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# Importance of melastatin-like transient receptor potential 7 and cations (magnesium, calcium) in human osteoblast-like cell proliferation

E. Abed and R. Moreau

*Laboratoire du Métabolisme Osseux, Centre BioMed, Département des Sciences Biologiques, Université du Québec à Montréal, Montréal, Québec, Canada*

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**Abstract.** Bone tissue in the adult is continuously being remodelled, and overall bone mass is maintained constant by the balance between osteoclastic bone resorption and osteoblastic bone formation. Adequate osteoblastic proliferation is essential for both appropriate formation and for regulation of resorption, and thereby the maintenance of bone remodelling equilibrium. *Objectives*: Here, we have investigated the roles of melastatin-like transient receptor potential 6 and 7 (TRPM6, TRPM7), which are calcium ($Ca^{2+}$) and magnesium ($Mg^{2+}$) conducting channels, during proliferation of human osteoblasts. *Results*: Genetic expression of TRPM6 and TRPM7 was shown in human osteoblast-like MG-63, SaOS and U2-OS cells, and reduction of extracellular $Mg^{2+}$ or $Ca^{2+}$ led to a decrease of cell proliferation. Concomitant reduction of both ions further accentuated reduction of cell proliferation. Expression of TRPM7 channels was increased under conditions of reduced extracellular $Mg^{2+}$ and $Ca^{2+}$ levels whereas expression of TRPM6 was not modified, suggesting compensatory mechanisms afforded by TRPM7 in order to maintain intracellular ion homeostasis. Pre-incubation of cells in reduced extracellular $Mg^{2+}$ conditions led to activation of $Ca^{2+}$ and $Mg^{2+}$ influx. Reduction of TRPM7 expression by specific siRNA prevented latter influx and inhibited cell proliferation. *Conclusions*: Our results indicate that extracellular $Mg^{2+}$ and $Ca^{2+}$ deficiency reduces the proliferation of human osteoblastic cells. Expression and activity of TRPM7 is modulated by extracellular $Mg^{2+}$ and $Ca^{2+}$ availability, indicating that TRPM7 channels are involved in intracellular ion homeostasis and proliferation of osteoblasts.

## INTRODUCTION

Bone is a dynamic tissue that is continuously remodeled at coordinated rates. Under normal conditions, special cells called osteoclasts are transiently breaking down old bone (the resorption process) at numerous sites, as other cells known as osteoblasts are replacing it by synthesis of

Correspondence: Robert Moreau, Département des sciences biologiques, Université du Québec à Montréal, C.P. 8888, Succ. Centre-Ville, Montreal, QC H3C 3P8, Canada. Tel.: (514) 987-3000; Fax: (514) 987-4647; E-mail: moreau.robert@uqam.ca

new tissue (bone formation). Osteoblastic cells ensure bone formation and mineralization through secretion of bone matrix components (type I collagen and non-collagenous proteins) and also provide factors essential for the differentiation of osteoclasts, such as macrophage colony-stimulating factor and receptor activator of nuclear factor kappa B ligand. By regulating osteoclast differentiation, osteoblasts play a central role not only in bone formation but also in the regulation of bone resorption (Mackie 2003). In this regard, adequate osteoblast proliferation, differentiation, secretory function or rate of apoptosis are essential for both adequate formation and for resorption processes, and thereby maintenance of bone remodelling equilibrium. Clinical and histomorphometric studies have demonstrated that ageing is associated with decreased bone mass and that reduced bone formation is an important pathogenetic factor (Kragstrup *et al*. 1983; Parfitt 1991; Brockstedt *et al*. 1993). In rodent models of ageing, studies have indicated a deficit of osteoprogenitor cells in the bone marrow, and a reduction in the number of osteoblasts (Liang *et al*. 1992; Roholl *et al*. 1994; Quarto *et al*. 1995; Bergman *et al*. 1996; Kotev-Emeth *et al*. 2000; Chen 2004). Similar observations have been obtained for humans, in addition with a defect in cell proliferation (Pfeilschifter *et al*. 1993; Kato *et al*. 1995; Neidlinger-Wilke *et al*. 1995; Bergman *et al*. 1996; D'Ippolito *et al*. 1999; Martinez *et al*. 1999). Cellular ageing and senescence have been reported *in vitro* with human osteoprogenitor cells from bone marrow and osteoblasts (Kassem *et al*. 1997; Stenderup *et al*. 2003; Peterson *et al*. 2004). Thus, it has been established that with ageing, the process of coupled bone formation is affected by the reduction of osteoblast proliferation and lifespan (Chan & Duque 2002). In view of bone disorders such as osteoporosis, research regarding osteoblast proliferation could bring new insights to therapeutic approaches.

Calcium influx is implicated in numerous cellular functions such as proliferation, differentiation, secretion and apoptosis (Berridge *et al*. 2000). In bone cells of the osteoblast lineage, calcium ($Ca^{2+}$) channels play fundamental roles in cellular responses to external stimuli including both mechanical forces and hormonal signals (Duncan *et al*. 1998; Iqbal & Zaidi 2005). Magnesium is also involved in the regulation of a large number of biochemical reactions and therefore influences important physiological functions such as nucleic acid metabolism, protein synthesis and energy production (Romani & Scarpa 2000). The 'membrane, magnesium mitosis' model of cell proliferation control suggests that upon mitogenic stimulus, cells are able to increase their intracellular magnesium ($Mg^{2+}$) content, likely by activating $Mg^{2+}$ influx, to levels optimum for the initiation of protein synthesis (Wolf *et al*. 2004; Rubin 2005). Therefore, influx of both extracellular $Ca^{2+}$ and $Mg^{2+}$ for proper intracellular ion homeostasis is likely solicited for cell proliferation.

Melastatin-like transient receptor potential (TRPM) is a recently emerging subfamily of the transient receptor potential protein, a diverse group of voltage-independent $Ca^{2+}$-permeable cation channels expressed in mammalian cells (Harteneck *et al*. 2000; Clapham *et al*. 2001; Minke & Cook 2002; Montell *et al*. 2002) that encompasses eight distinct members, designated TRPM1–8. TRPM7 combines structural elements of both an ion channel and a protein kinase (for recent reviews, see Fleig & Penner 2004; Harteneck 2005). Among TRPM members TRPM2, TRPM6 and TRPM7 uniquely possess an enzyme domain in their long C-termini, the latter two exhibiting spontaneously activated divalent cation ($Ca^{2+}$, $Mg^{2+}$ and other trace metals) entry, regulated by cytosolic $Mg^{2+}$ and ATP levels. Recently, TRPM7 channels have been associated with cell proliferation and survival (Nadler *et al*. 2001). TRPM7-deficient DT40 cells can be rescued from their cell growth defect by supplementary extracellular $Mg^{2+}$ (Schmitz *et al*. 2005) suggesting a residual capacity of these cells to maintain intracellular $Mg^{2+}$ homeostasis. Mutations in the TRPM6 gene have been shown in patients suffering from a hereditary form of hypomagnesaemia caused by impaired $Mg^{2+}$ resorption (Chubanov *et al*. 2004). Life-long dietary $Mg^{2+}$ supplementation of these patients is sufficient to rescue the phenotype of affected human beings. Furthermore, it has been shown that $Mg^{2+}$ deficiency leads to up-regulation of TRPM6 in mouse kidneys (Groenestege

*et al*. 2006). However, TRPM7 deficiency cannot be complemented by heterologously expressed TRPM6 (Schmitz *et al*. 2005) suggesting that both channels are not functionally redundant. In the present study, we investigated potential correlation between the $Ca^{2+}$- and $Mg^{2+}$-transporting activities of TRPM7 and the regulation of human osteoblast-like cell proliferation.

## MATERIALS AND METHODS

### Cell culture

Human osteoblast-like MG-63, SaOS and U2-OS cells were from the American Type Culture Collection (Rockville, MD, USA). MG-63 cells were grown in a 1 : 1 mixture of phenol-free Dulbeco's modified Eagle's medium (DMEM)/Ham's F12 medium (DMEM/F12; Sigma, Oakville, Ontario, Canada) and was supplemented with 10% foetal bovine serum (FBS; Cansera, Etobicoke, Ontario, Canada), L-glutamine (Invitrogen, Burlington, Ontario, Canada) and penicillin/streptomycin (Invitrogen). SaOS and U2-OS cells were cultured in McCoy's 5A medium (Hyclone, Logan, UT, USA) and were supplemented as described above. Cells were cultured in 5% $CO_2$ at 37 °C and were harvested once a week using Trypsin-EDTA solution (Invitrogen).

