nm laser at room temperature (25°C). For measurement of intracellular ATP, cells were plated at a density of 2.5 \(\times\)10\(^4\)/well on a 96-well plate and intracellular ATP levels were measured after 12 h using an EnzyLight™ ATP assay (BioAssay Systems) according to the manufacturer’s instructions. Luminescence was measured on a TECAN GENios Pro Microplate Reader and intracellular ATP levels were quantified using ATP standards.

**Cell cycle analysis**

Cells were cultured in DMEM with 10% (v/v) FBS and harvested at 70% confluence for cell cycle analysis. To enrich cells in the G0/G1 phase of the cell cycle, cells were serum-starved in medium containing 0.1% FBS and kept at 100% confluence for 48 h. Cells were harvested and washed with PBS and then fixed in 70% ethanol for 30 min. Cells were then washed again with PBS and then resuspended in PBS containing propidium iodide (10 \(\mu\)g/ml) and RNase (200 \(\mu\)g/ml) at room temperature for 30 min. Samples were analysed using a Beckman Coulter Cytomics FC500 flow cytometer.
Adenoviral transduction

To confirm the role of TRPM7 in cell survival and its control of ROS levels, we conducted gain-of-function studies by re-expressing TRPM7 in M7shRNA6 cells using a recombinant adenovirus that expresses SR-TRPM7 (silencing-resistant mouse TRPM7). To assess the contribution of TRPM7’s kinase domain and its ability to permeate Mg\(^2+\) to these processes, we also conducted gain-of-function studies using recombinant adenoviruses expressing a kinase-inactive SR-TRPM7 (SR-TRPM7-G1618D) and the FLAG-tagged Mg\(^2+\)-specific transporter SLC41A2. Expression of LacZ was used as the negative control for adenovirus transduction. Detailed information regarding the creation of these reagents has been described previously [24]. Viral titres were determined by a plaque-forming assay using HEK-293A cells (Invitrogen). For gain-of-function studies, M7shRNA6 cells were transduced with recombinant adenoviruses at an MOI (multiplicity of infection) ranging from 100 to 200 and analysed 5 days post-transduction.

RESULTS

Several investigations have indicated that the TRPM7 channel contributes to the demise of neurons in response to OGD and other forms of cellular stress. Depletion of the channel by RNA interference has been shown to decrease Ca\(^{2+}\) overload, Zn\(^{2+}\) influx, and lower cellular Mg\(^{2+}\) levels, all of which may be acting in concert to influence cell survival [17,20,21]. In a previous study we showed that HEK-293 cells could be rendered more resistant to several forms of apoptotic stimuli by reducing TRPM7 protein expression by RNA interference as well as by direct pharmacological blockade of the channel, indicating that the channel is influencing the cell death process in other cell types besides neurons [23]. Despite this progress, the molecular mechanism by which the channel affects cell survival has remained poorly understood.

Depletion of TRPM7 increases cell viability in response to apoptotic stimuli

To further understand the process by which TRPM7 influences cell survival, we employed a TRPM7-knockdown fibroblast cell line (M7shRNA6) derived from Swiss 3T3 cells, which we recently used to demonstrate a critical role for the channel in polarized cell movements [24]. M7shRNA6 cells express an shRNA that specifically targets the channel kinase, reducing TRPM7 expression by approximately 80% compared with a shRNA-C [24,25]. To determine whether M7shRNA6 cells are a good model system for investigating TRPM7’s role in cell death, we evaluated their resistance to apoptotic stimuli. TRPM7-knockdown cells were more resistant than WT (wild-type) Swiss 3T3 cells or control cells (shRNA-C) to cell death following treatment with the apoptotic inducers DOX, CHX and STS, as assessed by an MTT assay (Figure 1A). Similar results were obtained using the Trypan Blue exclusion assay (Supplementary Figure S1 at http://www.BiochemJ.org/bj/445/bj4450441add.htm). These results establish M7shRNA6 cells as a suitable system for investigating the mechanism by which TRPM7 affects cell survival.

