TRPM7 and MagT1 regulate the proliferation of osteoblast-like SaOS-2 cells through different mechanisms

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ABSTRACT

A correct Magnesium (Mg^{2+}) intake is essential for bone health. In particular, Mg^{2+} deficiency inhibits the proliferation of osteoblast-like SaOS-2 cells by increasing nitric oxide (NO) production through the upregulation of inducible NO synthase. At the moment, little is known about the expression and the role of TRPM7, a channel/enzyme involved in Mg^{2+} uptake, and MagT1, a Mg^{2+} selective transporter, in SaOS-2 cells. Here we demonstrate that TRPM7 expression is not modulated by different extracellular concentrations of Mg^{2+} and its silencing exacerbates growth inhibition exerted by low Mg^{2+} through the activation of inducible NO synthase and consequent accumulation of NO. Moreover, MagT1 is upregulated in SaOS-2 cultured in high Mg^{2+} and its silencing inhibits the growth of SaOS-2 cultured in media containing physiological or high Mg^{2+}, without any modulation of NO production. We propose that TRPM7 and MagT1 are both involved in regulating SaOS-2 proliferation through different mechanisms.

KEYWORDS

Magnesium, TRPM7, MagT1, osteoblasts, bone

RUNNING TITLE

MagT1, TRPM7 and osteoblast proliferation

INTRODUCTION

Osteoporosis is one of the most impactive age-related pathologies in Western countries and it is characterized by the deterioration of bone microarchitecture which leads to loss of bone mass and increased risk of fractures [1]. Given the social relevance of this disease, research on the mechanisms implicated in bone loss has increased significantly in recent years [2].

An important component of bones is Magnesium (Mg^{2+}), an essential nutrient with several metabolic, structural and regulatory functions that affects the growth of bone cells as well as the formation of hydroxyapatite crystals [3]. A tight control of Mg^{2+} homeostasis is crucial for bone health maintenance. Indeed, several studies assess that long-term low Mg^{2+} intake, a condition increasingly observed in Western population, is associated with a high risk of osteoporosis [4,5]. In particular, in rodents low Mg^{2+}-dependent decrease of bone formation is partly due to impaired osteoblastic activity, which is the result of both reduced proliferation and excessive apoptosis [6,7]. The inadequate osteoblastic activity, responsible for bone matrix deposition and mineralization, leads to a prevalence of osteoclast-dependent bone resorption and to the loss of bone mass.

We have previously demonstrated that low Mg^{2+} inhibits SaOS-2 osteoblast-like cells proliferation through the activation of the JNK signaling pathway which upregulates inducible nitric oxide synthase (iNOS), with the consequent increase of nitric oxide (NO) release [8]. Recently, we have shown that Mg^{2+} deprivation accelerates the osteogenic differentiation of human bone mesenchymal stem cells (hMSC) [9,10]. On the other hand, high extracellular Mg^{2+} inhibits osteoblast activity [11] as well as the osteogenic differentiation of hMSC [12]. Consequently, further investigations on the mechanisms involved in Mg^{2+}-dependent regulation of osteoblasts proliferation and activity are necessary.

Several transporters contribute to the maintenance of cellular Mg^{2+} homeostasis, among which the Transient Receptor Potential Cation Channel Subfamily M Member 7 (TRPM7) and Mg^{2+} transporter 1 (MagT1). While MagT1 transports Mg^{2+} across the plasma membrane in a highly specific manner, TRPM7 is characterized by an ion channel permeable to different divalent cations, primarily calcium (Ca) and Mg^{2+}, and also possesses an α-kinase domain that phosphorylates downstream substrates. These two Mg^{2+} transporters play important regulatory roles in the bone. TRPM7 is essential for the proliferation and survival of osteoblasts [13]. Moreover, both TRPM7 and MagT1 contribute to the osteogenic differentiation of hMSC, since the two Mg^{2+} transporters are upregulated during the hMSC osteogenic differentiation and their silencing accelerates the differentiation process [10,14].

On the basis of these considerations, we analyzed the expression and the role of TRPM7 and MagT1 in SaOS-2 cells cultured in media containing different concentrations of extracellular Mg^{2+}. The experiments were performed on SaOS-2 cells because they retain many features typical of mature osteoblasts [15,16], including the capability of differentiating into osteocyte-like cells [17].