### Cell proliferation assays

For the proliferation experiments, cells were seeded in 24- or 96-well plates (Sarstedt, Montréal, Québec, Canada) at 2500 cells/cm$^2$. After 4 days of culture in supplemented media, they were then incubated in $Ca^{2+}$- and $Mg^{2+}$-free DME/F12 (Sigma, Oakville, Ontario, Canada) medium without serum, and supplemented with different concentrations of calcium and magnesium for 48 h. Cell proliferation was determined by haematocytometer cell counting (24-well plates) or by the microtiter tetrazolium (MTT) reduction assay (96-well plates). Briefly for MTT assays, 1 h before the end of treatment, medium was replaced with DMEM/F12 containing 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide (MTT) (Sigma). At the end of the incubation, medium was aspired and formazan crystals were dissolved in dimethyl sulfoxide. Absorbance was measured at 575 nm and data are expressed as the ratio of absorbance of treated cells versus initial MTT absorbance corresponding to the level of MTT reduced to formazan crystals by cells, the initial day of treatment.

For evaluation of DNA synthesis, cells were seeded in 96-well plates (Sarstedt) at 2500 cells/cm$^2$ and were allowed to grow for 4 days in supplemented DMEM/F12. Thereafter, they were incubated in $Ca^{2+}$- and $Mg^{2+}$-free DME/F12 media containing different concentrations of $Ca^{2+}$ and $Mg^{2+}$ or with 25 ng/mL of platelet-derived growth factor-BB (Sigma) in DMEM/F12, as the positive control. DNA synthesis was determined by bromodeoxyuridine (BrdU) incorporation into cellular DNA using an ELISA kit (Roche Diagnostics, Laval, Québec, Canada), following the procedure described by the manufacturer. Briefly, cells were incubated with BrdU for the last 2 h of incubation, and then were incubated with nuclease solution. Incorporation of BrdU was determined by incubation with anti-BrdU-peroxidase for 30 min and revealed by peroxidase substrate application. Data are expressed as the mean ± SEM of the percentage of DNA synthesis compared to DMEM/F12.

### Alkaline phosphatase activity

Measurement of alkaline phosphatase activity was performed by colourimetric assay of enzyme activity as described previously (Moreau *et al*. 1997). Cell monolayers (24-well plates) were washed three times with phosphate-buffered saline buffer (0.1 g/L $CaCl_2$, 0.2 g/L KCl, 0.2 g/L

$KH_2PO_4$, 0.1 g/L $MgCl_26H_2O$, 8 g/L NaCl, 1.44 g/L $Na_2HPO_4$, pH 7.4) and then were scraped into assay buffer (100 mM glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 1% Triton X-100, pH 10.5). Assays were performed in 96-well plates with 75 μL of lysate mixed with 75 μL of freshly prepared colourimetric substrate *para*-nitrophenyl phosphate (Sigma) solubilized in the assay buffer. The enzymatic reaction was conducted for 1 h at 37 °C and was stopped by adding 100 μL of NaOH 0.2 N. Optical density of the yellow product *para*-nitrophenol was determined spectrophotometrically at 410 nm. Protein concentrations were quantified by MicroBCA protein assay (Pierce, Rockford, IL, USA) using bovine serum albumin as standard. Alkaline phosphatase activity was then deduced as *para*-nitrophenol produced in nmol/h/mg of cellular protein.

**PCR amplification**

Total RNA from cells was extracted using TriZol (Invitrogen) according to the manufacturer's instructions. Reverse transcription (RT) reactions were carried out with Omniscript RT kit (Qiagen, Mississauga, Ontario, Canada) using hexamers. PCR amplifications were conducted with *Taq* PCR core kit (Qiagen) using specific primer sets for human TRPM7 (sense: 5′-TGCACCTATACTAG-GAAACGTTTTCG-3′; antisense: 5′-CATGATAAAAGGCATAAAACTTTCGC-3′) and for TRPM6 (sense: 5′-TCACTGGACCTATGAGTACACTCGG-3′; antisense: 5′-GACGCTGATG-TAATCAACATCACG-3′). Each primer was designed in distinct exons to ensure specific transcript amplification. Briefly, amplifications were carried out for 40 cycles according to incubation of 1 min at 94 °C, 30 s at 58 °C and 1 min at 72 °C. Amplification products were resolved in 2% agarose gel with ethidium bromide staining.

For real-time quantitative PCR (RQ-PCR) analysis, RNA was extracted using the Rneasy kit (Qiagen) and cDNA was synthesized with Ominiscript RT kit using hexamers. RQ-PCR was performed with a LightCycler 1.5 system (Roche Diagnostics, Laval, Québec, Canada) using SYBR Premix Ex *Taq* solution (Takara Bio, Shiga, Japan), with specific primer sets for human TRPM7 (purchased from Qiagen) and expression levels were normalized to the housekeeping gene β-2-microglobulin expression (sense: 5′-ATCCAGCGTACTCCAAAGA-3′; antisense: 5′-GACAA-GTCTGAATGCTCCAC-3′). Amplifications were performed in LightCycler capillaries (20 μL) and data were analysed with the LightCycler software version 3.5 (Roche Diagnostics, Mannheim, Germany).

**Interference with siRNA**

Small interfering RNAs (siRNAs) directed against human TRPM7 [siTRPM7(1) and siTRPM7(2)], and a non-targeting control [siRNA(–)] were obtained from Qiagen. Transfection of the siRNAs was performed using HiPerFect reagent (Qiagen) following the manufacturer's instructions. Quantification of transcripts was performed by RQ-PCR 2 days post-transfection to evaluate TRPM7 expression level. To examine the role of TRPM7 in cell proliferation, cells grown in 96-well plates were transfected for 24 h, and thereafter were incubated in appropriate conditions for 48 h, with addition of MTT for the last 1 h of incubation.

**Measurements of intracellular $Ca^{2+}$ and $Mg^{2+}$ levels**

MG-63 cells were cultured in 4-well Labtek (Nalge Nunc, Naperville, IL, USA) plates for 5 days in supplemented media. They were then transferred to HEPES-buffered saline solution (HBSS (mM): 121 NaCl, 5.4 KCl, 0.8 $MgCl_2$, 25 HEPES, 1.8 $CaCl_2$ and 6.0 $NaHCO_3$ at pH 7.3) or $Ca^{2+}$-free and/or $Mg^{2+}$-free solution (HBSS without $Ca^{2+}$ and/or without $Mg^{2+}$) before being loaded with 2 μM Fluo-3 acetoxymethyl or Magnesium Green (Molecular Probes, Eugene, OR, USA), with an equivalent volume of 20% pluronic F127 (Molecular Probes), for 45 min at room temperature in the dark. Thereafter, cells were washed with corresponding HBSS and the loaded

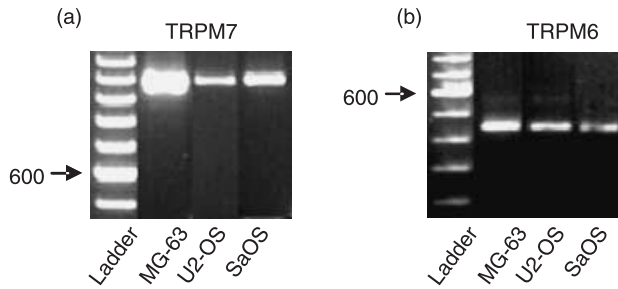


**Figure 1. Analysis of genetic expression of TRPM6 and TRPM7 channels in human osteoblast-like cells.** Total RNA from MG-63, SaOS and U2-OS cells was isolated and subjected to RT-PCR using specific primers for TRPM6 (NM_017662) and TRPM7 (NM_017672) channels. Results are representative data obtained from three independent isolations of RNA. Ladder of 100 bp.

dye was allowed to de-esterify for 45 min at room temperature in the dark. Following transfer to a $Ca^{2+}$- and $Mg^{2+}$-free HBSS, additions of $Ca^{2+}$ or $Mg^{2+}$ were made in an open chamber configuration at room temperature. The cells were examined with a laser scanning confocal (Bio-Rad Laboratories, Hercules, CA, USA) microscope (Nikon TE300, Tokyo, Japan) with an Apochromatic 40X N.A. 1.0 objective lens. Fluorescence was excited by an argon laser at 488 nm and emission was collected with a 515 filter. Data were analysed with Laser Sharp 2.1T, Time Course 1.0 software (Hemel Hempstead, UK).

### Statistical analysis

Statistical differences were evaluated using GraphPad Prism 3 software (San Diego, CA, USA). A level of $P < 0.05$ was considered significant.

## RESULTS

### Expression of TRPM6 and TRPM7 channels in human osteoblast-like cells

Because some members of the TRPM family, especially TRPM6 and TRPM7, have been identified as spontaneously activated $Ca^{2+}$- and $Mg^{2+}$-entry channels, suggested to be essential for cell proliferation, we evaluated their expression in human osteoblast-like cell lines. As shown in Fig. 1a, amplicons of expected size (976 bp) corresponding to the presence of messenger RNA for human TRPM7 were revealed by RT-PCR in MG-63, SaOS and U2-OS cells. Expression of TRPM6 was also shown in osteoblastic cell lines (Fig. 1b, amplicons of 441 bp). As for channels with enzyme domains, presence of transcripts for TRPM2 was not revealed by RT-PCR (data not shown). The identity of the amplicons was confirmed by restriction enzyme digestion (data not shown).