As TRPM7 has also been shown to be critical for cell growth and proliferation, we next investigated whether dysregulation of the cell cycle was affecting the resistance of M7shRNA6 cells to cell death. Consistent with previous reports, TRPM7-knockdown fibroblasts (M7shRNA6 cells) proliferated more slowly, with a higher percentage of cells in the G\(_0\)/G\(_1\) phase of the cell cycle, compared with shRNA-C control cells (Supplementary Figures S2A and S2B at http://www.BiochemJ.org/bj/445/bj4450441add.htm) [6–8]. However, M7shRNA6 cells grown to confluence and serum-starved, to enrich cells in the G\(_0\)/G\(_1\) phase of the cell cycle, showed a similar decreased sensitivity to apoptotic stimuli compared with arrested control shRNA-C cells, but showed increased amounts of ERK1/2 and ERK1/2 phosphorylation (p-ERK) following 24 h and 36 h of treatment with 2 \(\mu\)M DOX. \(\beta\)-Actin is shown as a loading control for the samples. Molecular mass in kDa is shown on the left-hand side.

![Figure 1 Depletion of TRPM7 increases cell resistance to apoptotic stimuli and reduces apoptotic signalling](Image)
M7shRNA6 cells’ decreased sensitivity to apoptotic stimuli was due to a disruption in apoptotic signalling, we next examined the cleavage of PARP and of caspase 3 following treatment of cells with the apoptosis inducer DOX. Caspase 3 is a proenzyme activated by proteolytic cleavage during apoptosis by both extrinsic (death ligand) and intrinsic (mitochondrial) pathways [26,27]. PARP is a substrate of caspase 3 that is inactivated by caspase-mediated proteolysis [28]. M7shRNA6 cells exhibited reduced PARP and caspase 3 cleavage following DOX treatment (Figure 1B). Conversely, levels of pro-survival ERK1/2 and its activated forms were elevated in TRPM7-knockdown cells compared with control shRNA-C cells (Figure 1B). As apoptosis is an ATP-dependent process, we investigated whether TRPM7-knockdown cells have lower levels of ATP compared with control cells, which could possibly account for the difference in apoptotic signalling we observed [29,30]. However, the concentration of ATP in the two cell lines was very similar (Supplementary Figure S4 at http://www.BiochemJ.org/bj/445/bj4450441add.htm). So although these results indicated that depletion of TRPM7 interferes with apoptotic signalling, the mechanism by which TRPM7 affects cell survival remained unclear.

Depletion of TRPM7 interferes with p38 MAPK and JNK activation and lowers ROS levels

In a previous study we showed that overexpression of TRPM7 in HEK-293 cells increased levels of ROS and concomitantly stimulated the activation of p38 MAPK and JNK [22]. These results prompted us to investigate whether depletion of TRPM7 has the opposite effect and diminishes p38 MAPK and JNK activation in response to oxidative stress. Application of H2O2, from which hydroxyl radicals are produced by the Fenton reaction, induced robust activation of p38 MAPK and JNK in shRNA-C cells compared with M7shRNA6 cells (Figure 2A). We next investigated whether depletion of TRPM7 lowers ROS levels by measuring the concentration of ROS by flow cytometry using the ROS indicator CM-H2DCFDA. TRPM7-knockdown cells (M7shRNA6) had a significantly lower concentration of ROS compared with shRNA-C cells, both in the absence of apoptotic stimuli and following apoptotic inducers (Figure 2B). These data indicate that TRPM7 is affecting ROS levels both in the absence and presence of cellular stress.