MATERIALS AND METHODS

Cell culture and proliferation

SaOS-2 cells were obtained from the American Type Culture Collection and cultured at 37 °C, 5% CO2 in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum and 2 mM glutamine. In all the experiments, the cells were seeded in growth medium. After 24 h the medium was replaced by Mg^{2+}-free MEM (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with $MgSO_4$ (Sigma-Aldrich, St. Louis, Missouri, USA) to obtain media containing 0.1, 1 or 5 mM Mg^{2+} [18]. For proliferation assays, the cells were seeded at low density and medium was replaced 24 h later to expose the cells to different Mg^{2+} regimens. After 96 h, the cells were trypsinized, stained with trypan blue (0,4%) and counted using a Luna Automated Cell Counter (Logos Biosystems, Anyang, South Korea).

TRPM7 or MagT1 silencing

To obtain a transient downregulation of MagT1 and TRPM7, we used Lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, Massachusetts, USA) in combination with the stealth siRNAs for *TRPM7* (Qiagen, Hilden, Germany) or for *MagT1* (Invitrogen, Carlsbad, California, USA). Non-silencing, scrambled sequences were used as controls (NS). After 96 h, cells were counted and lysed for protein extraction, while the medium was used to measure NO release.

Western blot

SaOS-2 were lysed in 10 mM Tris-HCl (pH 7.4) containing 3 mM $MgCl_2$, 10 mM NaCl, 0.1% SDS, 0.1% Triton X-100, 0.5 mM EDTA and protease inhibitors, separated by SDS-PAGE on Mini-PROTEAN TGX Stain-free Gels (Bio-Rad Laboratories, Hercules, California, USA) and transferred to nitrocellulose sheets by using Trans-Blot® Turbo™ Transfer Pack (Bio-Rad Laboratories). The immunoblot analysis was performed using primary antibodies against TRPM7 (Bethyl, Montgomery, USA), MagT1 (Abcam, Cambridge, UK) and β-actin (Tebu Bio-Santa Cruz, Magenta, Italy). After extensive washing, secondary antibodies labelled with horseradish peroxidase (Amersham Pharmacia Biotech Italia, Cologno Monzese, Italy) were used.

Immunoreactive proteins were detected with Clarity™ Western ECL substrate (Bio-Rad Laboratories). The experiments were performed 3 times and quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

NOS activity

NOS activity was measured in the conditioned media by using the Griess method for nitrite quantification as previously described [8]. The concentrations of NO in the samples were determined using a calibration curve generated with standard $NaNO_2$ solutions. The experiments were performed in triplicate and repeated three to five times with similar results.

NO release was inhibited by L-N^6-(1-Iminoethyl)lysine dihydrochloride (L-NIL, 100 µM) (Biotrend, Cologne, Germany), which inhibits iNOS, or by N_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME, 100 µM) (Sigma Aldrich), which inhibits eNOS.

Statistical analysis

Statistical significance was determined using the Student's t test and set at P values $\leq$ .05. In the figures, *p $\leq$ 0.05, **p $\leq$ 0.01 and ***p $\leq$ 0.001.

RESULTS

TRPM7 silencing inhibits the proliferation of Mg^{2+} deficient SaOS-2 cells.

We have previously demonstrated that extracellular Mg^{2+} affects both the proliferation and the differentiation of osteoblast like SaOS-2 cells [8,11]. Because TRPM7 also transports Mg^{2+} and has been suggested to be essential for cell proliferation [3], we evaluated TRPM7 levels in SaOS-2 cells cultured in different concentrations of Mg^{2+} for 96 h. No significant modulation of the total amounts of TRPM7 emerged by western blot (Figure 1A). To evaluate whether TRPM7 influenced SaOS-2 cell proliferation, the cells were transfected with specific siRNA against *TRPM7* and then cultured for 96 h in 0.1, 1.0 and 5.0 mM Mg^{2+}. Non-silencing scrambled sequences (NS) were used as a control. Western blot shows that siRNA targeting *TRPM7* markedly downregulated TRPM7 levels (Figure 1B). In agreement with previous work [11], we found that Mg^{2+}-deficient SaOS-2 cells are growth retarded when compared to cells cultured in media containing 1.0 and 5.0 mM Mg^{2+} (Figure 1C). Interestingly, silencing *TRPM7* did not affect SaOS-2 cell growth in 1.0 and 5.0 mM Mg^{2+}, while it exerted an additive inhibitory effect in Mg^{2+} deficient cells (Figure 1C, striped columns).

NO plays a pivotal role in regulating bone homeostasis [19]. We measured NO release in SaOS-2 cultured in different Mg^{2+} concentrations and found it increased in Mg^{2+} deficient cells (Figure 1D). Interestingly, *TRPM7* silencing exacerbated NO release by SaOS-2 cultured in low extracellular Mg^{2+} (Figure 1D, striped columns), thus establishing a connection between the amounts of NO and the inhibition of cell proliferation.

iNOS inhibition restores normal proliferation in Mg^{2+} deficient SaOS-2 cells.