### Influence of reduced extracellular $Mg^{2+}$ and $Ca^{2+}$ on osteoblast proliferation

Because expression of TRPM6 and TRPM7 occurred in osteoblasts, we determined the importance of extracellular $Ca^{2+}$ and $Mg^{2+}$ for their proliferation. MTT assays were performed on MG-63 cells maintained for 48 h in $Ca^{2+}$- and $Mg^{2+}$-free DME/F12 supplemented with various concentrations of both ions. Experiments were performed in the absence of serum in order to exclude addition of $Ca^{2+}$ and $Mg^{2+}$ from FBS. In serum-free conditions, MG-63 cells showed a basal level of cell proliferation ($2.12 \pm 0.21$ relative to the initial MTT activity after 48 h). As shown

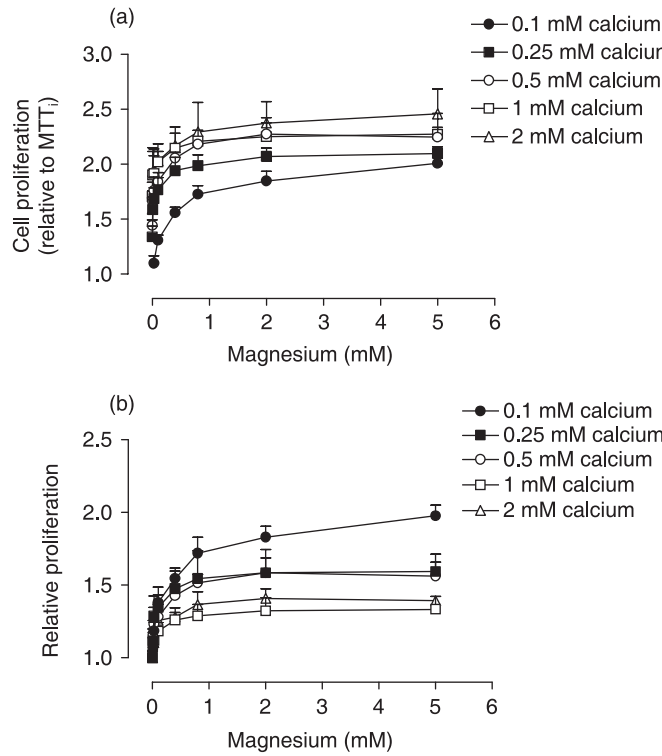


**Figure 2. Effect of extracellular $Mg^{2+}$ on MG-63 proliferation.** Cells were incubated for 48 h in culture media containing different concentrations of $Ca^{2+}$ and increasing concentrations of $Mg^{2+}$, and cell proliferation was determined by MTT assays. Results are expressed as (a) ratio of absorbance compared to initial MTT result or (b) as the relative effect of $Mg^{2+}$ compared to conditions without $Mg^{2+}$. Values for normal DMEM/F12 were 2.12 ± 0.21 relative to the initial MTT. Data are means ± SEM of at least three individual experiments.

in Fig. 2a, concentrations of $Mg^{2+}$ below 0.8 mM reduced cell proliferation at all concentrations of $Ca^{2+}$. No significant difference in cell proliferation was observed for concentrations of $Mg^{2+}$ between 0.8 and 5 mM. When expressed in terms of relative proliferation compared to conditions without $Mg^{2+}$ (Fig. 2b), relative stimulation in the presence of $Mg^{2+}$ was higher at 0.1 mM $Ca^{2+}$ compared to 0.5 mM $Ca^{2+}$ and 1 mM $Ca^{2+}$ ($P < 0.001$, ANOVA), and a significant difference between 0.5 and 1 mM $Ca^{2+}$ ($P < 0.05$, ANOVA) was still observed (Table 1). It should be noted that cell proliferation did not reach control levels (1 mM $Ca^{2+}$ and 0.8 mM $Mg^{2+}$) with higher concentrations of $Mg^{2+}$ (5 mM) under low $Ca^{2+}$ levels. As shown in Fig. 3a, concentrations of $Ca^{2+}$ below 0.5 mM reduced cell proliferation at all concentrations of $Mg^{2+}$. No difference in cell proliferation was observed for concentrations of 0.5 mM $Ca^{2+}$ and above. When expressed in terms of relative proliferation compared to those in low $Ca^{2+}$ conditions (0.1 mM) (Fig. 3b), relative stimulation by $Ca^{2+}$ was higher for conditions without $Mg^{2+}$ and 0.025 mM $Mg^{2+}$ ($P < 0.0001$, ANOVA), 0.1 mM $Mg^{2+}$ ($P < 0.0003$, ANOVA), 0.4 mM $Mg^{2+}$ ($P < 0.05$, ANOVA) compared to 0.8 mM (Table 1). It should be noted that cell proliferation did not reach control levels (1 mM $Ca^{2+}$ and 0.8 mM $Mg^{2+}$) with higher concentrations of $Ca^{2+}$ (2 mM) under conditions of zero extracellular $Mg^{2+}$. Protein quantification also confirmed relative reduction of cell proliferation by low extracellular $Ca^{2+}$ and $Mg^{2+}$ (data not shown). As shown in Table 2, osteoblast phenotype

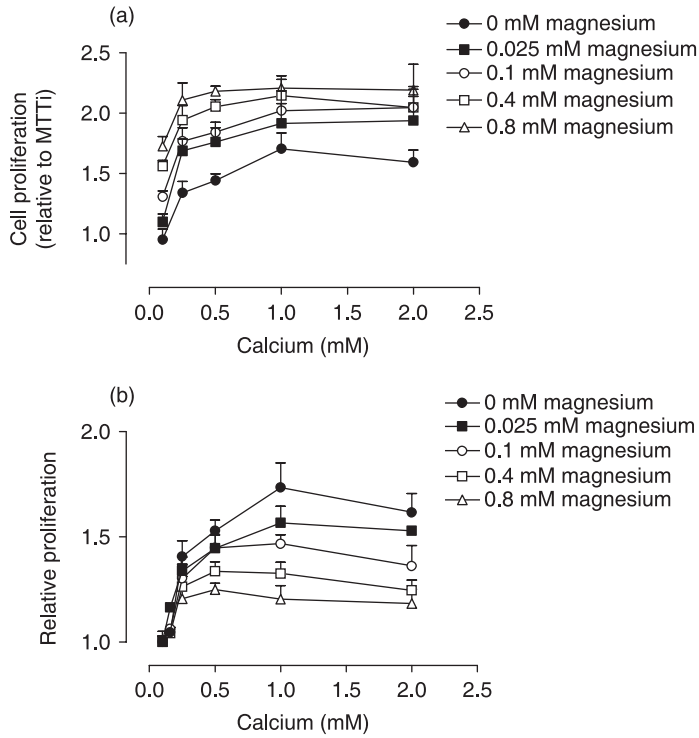


**Figure 3. Effect of extracellular $Ca^{2+}$ on MG-63 proliferation.** Cells were incubated for 48 h in culture media containing different concentrations of $Mg^{2+}$ and increasing concentrations of $Ca^{2+}$, and cell proliferation was determined by MTT assays. Results are expressed as (a) ratio of the absorbance compared to initial MTT value or (b) as relative effect of $Ca^{2+}$ compared to conditions with 0.1 mM $Ca^{2+}$. Values for normal DMEM/F12 were 2.12 ± 0.21. Data are means ± SEM of at least three individual experiments.