To confirm TRPM7’s impact on cellular ROS levels, we next investigated whether re-expression of TRPM7 can restore the concentration of ROS in M7shRNA6 cells to the level found in control cells. In a previous study we created a recombinant adenovirus that expresses SR-TRPM7, which was designed to evade RNA interference from expression of a TRPM7-specific shRNA (shRNA6) [24]. We recently employed SR-TRPM7 in gain-of-function studies with M7shRNA6 cells to demonstrate a role for TRPM7 in polarized cell movements [24]. Transduction of M7shRNA6 cells with the recombinant SR-TRPM7 adenovirus increased the concentration of ROS to a similar level as detected in control shRNA-C cells (Figure 3). M7shRNA6 cells transduced with a recombinant LacZ-expressing adenovirus as a negative control had a similar concentration of ROS compared with non-transduced M7shRNA6 cells. We next investigated whether TRPM7’s kinase was affecting cellular ROS levels by transducing a recombinant kinase-inactive TRPM7 (SR-TRPM7-G1618D) adenovirus into M7shRNA6 cells and determining whether expression of the kinase-dead mutant could also increase the concentration of ROS [24]. Re-expression of SR-TRPM7-G1618D in M7shRNA6 cells was as effective in boosting ROS levels as expression of the WT channel (Figure 3). These results indicate that loss of TRPM7’s channel activity is responsible for the decrease in ROS in TRPM7 knockdown cells. However, the mechanism by which the channel affects the concentration of ROS remained unclear.

Expression of the Mg2+ transporter SLC41A2 increases ROS levels and restores sensitivity to apoptotic stimuli

Deletion of TRPM7 in the DT40 B-cell line causes proliferation arrest in G0/G1 and G2 phases of the cell cycle that can be reversed by supplementation of the growth medium with
10–15 mM Mg$^{2+}$ or by expression of the Mg$^{2+}$ transporter SLC41A2 [6,7,31]. We have recently shown that depletion of TRPM7 during early embryonic development can interfere with convergent extension cell movements during gastrulation [10]. The gastrulation defect can be prevented by Mg$^{2+}$ supplementation as well as by co-expression of the Mg$^{2+}$ transporter SLC41A2 with antisense TRPM7 morpholinos that target X. laevis TRPM7 [10]. As mentioned above, reducing TRPM7 expression in Swiss 3T3 fibroblasts impairs the ability of cells to execute polarized cell movements [24]. The defect in cell polarity in TRPM7-knockdown cells can also be rescued by expression of the Mg$^{2+}$ transporter SLC41A2 [24]. We therefore investigated whether expression of SLC41A2 could increase the concentration of ROS in M7shRNA6 cells. Viral transduction of the recombinant Mg$^{2+}$ transporter SLC41A2 adenovirus into M7shRNA6 cells increased ROS levels in the TRPM7-knockdown cells and elevated them to a level similar to that measured in shRNA-C cells. As expected, expression of LacZ in M7shRNA6 cells did not change the concentration of ROS (Figure 4). Interestingly, supplementation of the growth medium with 10 mM Mg$^{2+}$ increased ROS levels in shRNA-C cells, but not in the TRPM7-knockdown cell line (Figure 4). Moreover, expression of SLC41A2 in M7shRNA6 cells restored the ability of Mg$^{2+}$ supplementation to increase ROS levels. These data strongly suggest that changes in intracellular Mg$^{2+}$ affect the concentration of ROS in cells.

To confirm that depletion of TRPM7 is affecting intracellular Mg$^{2+}$, we measured the concentration of free Mg$^{2+}$ in the WT Swiss 3T3 cells, TRPM7-knockdown M7shRNA6 cells, and control shRNA-C cells by flow cytometry using the Mg$^{2+}$ indicator mag-fluo-4 (Supplementary Figure S5 at http://www.BiochemJ.org/bj/445/bj4450441add.htm). To evaluate whether re-expression of TRPM7 was able to restore intracellular Mg$^{2+}$ in M7shRNA6 cells to a level similar to that in control cells, we transduced the recombinant adenovirus expressing SR-TRPM7 into TRPM7-knockdown cells. Re-expression of SR-TRPM7 in M7shRNA6 cells increased the concentration of intracellular Mg$^{2+}$ concentration to a similar level as that observed in WT and control cells (shRNA-C) (Figure 5). Similar results were obtained using the Mg$^{2+}$ indicator mag-6FAM (Supplementary Figure S5 at http://www.BiochemJ.org/bj/445/bj4450441add.htm).