Since i) low Mg^{2+} impairs SaOS-2 proliferation through increased NO release by upregulated iNOS [8] and ii) *TRPM7* silencing exacerbates these effects, we inhibited NO production by treating the cells with the iNOS inhibitor L-NIL (100 μM). Therefore, we cultured Mg^{2+} deficient SaOS-2 silencing or not *TRPM7* with or without L-NIL and found that the inhibition of iNOS prevents NO increase and rescues SaOS-2 cell growth (Figure 2A-B).

These results suggest that the inhibition of cell proliferation induced by the combined action of low extracellular Mg^{2+} and *TRPM7* silencing is mediated by the increase of NO induced by iNOS.

MagT1 silencing inhibits SaOS-2 proliferation in physiological and high Mg^{2+} conditions.

In addition to TRPM7, also the Mg^{2+} transporter MagT1 has a role in bone homeostasis since it is modulated in the osteogenic differentiation of hMSC [10]. To our best knowledge no data are available about MagT1 in mature osteoblasts.

Therefore, we evaluated the total amounts of MagT1 in SaOS-2 cells cultured in media containing different Mg^{2+} concentrations. By western blot we found that 5 mM Mg^{2+} upregulates MagT1 (Figure 3A). We then silenced *MagT1* in SaOS-2 cultured in 0.1, 1.0 and 5.0 mM Mg^{2+} and analyzed cell proliferation and NO production. Figure 3B shows the downregulation of MagT1 levels by specific siRNAs compared to the cells treated with non-silencing sequences. The downregulation of MagT1 significantly inhibited the proliferation of the cells cultured in media containing physiological and high concentrations of Mg^{2+}, while it had no effect on the proliferation of Mg^{2+}-deficient SaOS-2 cells (Figure 3C). The inhibition of cell proliferation was not coupled with an increase of NO synthesis in *MagT1* silencing SaOS-2 cells in all the conditions tested (Figure 3D).

iNOS and eNOS inhibition does not restore normal proliferation in SaOS-2 cells silencing MagT1. To confirm that NO synthesis was not involved in inhibiting the proliferation of *MagT1* silencing cells, we inhibited both iNOS and eNOS using L-NIL and L-NAME, respectively, and analyzed cell proliferation. Figure 4 shows that the inhibition of NO production did not restore normal proliferation in MagT1 silencing cells cultured in physiological and high Mg^{2+} containing media. It is noteworthy that the both L-NIL and L-NAME prevent growth inhibition by low Mg^{2+}.

DISCUSSION

Osteoporosis is the result of an imbalance of the activities of osteoblasts, the cells that form bone, and osteoclasts, the cells that resorb bone [1]. Importantly, the number of osteoblasts is reduced in osteoporosis [20].

Mg^{2+} contributes to bone health. It has a structural role in the apatite crystals, it modulates the differentiation of the precursors of osteoclasts and osteoblasts, it is essential in controlling the proliferation and the activity of osteoblasts [3,21]. TRPM7 has a fundamental role in skeletogenesis [22], is critical for the survival of MSC [23] and plays a role in their osteogenic differentiation [10,14]. Also the Mg^{2+} transporter MagT1 contributes to the osteogenic differentiation of MSC [14,24], while no data are available at the moment about the expression and the role of MagT1 in mature osteoblasts. In this paper we focused our attention on mature osteoblast-like SaOS-2 cells [15–17]. We have previously demonstrated that Mg^{2+} deficiency inhibits the growth of these cells by increasing NO production through the upregulation of iNOS [8]. Here we demonstrate that SaOS-2 cells express both TRPM7 and MagT1, which are both involved in regulating their proliferation, although through different mechanisms.

The total amounts of TRPM7 are not modulated by the extracellular concentrations of Mg^{2+}. On the contrary, human macrovascular endothelial cells upregulate TRPM7 when exposed to 0.1 mM Mg^{2+} and downregulate it when cultured in 5.0 mM Mg^{2+}, and this is due to post-transcriptional mechanisms [25]. Microvascular endothelial cells behave differently, since under low Mg^{2+} conditions they show reduced amounts of TRPM7 [26]. We conclude that the concentrations of extracellular Mg^{2+} differently impact on TRPM7 levels in various cell types.

TRPM7 is known to contribute to the regulation of cell growth in many cell types [3]. In SaOS-2 silencing *TRPM7* exacerbates growth inhibition exerted by low Mg^{2+}, while no differences in proliferative behavior occur in cells maintained in 1.0 or 5.0 mM Mg^{2+}. We hypothesize that the downregulation of TRPM7 might severely impair Mg^{2+} homeostasis of SaOS-2 cultured in low Mg^{2+} to the point that cells stop dividing. On the contrary, physiological or high concentrations of extracellular Mg^{2+} might hinder the inhibitory effect of *TRPM7* silencing by providing sufficient Mg^{2+} to the cells through the involvement of other transporters/channels or the inhibition of Mg^{2+} extruders.