**Table 1.** Relative stimulation of cell proliferation as a function of $Ca^{2+}$ and $Mg^{2+}$ levels in the culture media

| Conditions | 0.8 mM $Mg^{2+}$ versus 0 mM $Mg^{2+}$ | Conditions | 1 mM $Ca^{2+}$ versus 0.1 mM $Ca^{2+}$ |
|---|---|---|---|
| 0.1 mM $Ca^{2+}$ | 1.79 ± 0.17[a] | 0 mM $Mg^{2+}$ | 1.66 ± 0.16[c] |
| 0.5 mM $Ca^{2+}$ | 1.47 ± 0.05[b] | 0.025 mM $Mg^{2+}$ | 1.55 ± 0.14[c] |
| 1 mM $Ca^{2+}$ | 1.35 ± 0.10 | 0.1 mM $Mg^{2+}$ | 1.50 ± 0.04[d] |
| | | 0.4 mM $Mg^{2+}$ | 1.33 ± 0.10[e] |
| | | 0.8 mM $Mg^{2+}$ | 1.24 ± 0.09 |

[a]$P < 0.0001$, [b]$P < 0.05$ compared to condition with 1 mM $Ca^{2+}$ (Student's *t*-test).
[c]$P < 0.0001$, [d]$P < 0.0003$, [e]$P < 0.05$ compared to condition with 0.8 mM $Mg^{2+}$ (Student's *t*-test).

markers such as alkaline phosphatase activity was not different between conditions that reduced cell proliferation. Similar reduction in cell proliferation under extracellular low $Mg^{2+}$ and $Ca^{2+}$ conditions was obtained with two other human osteoblast-like cells, namely SaOs and U2-OS (data not shown). The effects of reducing extracellular $Ca^{2+}$ and $Mg^{2+}$ on cell proliferation were reversible, because exposing $Ca^{2+}$- and $Mg^{2+}$-depleted cells, which showed growth arrest after

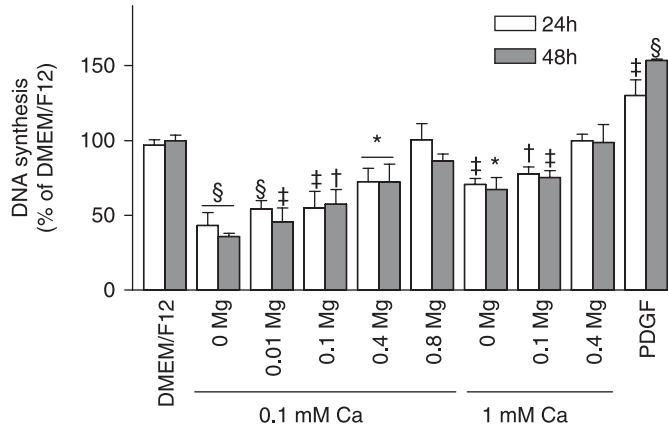


**Figure 4. Effect of extracellular $Mg^{2+}$ and $Ca^{2+}$ on DNA synthesis.** Cells were incubated for 24 or 48 h in DME/F12 containing different concentrations of $Mg^{2+}$ and $Ca^{2+}$. The evaluation of DNA synthesis was performed by BrdU incorporation as described in the Materials and Methods section. As positive control for the stimulation of DNA synthesis, cells were incubated with 25 ng/mL PDGF-BB in DMEM/F12. Values are the mean ± SEM of percentages compared to DMEM/F12 medium from three to four individual experiments. $*P < 0.05$, $\dagger P < 0.02$, $\ddagger P < 0.01$, $\S P < 0.001$ compared to DMEM/F12.

**Table 2.** Effect of extracellular $Mg^{2+}$ and $Ca^{2+}$ on alkaline phosphatase activity

| | Alkaline phosphate[a] (nmol PNP/h/mg protein) | | | | |
|---|---|---|---|---|---|
| Condition (calcium) | 0.1 mM | 0.25 mM | 0.5 mM | 1 mM | 2 mM |
| 0 mM $Mg^{2+}$ | 591.6 ± 48.4 | 559.4 ± 85.9 | 539.2 ± 78.9 | 678.6 ± 115.6 | 579.1 ± 66.8 |
| Condition[b] (magnesium) | 0 mM | 0.1 mM | 0.4 mM | 0.8 mM | 5 mM |
| 0.1 mM $Ca^{2+}$ | 591.6 ± 48.4 | 658.8 ± 56.4 | 624.8 ± 53.4 | 583.4 ± 47.4 | 615.4 ± 126.2 |

[a]Mean ± SD of alkaline phosphatase activity.
[b]Alkaline phosphatase activity from condition with 0.8 mM $Mg^{2+}$ and 1 mM $Ca^{2+}$ (519.9±90.4 nmol PNP/h/mg protein) not different from DMEM/F12 (496.2±61.6 nmol PNP/h/mg protein).

48 h of incubation (as shown in the Figs 2 and 3), to DMEM/F12 (1 mM $Ca^{2+}$ and 0.8 mM $Mg^{2+}$) for a further culture period of 96 h with FBS, restored cell proliferation (relative MTT activity of 1.97 ± 0.29 versus 1.06 ± 0.12).

To confirm that reduction of $Ca^{2+}$ and $Mg^{2+}$ in culture media lead to a decrease in osteoblast proliferation, we measured DNA synthesis of MG-63 cells under representative culture conditions, which allowed reduction of cell proliferation evaluated by MTT assay. Figure 4 shows that DNA synthesis was significantly decreased at 24 and 48 h under culture conditions of low $Mg^{2+}$ for both 0.1 and 1 mM $Ca^{2+}$ while significant stimulation of DNA synthesis was obtained (with platelet-derived growth factor used as positive control) for cell proliferation. DNA synthesis was not different from control conditions of DMEM/F12, for concentrations of $Mg^{2+}$ of 0.8 mM with 0.1 mM $Ca^{2+}$ and of 0.4 mM $Mg^{2+}$ with 1 mM $Ca^{2+}$. Thus, our DNA synthesis data agree with the MTT assays. Furthermore, the effect of low $Mg^{2+}$ and $Ca^{2+}$ on cell proliferation was confirmed by cell counts (Table 3).

**Table 3** Effect of low concentrations of extracellular $Mg^{2+}$ and $Ca^{2+}$ on cell counts

| | Cell counts | | | | |
|---|---|---|---|---|---|
| Condition[a] (magnesium) | 0 mM | 0.025 mM | 0.1 mM | 0.8 mM | 2 mM |
| 0.1 mM $Ca^{2+}$ | 33.6 ± 4.6[a] | 52.5 ± 7.6[d] (1.6) | 57.5 ± 18.3[e] (1.7) | 71.8 ± 8.8[c] (2.1) | 73.5 ± 17.7[b] (2.2) |
| Condition (calcium) | 0.1 mM | 0.25 mM | 1 mM | | |
| 0 mM $Mg^{2+}$ | 33.6 ± 4.6 | 54.0 ± 5.3[f] [1.6] | 60.4 ± 6.9[f] [1.8] | | |

[a]cells ± SD ×1000 after 48 h of incubation.
[b]$P < 0.01$, [c]$P < 0.001$, [d]$P < 0.02$, [e]$P < 0.05$ when compared to condition without $Mg^{2+}$.
[f]$P < 0.001$ when compared to condition with 0.1 mM $Ca^{2+}$.
() ratio versus 0 mM $Mg^{2+}$.
[] ratio versus 0.1 mM $Ca^{2+}$.

**Expression of TRPM and channel activation under low extracellular $Mg^{2+}$ and $Ca^{2+}$ levels**

In order to investigate the potential implication of TRPM6 and TRPM7 in compensatory mechanisms to maintain cellular ion homeostasis, we quantified their expression levels from cells maintained for 48 h under low concentrations of $Mg^{2+}$ and/or $Ca^{2+}$. As shown in Fig. 5, expression of TRPM7 increased by approximately 2-fold in the absence of $Mg^{2+}$ and low concentration of $Ca^{2+}$ while no difference was noted for expression of TRPM6.

In order to determine the influence of low extracellular levels of $Ca^{2+}$ and $Mg^{2+}$ on the activation of channels for the maintenance of cellular ion homeostasis, we performed intracellular $Ca^{2+}$ and $Mg^{2+}$ measurements with Fluo-3 and Magnesium Green. As shown in Fig. 6a (left panel: inset), addition of calcium to cells after prior incubation for 2 h in $Ca^{2+}$-free and 0.8 mM $Mg^{2+}$ incubation medium had no effect on intracellular $Ca^{2+}$ level in MG-63 cells. Similar results were obtained for cells after prior incubation in normal 1 mM $Ca^{2+}$ and 0.8 mM $Mg^{2+}$ incubation medium (data not shown). On the other hand, prior incubation of cells in 1 mM $Ca^{2+}$- and $Mg^{2+}$-free media for 2 h resulted in $Ca^{2+}$ influx upon addition of the ion to the incubation medium (Fig. 6a, left panel), suggesting that channels were activated by previous incubation conditions without $Mg^{2+}$. Accordingly, addition of extracellular $Mg^{2+}$ resulted in an increase of intracellular $Mg^{2+}$ in cells pre-incubated for 2 h in 1 mM $Ca^{2+}$- and $Mg^{2+}$-free medium (Fig. 6a, right panel). Furthermore, no significant augmentation of intracellular $Mg^{2+}$ was observed upon addition of extracellular $Mg^{2+}$ with cells pre-incubated in $Ca^{2+}$-free and 0.8 mM $Mg^{2+}$ incubation medium (Fig. 6a, right panel: inset) or in normal 1 mM $Ca^{2+}$ and 0.8 mM $Mg^{2+}$ medium (data not shown). We took advantage of the interference strategy by siRNA to evaluate the importance of TRPM7 on the $Ca^{2+}$ and $Mg^{2+}$ influx. Figure 6b shows that two specific siRNA for TRPM7 [siTRPM7(1) and siTRPM7(2)] reduced the expression by approximately 60% after 48 h of incubation, while no modification in expression of TRPM6 was noted. Reduction of TRPM7 expression prevented $Ca^{2+}$ influx induced by prior incubation of MG-63 cells in $Mg^{2+}$-free conditions (Fig. 6c, left panel). On the other hand, store-operated $Ca^{2+}$ channel antagonist SKF-96365 was without effect on $Ca^{2+}$ influx, indicating that canonical transient receptor potential channels are not involved (Fig. 6c, right panel). Similarly, $Mg^{2+}$ influx was prevented when cells were transfected with siRNA against TRPM7 (data not shown).