**Figure 3** Re-expression of TRPM7 restores ROS production in TRPM7-knockdown cells

ROS production in fibroblasts was measured by FACS analysis labelled with the fluorescent ROS indicator CM-H$_2$DCFDA. Adenovirus-mediated re-expression of SR-TRPM7 or of the kinase-inactive SR-TRPM7 (SR-TRPM7-G1618D) in TRPM7-knockdown cells (M7shRNA6) increased ROS production to a level similar to that of control fibroblasts (shRNA-C), whereas expression of LacZ had no effect. Results are means ± S.D. for two independent experiments. The asterisk (*) indicates a significant difference in ROS production in TRPM7-knockdown cells and TRPM7-knockdown cells expressing LacZ compared with control cells (shRNA-C) by Student’s t test (P < 0.05).

**Figure 4** Expression of Mg$^{2+}$ transporter SLC41A2 restores ROS production in M7shRNA6 cells

Expression of the Mg$^{2+}$ transporter SLC41A2 in TRPM7-knockdown cells (M7shRNA6) increased ROS production to a similar level as control cells (shRNA-C) expressing LacZ. In addition, supplementation of the growth medium with excess 10 mM Mg$^{2+}$ augmented ROS production in shRNA-C cells and SLC41A2-expressing M7shRNA6 cells, but not in TRPM7-knockdown cells (M7shRNA6) expressing LacZ. Results are means ± S.D. for two independent experiments. H, cells grown in high Mg$^{2+}$ (10 mM); N, cells grown in normal Mg$^{2+}$ (0.95 mM). The hash symbol (#) indicates a significant difference in ROS production between M7shRNA6 cells and M7shRNA6 cells expressing SLC41A2, and the asterisk (*) indicates a significant difference in the ROS production between samples grown in normal growth medium and samples grown in the medium supplemented with 10 mM Mg$^{2+}$ by Student’s t test (P < 0.05).
To further examine the effect of Mg$^{2+}$ on the cellular response to apoptotic stimuli, we next investigated whether expression of the Mg$^{2+}$ transporter SLC41A2 in TRPM7-knockdown M7shRNA6 cells decreases cell viability in response to apoptotic stimuli. M7shRNA6 cells transduced with the recombinant SLC41A2 adenovirus restored TRPM7 knockdown cells more sensitive to cell death induced by the apoptotic inducers DOX, CHX and STS (Figure 6A). In addition, following treatment with apoptotic inducers, levels of ROS were significantly elevated in M7shRNA6 cells expressing SLC41A2 compared with M7shRNA6 cells expressing LacZ (Figure 6B). Collectively, these results indicate that intracellular Mg$^{2+}$ potently regulates ROS levels as well as the cellular response to apoptotic stimuli, revealing a heretofore unrecognized connection between TRPM7’s control of Mg$^{2+}$ homoeostasis and the channel’s impact on cell survival.

