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

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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

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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 Dulbecco'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/cm². 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 aspirated 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/cm² 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 $\pm$ 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 $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 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-GAAACGTTTTTCG-3'; antisense: 5'-CATGATAAAAGGCATAAACTTTTCGC-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 Omniscript 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 ± 0.21 relative to the initial MTT activity after 48 h). As shown

Figure 2. Effect of extracellular Mg²⁺ on MG-63 proliferation. Cells were incubated for 48 h in culture media containing different concentrations of Ca²⁺ and increasing concentrations of Mg²⁺, 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 Mg²⁺ compared to conditions without Mg²⁺. Values for normal DMEM/F12 were 2.12 ± 0.21 relative to the initial MTT. Data are means $\pm$ SEM of at least three individual experiments.

in Fig. 2a, concentrations of Mg²⁺ below 0.8 mM reduced cell proliferation at all concentrations of Ca²⁺. No significant difference in cell proliferation was observed for concentrations of Mg²⁺ between 0.8 and 5 mM. When expressed in terms of relative proliferation compared to conditions without Mg²⁺ (Fig. 2b), relative stimulation in the presence of Mg²⁺ was higher at 0.1 mM Ca²⁺ compared to 0.5 mM Ca²⁺ and 1 mM Ca²⁺ ($P < 0.001$, ANOVA), and a significant difference between 0.5 and 1 mM Ca²⁺ ($P < 0.05$, ANOVA) was still observed (Table 1). It should be noted that cell proliferation did not reach control levels (1 mM Ca²⁺ and 0.8 mM Mg²⁺) with higher concentrations of Mg²⁺ (5 mM) under low Ca²⁺ levels. As shown in Fig. 3a, concentrations of Ca²⁺ below 0.5 mM reduced cell proliferation at all concentrations of Mg²⁺. No difference in cell proliferation was observed for concentrations of 0.5 mM Ca²⁺ and above. When expressed in terms of relative proliferation compared to those in low Ca²⁺ conditions (0.1 mM) (Fig. 3b), relative stimulation by Ca²⁺ was higher for conditions without Mg²⁺ and 0.025 mM Mg²⁺ ($P < 0.0001$, ANOVA), 0.1 mM Mg²⁺ ($P < 0.0003$, ANOVA), 0.4 mM Mg²⁺ ($P < 0.05$, ANOVA) compared to 0.8 mM (Table 1). It should be noted that cell proliferation did not reach control levels (1 mM Ca²⁺ and 0.8 mM Mg²⁺) with higher concentrations of Ca²⁺ (2 mM) under conditions of zero extracellular Mg²⁺. Protein quantification also confirmed relative reduction of cell proliferation by low extracellular Ca²⁺ and Mg²⁺ (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 $\pm$ 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 $\pm$ SEM of percentages compared to DMEM/F12 medium from three to four individual experiments. * $P < 0.05$, † $P < 0.02$, ‡ $P < 0.01$, § $P < 0.001$ 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 $\pm$ 48.4	559.4 $\pm$ 85.9	539.2 $\pm$ 78.9	678.6 $\pm$ 115.6	579.1 $\pm$ 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 $\pm$ 48.4	658.8 $\pm$ 56.4	624.8 $\pm$ 53.4	583.4 $\pm$ 47.4	615.4 $\pm$ 126.2

^aMean $\pm$ SD of alkaline phosphatase activity.

^bAlkaline phosphatase activity from condition with 0.8 mM Mg^{2+} and 1 mM Ca^{2+} (519.9 $\pm$ 90.4 nmol PNP/h/mg protein) not different from DMEM/F12 (496.2 $\pm$ 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 ^c (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]		

^acells ± 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 $\pm$ 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 Mg²⁺ levels. (a) Cells were loaded for a 2-h incubation period with Fluo-3 or Magnesium Green in HBSS with 1 mM Ca²⁺ without Mg²⁺ or HBSS without Ca²⁺ with 0.8 mM Mg²⁺ (right panel: inset). Thereafter, cells were transferred to Ca²⁺-free and Mg²⁺-free HBSS and Ca²⁺ (left panel) or Mg²⁺ (right panel) were added to the incubation medium (final concentration of 3 mM and 5 mM respectively). Measurements of intracellular Ca²⁺ or Mg²⁺ 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 Ca²⁺ without Mg²⁺. Thereafter, cells were transferred to Ca²⁺- and Mg²⁺-free HBSS and Ca²⁺ 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 Ca²⁺ without Mg²⁺ were first incubated with 30 μ M SKF-96365 and Ca²⁺ was added to the incubation medium. Data are means $\pm$ 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 $\pm$ 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 hypomagnesian 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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