**Cell proliferation under condition of reduced TRPM7 expression**

In order to determine the importance of TRPM7 channels in the proliferation of osteoblastic cells, MTT assays were performed with cells transfected with specific siRNA against TRPM7.

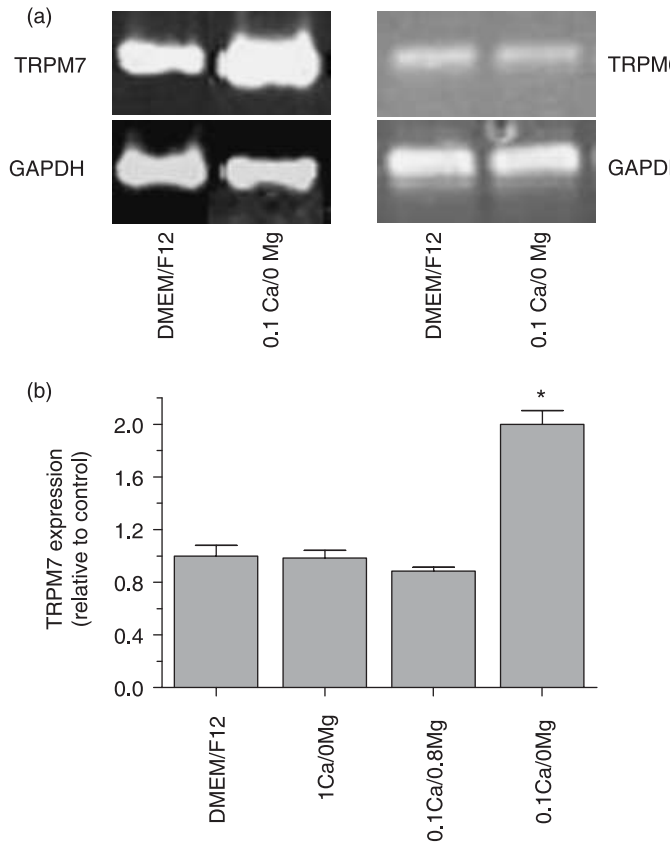


**Figure 5. Expression of TRPM6 and TRPM7 under $Mg^{2+}$- and/or $Ca^{2+}$- reduced culture conditions.** MG-63 cells were incubated for 48 h in DME/F12 containing 1 mM $Ca^{2+}$ without $Mg^{2+}$, in DME/F12 containing 0.8 mM $Mg^{2+}$ and 0.1 mM $Ca^{2+}$, in DME/F12 containing 0.1 mM $Ca^{2+}$ without $Mg^{2+}$ or in DMEM/F12 (1 mM $Ca^{2+}$ and 0.8 mM $Mg^{2+}$). Total RNA was isolated from cells cultured under previous conditions, and levels of TRPM6 and TRPM7 transcripts were determined by semiquantitative RT-PCR (a) or real-time PCR (b) as described in the Materials and Methods section. Relative levels of TRPM7 expression compared to those under control conditions (DMEM/F12) are expressed as the mean ± SEM of three independent experiments. $*P < 0.008$, Student's *t*-test.

Figure 7 shows that the proliferation of osteoblastic-like MG-63 and U2-OS cells, respectively, was inhibited by 60–75% with specific siRNAs for TRPM7.

## DISCUSSION

Influx of both $Ca^{2+}$ and $Mg^{2+}$ is solicited for numerous processes implicated in cell proliferation (Berridge *et al*. 2000; Rubin 2005). In contrast to agonist-stimulated ion influx that is generally rapid and transient, spontaneously activated ion channels, regulated by intracellular ion availability, are more likely involved in the progression of cell cycle. Accordingly, TRPM6 and TRPM7 channel activity has been shown to be regulated by intracellular magnesium (Fleig & Penner 2004). Therefore, we investigated the involvement of such channels in the proliferation of osteoblastic cells.

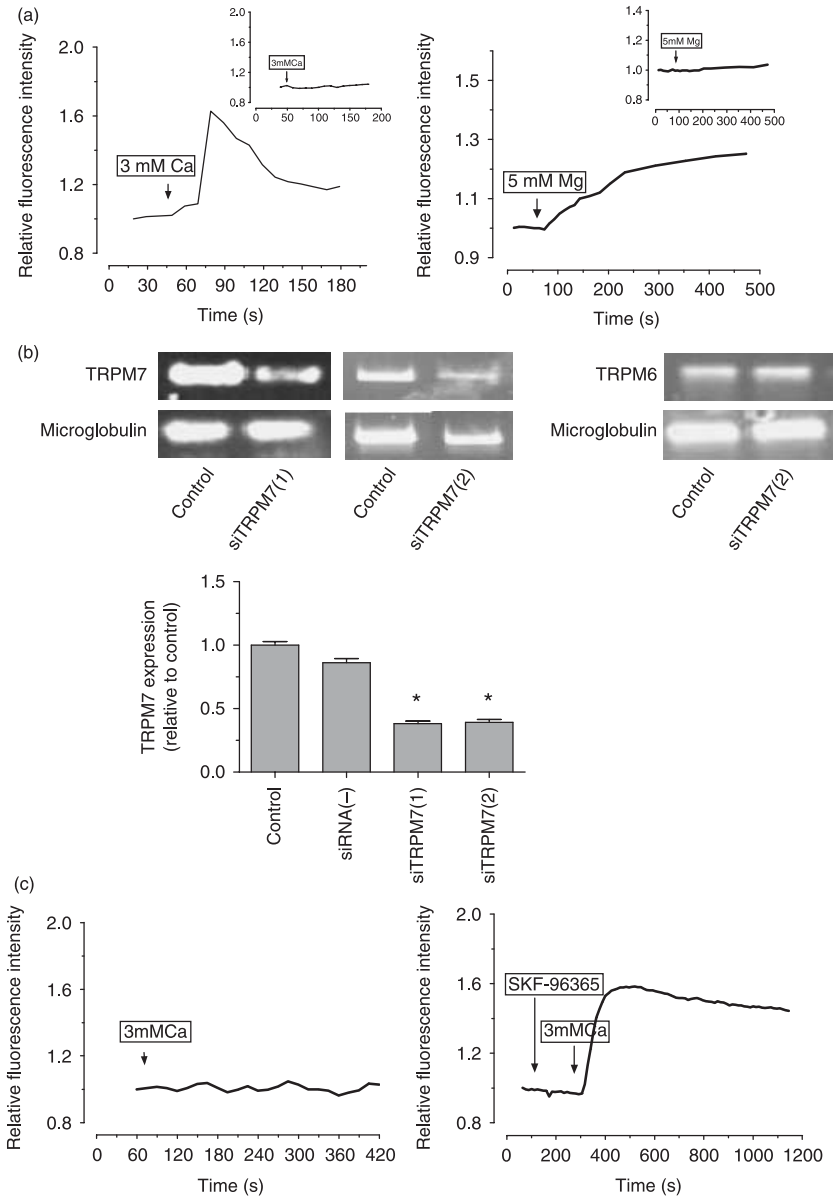


**Figure 6. Activation of TRPM7 under low extracellular $Mg^{2+}$ levels.** (a) Cells were loaded for a 2-h incubation period with Fluo-3 or Magnesium Green in HBSS with 1 mM $Ca^{2+}$ without $Mg^{2+}$ or HBSS without $Ca^{2+}$ with 0.8 mM $Mg^{2+}$ (right panel: inset). Thereafter, cells were transferred to $Ca^{2+}$-free and $Mg^{2+}$-free HBSS and $Ca^{2+}$ (left panel) or $Mg^{2+}$ (right panel) were added to the incubation medium (final concentration of 3 mM and 5 mM respectively). Measurements of intracellular $Ca^{2+}$ or $Mg^{2+}$ were performed as described in the Materials and Methods section. (b) MG-63 cells were transfected with specific siRNA against TRPM7 for 48 h. RT-PCR and RQ-PCR were performed to evaluate expression levels of TRPM6, TRPM7 and β-2-microglobulin. $*P < 0.001$. (c) Cells were transfected with siTRPM7(2) for 48 h and were loaded for a 2-h incubation period with Fluo-3 in HBSS with 1 mM $Ca^{2+}$ without $Mg^{2+}$. Thereafter, cells were transferred to $Ca^{2+}$- and $Mg^{2+}$-free HBSS and $Ca^{2+}$ was added to the incubation medium (final concentration of 3 mM). Right panel: non-transfected cells loaded with Fluo-3 in HBSS with 1 mM $Ca^{2+}$ without $Mg^{2+}$ were first incubated with 30 μM SKF-96365 and $Ca^{2+}$ was added to the incubation medium. Data are means ± SEM of three to four individual experiments with cumulating analysis of between 10 and 20 cells per field.