**DISCUSSION**

Ca$^{2+}$ has a clearly established role in apoptotic signalling pathway; however, there is very little evidence linking Mg$^{2+}$ to this important cellular process [27]. In the present study, we have uncovered a role for TRPM7’s control of cellular Mg$^{2+}$ homoeostasis in regulating ROS production during cell stress. In the present study, we showed that knockdown of TRPM7 in Swiss 3T3 fibroblasts increased cellular viability in response to a range of apoptotic stimuli. This result parallels the increased resistance to apoptotic stimuli detected in HEK-293 cells depleted of the channel kinase [23]. Reduction of TRPM7 expression in fibroblasts decreased apoptotic signalling, as revealed by reduced proteolytic cleavage of caspase 3 and PARP. Moreover, knockdown of TRPM7 diminished the activation of p38 MAPK and of JNK in response to cell stress. TRPM7-knockdown fibroblasts had lower amounts of ROS than control cells. The decrease in ROS caused by depletion of the channel was reversed by re-expression of TRPM7. Surprisingly, expression of the Mg$^{2+}$-specific transporter SLC41A2 in TRPM7-knockdown cells was equally effective in restoring levels of ROS as well as in...
re-sensitizing cells to apoptotic stimuli. Another important finding of the present study was that supplementation of the growth medium with 10 mM Mg$^{2+}$ increased ROS levels for shRNA-C cells and for TRPM7-knockdown M7shRNA6 cells expressing SLC41A2. In contrast, Mg$^{2+}$ supplementation of the growth medium did not affect the concentration of intracellular free Mg$^{2+}$ in M7shRNA6 cells nor did it elevate ROS levels, indicating that TRPM7 probably constitutes the major pathway for Mg$^{2+}$ entry in Swiss 3T3 fibroblasts. This finding explains why in our previous study we found that in defected in polarized cell movements by TRPM7-knockdown fibroblasts could be rescued by expression of the Mg$^{2+}$-transporter SLC41A2, but not by Mg$^{2+}$ supplementation [24]. Mg$^{2+}$ supplementation is, however, able to rescue the proliferation arrest caused by depletion of TRPM7 in DT40 B-cells [6,7]. The most parsimonious explanation for this difference is that Swiss 3T3 fibroblasts rely primarily on TRPM7 for Mg$^{2+}$ entry, whereas DT40 cells employ TRPM7 as well as another Mg$^{2+}$ transporter, such as SLC41A2, for Mg$^{2+}$ transport, and are thus able to compensate for loss of the channel when cells are cultured in high Mg$^{2+}$. Thus the failure to rescue a phenotype caused by loss of TRPM7 using Mg$^{2+}$ supplementation should be interpreted cautiously, as the success of this approach is probably dependent upon the presence of an alternate pathway for cellular Mg$^{2+}$ entry.

Our observation that Mg$^{2+}$ influences the concentration of ROS is consistent with the findings of Maier and colleagues, who reported that HC11 mouse mammary epithelial cells cultured in medium containing low extracellular Mg$^{2+}$ had decreased ROS levels [32]. Adding Mg$^{2+}$ back into the growth medium was able to return the concentration of ROS in HC11 cells to a normal level. The mechanism by which Mg$^{2+}$ controls cellular ROS levels remains unclear. Lower intracellular Mg$^{2+}$ may be slowing ROS production, increasing ROS turnover, or some combination of both. For example, HC11 cells cultured in low Mg$^{2+}$ had increased glutathione transferase activity [32]. Glutathione transferease are a family of scavenger enzymes involved in the detoxification of free radical species [33]. In addition, other studies have reported that Mg$^{2+}$ can stimulate the activity of NADPH oxidase, a major source of ROS within cells [34,35]. Mg$^{2+}$ may also affect ROS production by mitochondria [36]. At this point, the role of Mg$^{2+}$ in controlling ROS levels still remains undefined, and additional work is needed to give a clearer picture of how Mg$^{2+}$ regulates oxidative stress. Nevertheless, the present study has shown that changes in Mg$^{2+}$ homoeostasis can affect ROS levels, which in turn can potentiate influence the cellular response to apoptotic stimuli. Thus the mechanism by which TRPM7 controls cell survival is more complex than originally envisioned.

Tymianski, MacDonald and colleagues were the first to identify a critical role for TRPM7 in neuronal cell death during ischaemia, showing that knockdown of TRPM7 expression by RNA interference decreased neuronal death and Ca$^{2+}$ overload in response to OGD [17]. The connection between TRPM7, Ca$^{2+}$ and cell stress was also revealed by another study, which found that suppression of TRPM7 expression in PC12 cells reduced the increase in intracellular Ca$^{2+}$ caused by LPS (lipopolysaccharide) treatment [37]. Undoubtedly, intracellular Ca$^{2+}$ plays a key role in the apoptotic pathway and is important to TRPM7’s control over cell survival. However, evidence suggests that other cations contribute to neuronal cell death via TRPM7. For example, Zn$^{2+}$- induced neurotoxicity was revealed to be dependent on TRPM7’s ability to transport Zn$^{2+}$ [20]. In addition, TRPM7 has been shown to contribute to increases in intracellular Mg$^{2+}$ in rat hippocampal neurons following hypoxia [21]. The results of the present study suggest that, during ischaemia, changes in intracellular Mg$^{2+}$ levels could be affecting ROS levels and susceptibility to neuronal cell death. Additional studies are required to evaluate this hypothesis as well as to determine whether Mg$^{2+}$ homoeostasis affects ROS levels in other systems, especially as different cell types have their own distinctive response to cell stress in addition to their own unique ion transport mechanisms. It is becoming increasingly clear that different ions act in concert to regulate cell death, and that additional studies are required to understand how various divalent cations function together. TRPM7, with its ability to permeate multiple species of cations, is uniquely adapted to orchestrate their collective actions.