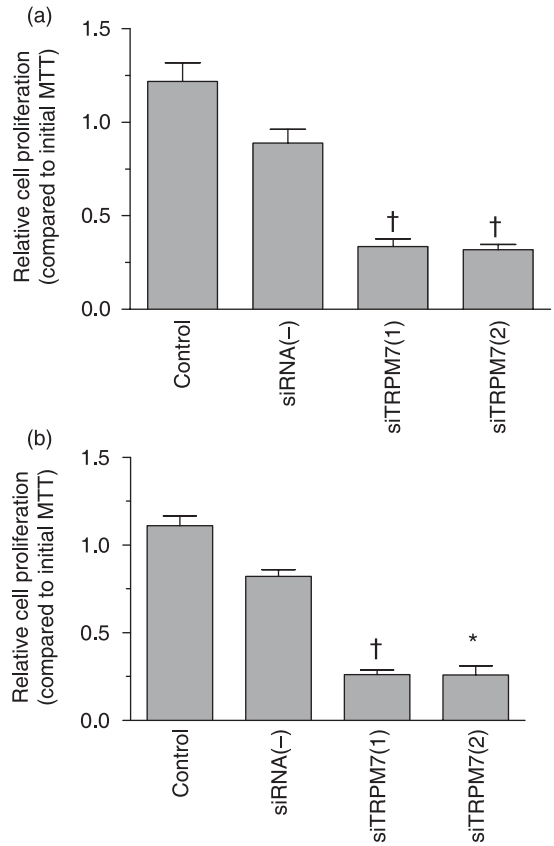


**Figure 7. Effect of reducing TRPM7 expression on cell proliferation.** Cells were transfected with specific siRNAs against TRPM7 [siTRPM7(1) and siTRPM7(2)] or control negative siRNA [siRNA(–)] and cell proliferation was determined after 72 h for MG-63 cells (a) or U2-OS cells (b) and compared to MTT activity following 24 h of incubation with siRNA (MTT initial). Data are means ± SEM of three individual experiments. *$P < 0.05$ and †$P < 0.001$ compared to cell proliferation with siRNA(–).

Here, we show for the first time, expression of TRPM6 and TRPM7 channels in human osteoblast-like cells. Expression was revealed for cells of recognized pre-osteoblast phenotype (MG-63 and U2-OS) and for more mature osteoblastic cells (SaOS). Expression of TRPM7 has been reported in neurones (Aarts *et al*. 2003), lymphocytes (Perraud *et al*. 2004), cardiac myocytes (Gwanyanya *et al*. 2004) and vascular smooth muscle cells (Beech 2005), and is thought to be widely expressed. TRPM7 channels have been associated with cell viability (Nadler *et al*. 2001), cellular magnesium homeostasis (Schmitz *et al*. 2003), anoxic neuronal cell death (Aarts *et al*. 2003), intestinal pacemaking activity (Kim *et al*. 2005), cell proliferation of retinoblastoma cells (Hanano *et al*. 2004), vascular smooth muscle cell proliferation and response to fluid flow (Beech 2005).

As $Ca^{2+}$- and $Mg^{2+}$-conducting channels, TRPM6 and TRPM7 may be involved in intracellular homeostasis of both ions that have been associated with important cell functions. We observed significant reduction of osteoblastic cell proliferation under culture conditions of low extracellular $Mg^{2+}$ or $Ca^{2+}$ concentrations. Decrease in cell proliferation was amplified by the concomitant reduction of extracellular $Mg^{2+}$ and $Ca^{2+}$ in the culture media. Accumulating evidence, from

bacteria to human cells, points to a universal role for $Mg^{2+}$ in controlling numerous processes associated with the cell cycle (for a review, see Rubin 2005). It has been proposed that $Ca^{2+}$ and $Mg^{2+}$ act in concert *via* a common mechanism. Although our results agree with the important role of $Mg^{2+}$ in cell proliferation, both ions seem essential for optimal cell proliferation because higher $Mg^{2+}$ concentration (5 mM) or higher $Ca^{2+}$ concentration (2 mM) did not compensate each other. Therefore, part of the progression of cell proliferation is ensured by both ions acting, probably together *via* a common mechanism, but distinct mechanisms are revealed for each ion to reach optimal cell proliferation. In that regard, TRPM6 and TRPM7 channels may promote influx of both $Ca^{2+}$ and $Mg^{2+}$ to assure the accomplishment of respective processes of both ions for cell proliferation. Accordingly, the reduction of TRPM7 expression by 60% in osteoblastic cells using interference by siRNA, led to a drastic reduction (around 60–75%) of cell proliferation. Similar correlation of TRPM7 and cell proliferation was reported by Hanano *et al*. (2004) in retinoblastoma cells, and by He *et al*. (2005) in vascular smooth muscle cells. Therefore, TRPM6 could not compensate for the reduction of TRPM7 expression for osteoblastic cell proliferation. Schmitz *et al*. (2005) have reported heteromeric formation of TRPM6 channels with TRPM7, and that TRPM6 requires TRPM7 for cell surface expression. In addition, it was shown that TRPM7 deficiency cannot be complemented by TRPM6. Moreover, Chubanov *et al*. (2004) reported similar requirement of TRPM6/TRPM7 association.

In view of the increase in TRPM7 expression that we have revealed under low $Mg^{2+}$ and $Ca^{2+}$ levels, TRPM7 may be part of a compensatory mechanism for gradual intracellular deficit that arises from low concentrations of extracellular $Mg^{2+}$ and $Ca^{2+}$, which agree with the reported ionic conductance of TRPM7 for $Ca^{2+}$ and $Mg^{2+}$. Surprisingly, osteoblast TRPM6 expression was not modified by reducing extracellular concentrations of both ions. Up-regulation of renal TRPM6 mRNA levels by low $Mg^{2+}$ diet has been reported, while similar up-regulation of intestinal TRPM6 expression was observed by enriched $Mg^{2+}$ diet (Groenestege *et al*. 2006). On the other hand, TRPM7 expression was not modified under either condition. However, it could not be excluded that renal and intestinal expression of TRPM6 may be regulated *in vivo* by indirect mechanisms and not by $Mg^{2+}$ directly. Such indirect mechanisms may implicate endocrine regulation because expression of TRPM6 has been shown to increase by 17β-estradiol treatment (Groenestege *et al*. 2006). Recently, Goytain and Quamme have described the cloning and the characterization of numerous plasma membrane $Mg^{2+}$ transporters designated SLC41A1 (Goytain & Quamme 2005b), SLC41A2 (Goytain & Quamme 2005c), MagT1 (Goytain & Quamme 2005d) and ACDP2 (Goytain & Quamme 2005a). Renal expression of SLC41A1, MagT1 and ACDP2 has been shown to be up-regulated in mice under hypomagnesic conditions. Furthermore, expression of MagT1 and ACDP2 was up-regulated in distal tubule kidney cells by reduced extracellular $Mg^{2+}$ level. Our current results indicate that TRPM7 could be added to this list of $Mg^{2+}$ transporters that are up-regulated by reducing extracellular $Mg^{2+}$ levels. No information has been available until now concerning expression of these $Mg^{2+}$ transporters in osteoblasts. However, given that reducing TRPM7 expression in osteoblastic cells results in reduction in cell proliferation, it is proposed that these $Mg^{2+}$ transporters are not expressed in osteoblasts or even in their presence, the cells are unable to compensate for reduction of TRPM7 expression. Accordingly, Sahni *et al*. (2007) have reported that although TRPM7-deficient DT40 cells express the SLC41A2 transporter, its endogenous expression level does not compensate for the reduction in cell proliferation.

Although conditions of restricted $Mg^{2+}$ levels have been shown to modify eukaryotic gene expression, the mechanism is unknown. In prokaryotes, a two-component system, namely PhoP-PhoQ, has been documented to mediate adaptation to $Mg^{2+}$-limited environments, and regulates numerous cellular activities in several gram-negative species (reviewed by Groisman 2001).

PhoP-PhoQ constitutes the first example of a regulatory system that uses extracellular $Mg^{2+}$ as a primary signal. Growth in micromolar concentrations of $Mg^{2+}$ promotes transcription of PhoP-activated genes in a PhoP- and PhoQ-dependent manner. In addition to $Mg^{2+}$and $Ca^{2+}$, manganese can repress transcription of PhoP-activated genes. Consistent with $Mg^{2+}$ and $Ca^{2+}$ being the physiological signals controlling the PhoP-PhoQ system, several PhoP-dependent phenotypes are regulated by these divalent cations in wild-type micro-organisms. Especially, the PhoP-PhoQ system regulates transcription of *mgtA*, *mgtB* and *mgtC* genes, which encode P-type ATPases that transport $Mg^{2+}$. The existence of homologous systems in eukaryotic cells has to be investigated. Maier *et al*. (2004) have identified (by cDNA array), several transcripts modulated by exposure to low $Mg^{2+}$, such as c-src, ezrin, CD9, cytohesin and zyxin. Such cellular response to $Mg^{2+}$ reduction was suggested to alter endothelial adhesion to substrates and migration, thereby promoting atherosclerosis, thrombosis and hypertension associated to $Mg^{2+}$ deficiency. Thus, conditions of restricted $Mg^{2+}$ levels potentially can modify eukaryotic cell phenotype. That $Ca^{2+}$ and $Mg^{2+}$ may act as independent repressors for transcription of TRPM7 channels in osteoblastic cells is suggested by our results (Fig. 5). Given that cell proliferation was restored, although without being optimal, by $Ca^{2+}$ and $Mg^{2+}$ when the extracellular concentration of the counterpart ion was reduced, independent repression of TRPM7 expression by $Ca^{2+}$ and $Mg^{2+}$ may be the result of the $Ca^{2+}$- and $Mg^{2+}$-transporting capacity of TRPM7 bearing in mind that both ions influence cell proliferation, in part *via* common mechanisms.

Electrophysiological studies have indicated that TRPM7-mediated currents (Monteilh-Zoller *et al*. 2003), also known as MIC ($Mg^{2+}$-inhibited cation) current or MagNum ($Mg^{2+}$-nucleotide-inhibited metal) current (Nadler *et al*. 2001; Prakriya & Lewis 2003), are regulated by the cytosolic $Mg^{2+}$. When osteoblastic cells were incubated in $Mg^{2+}$-deficient medium for 2 h, $Ca^{2+}$ and $Mg^{2+}$ influx were observed upon its addition to the incubation medium, indicating that channels were activated in the latter condition. Similar $Ca^{2+}$ and $Mg^{2+}$ influxes were not seen for cells transfected with specific siRNA against TRPM7, suggesting that TRPM7 channels in osteoblastic cells were activated by low concentrations of extracellular $Mg^{2+}$. Although transfection with siRNA against TRPM7 led to a 60% reduction of TRPM7 expression, $Ca^{2+}$ and $Mg^{2+}$ influxes induced by prior incubation of cells in $Mg^{2+}$-deficient medium were mostly prevented. Because transient receptor potential channels are usually formed by tetramerization, a threshold of TRPM7 expression may be essential to obtain functional channels at the plasma membrane. Activation of TRPM7 was not instantaneous following reduction of extracellular $Mg^{2+}$, because no $Ca^{2+}$ influx was seen upon addition of extracellular $Ca^{2+}$ to cells previously transferred in $Ca^{2+}$- and $Mg^{2+}$-free HBSS for few minutes. In addition, absence of $Ca^{2+}$ influx from cells incubated in HBSS without $Ca^{2+}$ with 0.8 mM $Mg^{2+}$ further supports the notion that $Mg^{2+}$ is the main ion that regulates TRPM7 activity. The latter result agrees with activation of TRPM7 following gradual reduction of intracellular $Mg^{2+}$.

Magnesium deficiency is not uncommon among the general population: its intake has decreased over the years in a significant proportion of the population, especially in the Western world. Many important human pathologies such as hypertension, heart failure, several nervous system complaints, muscle diseases and atherosclerosis have been associated with a decrease in $Mg^{2+}$ availability (Laires *et al*. 2004). In addition, coexisting disorders that impair intestinal $Mg^{2+}$ absorption and/or are associated with renal $Mg^{2+}$ loss, such as malabsorption syndromes, alcoholism, diabetes mellitus and drugs (e.g. diuretics), would place an individual at even greater risk. It should be noted, however, that serum $Mg^{2+}$ levels do not reflect actual body stores of $Mg^{2+}$ because blood levels are kept within the normal range at the expense of other tissues, especially bones. In this regard, epidemiological studies provide a link associating insufficient dietary $Mg^{2+}$ intake in humans with low bone mass and osteoporosis (for a review, see Rude &

Gruber 2004). Experimental $Mg^{2+}$ deficiency in animal models has resulted in impaired bone growth, osteopaenia, and increased skeletal fragility. Magnesium depletion causes a decrease in both osteoblast number and osteoclast activity with development of a form of aplastic bone disease. Moreover, genetic hypomagnesaemia with renal $Mg^{2+}$ wasting leads to low bone mass (Kantorovich *et al*. 2002). Our current results indicate that extracellular $Mg^{2+}$ deficit *in vitro* reduces osteoblast proliferation, an effect amplified by low extracellular $Ca^{2+}$ concentrations. Such reduction in osteoblast cell proliferation would lead to inadequate bone formation and poor regulation of resorption, resulting in the development of osteoporosis.

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## REFERENCES

Aarts M, Iihara K, Wei WL, Xiong ZG, Arundine M, Cerwinski W, MacDonald JF, Tymianski M (2003) A key role for TRPM7 channels in anoxic neuronal death. *Cell* **115**, 863–877.

Beech DJ (2005) Emerging functions of 10 types of TRP cationic channel in vascular smooth muscle. *Clin. Exp. Pharmacol. Physiol*. **32**, 597–603.

Bergman RJ, Gazit D, Kahn AJ, Gruber H, McDougall S, Hahn TJ (1996) Age-related changes in osteogenic stem cells in mice. *J. Bone Miner. Res*. **11**, 568–577.

Berridge MJ, Lipp P, Bootman MD (2000) The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol*. **1**, 11–21.

Brockstedt H, Kassem M, Eriksen EF, Mosekilde L, Melsen F (1993) Age- and sex-related changes in iliac cortical bone mass and remodeling. *Bone* **14**, 681–691.

Chan GK, Duque G (2002) Age-related bone loss: old bone, new facts. *Gerontology* **48**, 62–71.

Chen TL (2004) Inhibition of growth and differentiation of osteoprogenitors in mouse bone marrow stromal cell cultures by increased donor age and glucocorticoid treatment. *Bone* **35**, 83–95.

Chubanov V, Waldegger S, Schnitzler M, Vitzthum H, Sassen MC, Seyberth HW, Konrad M, Gudermann T (2004) Disruption of TRPM6/TRPM7 complex formation by a mutation in the TRPM6 gene causes hypomagnesemia with secondary hypocalcemia. *Proc. Natl. Acad. Sci. USA* **101**, 2894–2899.

Clapham DE, Runnels LW, Strubing C (2001) The TRP ion channel family. *Nat. Rev. Neurosci*. **2**, 387–396.

D'Ippolito G, Schiller PC, Ricordi C, Roos BA, Howard GA (1999) Age-related osteogenic potential of mesenchymal stromal stem cells from human vertebral bone marrow. *J. Bone Miner. Res*. **14**, 1115–1122.

Duncan RL, Akanbi KA, Farach-Carson MC (1998) Calcium signals and calcium channels in osteoblastic cells. *Semin. Nephrol*. **18**, 178–190.

Fleig A, Penner R. (2004) The TRPM ion channel subfamily: molecular, biophysical and functional features. *Trends Pharmacol. Sci*. **25**, 633–639.

Goytain A, Quamme GA (2005a) Functional characterization of ACDP2 (ancient conserved domain protein), a divalent metal transporter. *Physiol. Genomics* **22**, 382–389.

Goytain A, Quamme GA (2005b) Functional characterization of human SLC41A1, a $Mg^{2+}$ transporter with similarity to prokaryotic MgtE $Mg^{2+}$ transporters. *Physiol. Genomics* **21**, 337–342.

Goytain A, Quamme GA (2005c) Functional characterization of the human solute carrier, SLC41A2. *Biochem. Biophys. Res. Commun*. **330**, 701–705.

Goytain A, Quamme GA (2005d) Identification and characterization of a novel mammalian $Mg^{2+}$ transporter with channel-like properties. *BMC Genomics* **6**, 48.

Groenestege WM, van den Hoenderop JGHL, Knoers N, Bindels RJ (2006) The epithelial $Mg^{2+}$ channel transient receptor potential melastatin 6 is regulated by dietary $Mg^{2+}$ content and estrogens. *J. Am. Soc. Nephrol*. **17**, 1035–1043.