**Author Contribution**

Hsia-Chin Chen, Li-Ting Su, Omaya González-Pagán and Jeffrey Overton performed the experiments. Loren Runnels and Hsia-Chin Chen planned the experiments, analysed the experimental data and wrote the paper.

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SUPPLEMENTARY ONLINE DATA

A key role for Mg$^{2+}$ in TRPM7's control of ROS levels during cell stress

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Figure S1 Depletion of TRPM7 increases cell resistance to apoptotic stimuli

Manual cell counting using a Trypan Blue exclusion assay showed increased resistance of TRPM7-knockdown Swiss 3T3 fibroblasts (M7shRNA6) to the apoptotic stimuli DOX, CHX and STS compared with WT or control cells (expressing shRNA-C). Cells were treated with DOX (2 μM), CHX (10 μg/ml) and STS (2 μM) for 48 h. Results are means ± S.D. for three independent experiments. The asterisk (*) indicates a significant difference in cell viability between TRPM7-knockdown cells and WT and control cells by Student's t test ($P < 0.05$).

Figure S2 TRPM7-knockdown fibroblasts proliferate more slowly

(A) Cell cycle distribution of TRPM7-knockdown cells (M7shRNA6), WT and control cells (shRNA-C). Cell cycle distributions were obtained by FACS analysis and propidium iodide staining. TRPM7-knockdown fibroblasts (M7shRNA6) cells are more enriched in the G$_0$/G$_1$ phase of the cell cycle compared with WT and control cells (shRNA-C). (B) TRPM7-knockdown cells (M7shRNA6) proliferate more slowly than WT and control cells (shRNA-C). Cells were manually counted every 24 h for 3 days ($n = 6$).

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Figure S3  TRPM7-knockdown cells enriched in the G₀/G₁ phase of the cell cycle exhibited decreased cell death following treatment with apoptotic inducers

(A) Cell cycle distribution of TRPM7-knockdown cells (M7shRNA6) and control cells (shRNA-C) that were serum-starved for 48 h at 100% confluence to enrich cells in the G₀/G₁ phase of the cell cycle. Cells were untreated or treated with the apoptotic inducers DOX (2 μM), CHX (10 μg/ml) and STS (2 μM) for 48 h after proliferation arrest. (B) MTT assay demonstrates that TRPM7-knockdown cells exhibited more resistance to apoptotic stimuli when enriched in the G₀/G₁ phase of the cell cycle compared with shRNA-C cells. Cells were treated with DOX (2 μM), CHX (10 μg/ml) and STS (2 μM) for 48 h after proliferation arrest.

Figure S4  shRNA-C and M7shRNA6 cells have similar levels of ATP

Measurement of ATP levels in shRNA-C and M7shRNA6 cells using a bioluminescent assay indicated that M7shRNA6 cells had approximately 5% less ATP compared with shRNA-C cells. Results are means ± S.D. for three independent experiments.

Figure S5  TRPM7-knockdown fibroblasts have lower intracellular Mg²⁺

Measurement of free Mg²⁺ concentration in TRPM7-knockdown M7shRNA cells and control shRNA-C cells by FACS analysis using the Mg²⁺ indicator Mag-fluo-4 indicated that M7shRNA6 cells have a lower Mg²⁺ concentration than control cells. Results are means ± S.D. for three independent experiments; *P < 0.05.