Groisman EA (2001) The pleiotropic two-component regulatory system PhoP-PhoQ. *J. Bacteriol*. **183**, 1835–1842.

Gwanyanya A, Amuzescu B, Zakharov SI, Macianskiene R, Sipido KR, Bolotina VM, Vereecke J, Mubagwa K (2004) Magnesium-inhibited, TRPM6/7-like channel in cardiac myocytes: permeation of divalent cations and pH-mediated regulation. *J. Physiol*. **559**, 761–776.

Hanano T, Hara Y, Shi J, Morita H, Umebayashi C, Mori E, Sumimoto H, Ito Y, Mori Y, Inoue R. (2004) Involvement of TRPM7 in cell growth as a spontaneously activated $Ca^{2+}$ entry pathway in human retinoblastoma cells. *J. Pharmacol. Sci*. **95**, 403–419.

Harteneck C (2005) Function and pharmacology of TRPM cation channels. *Naunyn Schmiedebergs Arch. Pharmacol*. **371**, 307–314.

Harteneck C, Plant TD, Schultz G (2000) From worm to man: three subfamilies of TRP channels. *Trends Neurosci*. **23**, 159–166.

He Y, Yao G, Savoia C, Touyz RM (2005) Transient receptor potential melastatin 7 ion channels regulate magnesium homeostasis in vascular smooth muscle cells: role of angiotensin II. *Circ. Res*. **96**, 207–215.

Iqbal J, Zaidi M (2005) Molecular regulation of mechanotransduction. *Biochem. Biophys. Res. Commun*. **328**, 751–755.

Kantorovich V, Adams JS, Gaines JE, Guo X, Pandian MR, Cohn DH, Rude RK (2002) Genetic heterogeneity in familial renal magnesium wasting. *J. Clin. Endocrinol. Metab*. **87**, 612–617.

Kassem M, Ankersen L, Eriksen EF, Clark BF, Rattan SI (1997) Demonstration of cellular aging and senescence in serially passaged long-term cultures of human trabecular osteoblasts. *Osteoporos. Int*. **7**, 514–524.

Kato H, Matsuo R, Komiyama O, Tanaka T, Inazu M, Kitagawa H, Yoneda T (1995) Decreased mitogenic and osteogenic responsiveness of calvarial osteoblasts isolated from aged rats to basic fibroblast growth factor. *Gerontology* **41** (Suppl. 1), 20–27.

Kim BJ, Lim HH, Yang DK, Jun JY, Chang IY, Park CS, So I, Stanfield PR, Kim KW (2005) Melastatin-type transient receptor potential channel 7 is required for intestinal pacemaking activity. *Gastroenterology* **129**, 1504–1517.

Kotev-Emeth S, Savion N, Pri-chen S, Pitaru S (2000) Effect of maturation on the osteogenic response of cultured stromal bone marrow cells to basic fibroblast growth factor. *Bone* **27**, 777–783.

Kragstrup J, Melsen F, Mosekilde L (1983) Thickness of bone formed at remodeling sites in normal human iliac trabecular bone: variations with age and sex. *Metab. Bone Dis. Relat. Res*. **5**, 17–21.

Laires MJ, Monteiro CP, Bicho M (2004) Role of cellular magnesium in health and human disease. *Front. Biosci*. **9**, 262–276.

Liang CT, Barnes J, Seedor JG, Quartuccio HA, Bolander M, Jeffrey JJ, Rodan GA (1992) Impaired bone activity in aged rats: alterations at the cellular and molecular levels. *Bone* **13**, 435–441.

Mackie EJ (2003) Osteoblasts: novel roles in orchestration of skeletal architecture. *Int. J. Biochem. Cell Biol*. **35**, 1301–1305.

Maier JA, Malpuech-Brugere C, Zimowska W, Rayssiguier Y, Mazur A (2004) Low magnesium promotes endothelial cell dysfunction: implications for atherosclerosis, inflammation and thrombosis. *Biochim. Biophys. Acta* **1689**, 13–21.

Martinez ME, del Campo MT, Medina S, Sanchez M, Sanchez-Cabezudo MJ, Esbrit P, Martinez P, Moreno I, Rodrigo A, Garces MV, Munuera L (1999) Influence of skeletal site of origin and donor age on osteoblastic cell growth and differentiation. *Calcif. Tissue Int*. **64**, 280–286.

Minke B, Cook B (2002) TRP channel proteins and signal transduction. *Physiol. Rev*. **82**, 429–472.

Monteilh-Zoller MK, Hermosura MC, Nadler MJ, Scharenberg AM, Penner R, Fleig A (2003) TRPM7 provides an ion channel mechanism for cellular entry of trace metal ions. *J. Gen. Physiol*. **121**, 49–60.

Montell C, Birnbaumer L, Flockerzi V (2002) The TRP channels, a remarkably functional family. *Cell* **108**, 595–598.

Moreau R, Aubin R, Lapointe JY, Lajeunesse D (1997) Pharmacological and biochemical evidence for the regulation of osteocalcin secretion by potassium channels in human osteoblast-like MG-63 cells. *J. Bone Miner. Res*. **12**, 1984–1992.

Nadler MJ, Hermosura MC, Inabe K, Perraud AL, Zhu Q, Stokes AJ, Kurosaki T, Kinet JP, Penner R, Scharenberg AM, Fleig A (2001) LTRPC7 is a Mg.ATP-regulated divalent cation channel required for cell viability. *Nature* **411**, 590–595.

Neidlinger-Wilke C, Stalla I, Claes L, Brand R, Hoellen I, Rubenacker S, Arand M, Kinzl L (1995) Human osteoblasts from younger normal and osteoporotic donors show differences in proliferation and TGF beta-release in response to cyclic strain. *J. Biomech*. **28**, 1411–1418.

Parfitt AM (1991) Bone forming cells in clinical conditions. In: Hall BK, ed. *Bone, the Osteoblast and Osteocyte*. London: Telford Press, pp. 351–426.

Perraud AL, Knowles HM, Schmitz C (2004) Novel aspects of signaling and ion-homeostasis regulation in immunocytes. The TRPM ion channels and their potential role in modulating the immune response. *Mol. Immunol*. **41**, 657–673.

Peterson WJ, Tachiki KH, Yamaguchi DT (2004) Serial passage of MC3T3–E1 cells down-regulates proliferation during osteogenesis *in vitro*. *Cell Prolif*. **37**, 325–336.

Pfeilschifter J, Diel I, Pilz U, Brunotte K, Naumann A, Ziegler R. (1993) Mitogenic responsiveness of human bone cells *in vitro* to hormones and growth factors decreases with age. *J. Bone Miner. Res*. **8**, 707–717.

Prakriya M, Lewis RS (2003) CRAC channels: activation, permeation, and the search for a molecular identity. *Cell Calcium* **33**, 311–321.

Quarto R, Thomas D, Liang CT (1995) Bone progenitor cell deficits and the age-associated decline in bone repair capacity. *Calcif. Tissue Int*. **56**, 123–129.

Roholl PJ, Blauw E, Zurcher C, Dormans JA, Theuns HM (1994) Evidence for a diminished maturation of preosteoblasts into osteoblasts during aging in rats: an ultrastructural analysis. *J. Bone Miner. Res*. **9**, 355–366.

Romani AM, Scarpa A (2000) Regulation of cellular magnesium. *Front. Biosci*. **5**, D720–D734.

Rubin H (2005) The membrane, magnesium, mitosis (MMM) model of cell proliferation control. *Magnes. Res*. **18**, 268–274.

Rude RK, Gruber HE (2004) Magnesium deficiency and osteoporosis: animal and human observations. *J. Nutr. Biochem*. **15**, 710–716.

Sahni J, Nelson B, Scharenberg AM (2007) SLC41A2 encodes a plasma-membrane $Mg^{2+}$ transporter. *Biochem. J*. **401**, 505–513.

Schmitz C, Dorovkov MV, Zhao X, Davenport BJ, Ryazanov AG, Perraud AL (2005) The channel kinases TRPM6 and TRPM7 are functionally nonredundant. *J. Biol. Chem*. **280**, 37763–37771.

Schmitz C, Perraud AL, Johnson CO, Inabe K, Smith MK, Penner R, Kurosaki T, Fleig A, Scharenberg AM (2003) Regulation of vertebrate cellular $Mg^{2+}$ homeostasis by TRPM7. *Cell* **114**, 191–200.

Stenderup K, Justesen J, Clausen C, Kassem M (2003) Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. *Bone* **33**, 919–926.

Wolf FI, Fasanella S, Tedesco B, Torsello A, Sgambato A, Faraglia B, Palozza P, Boninsegna A, Cittadini A (2004) Regulation of magnesium content during proliferation of mammary epithelial cells (HC-11). *Front. Biosci*. **9**, 2056–2062.