Interestingly, growth inhibition is associated with the increased release of NO through the activation of iNOS. It is known that low concentrations of NO are necessary for the proliferation and activity

of osteoblasts, whereas too much NO is responsible for the inhibition of osteoblast function *in vitro* and the induction of bone resorption [19]. In addition to function as a channel for divalent cations, TRPM7 also contributes to signal transduction because it possesses an α-kinase domain [3]. Consequently, it is feasible that TRPM7 might participate to the regulation of the expression of iNOS eventually impacting on the activation of JNK. More studies are necessary on these issues.

Our results provide the first evidence about the expression and the role of MagT1 in osteoblasts. We show that MagT1 is expressed in SaOS-2 cells and slightly upregulated in cells cultured in high Mg^{2+}. While MagT1 silencing exerts no effect in Mg^{2+} deficient cells, it inhibits the growth of SaOS-2 cells cultured in media containing 1.0 or 5.0 mM Mg^{2+}. It is feasible to propose that, upon its silencing, the remaining channels/transporters do not suffice to guarantee Mg^{2+} homeostasis, so that the cells reduce their proliferation rate. These results point to a critical role of MagT1 in mediating Mg uptake in osteoblasts exposed to physiological or high extracellular Mg, as it might happen when biocompatible magnesium alloys are utilized for temporary bone implants [27]. We therefore hypothesize that MagT1 might emerge as an important player in bone remodeling and repair. Considering that neither MagT1 silencing modulates NO release nor iNOS or eNOS inhibition rescues proliferation of MagT1 silencing cells, we can rule out any involvement of NO in inhibiting the proliferation of SaOS-2. More studies are necessary to individuate the links between osteoblast proliferation and MagT1 expression.

Since MagT1 is also involved in protein glycosylation as it is a subunit of the oligosaccharyltransferase complex [28], it is feasible that MagT1 downregulation affects post-translational glycosylation of proteins which control either the proliferation of SaOS-2 or Mg^{2+} handling. Accordingly, loss-of-function mutations of the *MagT1* gene, which causes the primary immunological deficiency named XMEN (X-linked immunodeficiency with magnesium defect, Epstein-Barr virus (EBV) infection, and neoplasia)-disease, impair N-linked glycosylation of genes involved in immune responses [29,30]. In addition, low Mg^{2+} exacerbates the deficit of N-glycosylation [29].

In conclusion, our studies outline that both TRPM7 and MagT1 contribute to osteoblast proliferation and, consequently, to bone health.

ACKNOWLEDGEMENTS

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LEGENDS TO THE FIGURES

Figure 1. TRPM7 levels and its role in SaOS-2 cells cultured in different concentrations of Mg^{2+}. SaOS-2 cells were cultured in 0.1, 1.0 and 5.0 mM Mg^{2+}.

(A) After 96 h western blot was performed on protein lysates and antibodies against TRPM7 were utilized. Anti-β-actin antibodies were used as control of equal loading. A representative blot is shown.

(B) SaOS-2 were silenced for *TRPM7* with specific siRNAs. Scrambled, non-silencing sequences (NS) were used as controls. Western blot was performed as in (A). The histogram shows the densitometry (TRPM7 vs β-actin) of three separate experiments ± standard deviation (*$p \leq 0.05$).

(C) SaOS-2 silenced or not for *TRPM7* were counted after 96 h. The bars are the mean ± standard deviation of four separate experiments. p was calculated vs NS (**$p \leq 0.01$).

(D) NO release was measured with the Griess method. Data are expressed as the mean ± standard deviation of three separate assays (**$p \leq 0.01$, *** $p \leq 0.001$).

Figure 2. The effect of iNOS inhibitor on the release of NO and on the proliferation of Mg^{2+} deficient SaOS-2 cells.

SaOS-2 silenced or not for *TRPM7*, were cultured in 0.1 mM Mg^{2+} for 96 h, in the presence or not of L-NIL.

(A) The cells were counted as described. The bars are the mean ± standard deviation of four separate experiments (*$p \leq 0.05$, **$p \leq 0.01$).

(B) Nitric oxide release was measured with the Griess method. Data are expressed as the mean ± standard deviation of three separate assays (**$p \leq 0.01$).

Figure 3. MagT1 levels and its role in SaOS-2 cells cultured in different concentrations of Mg^{2+}. SaOS-2 cells were cultured in 0.1, 1.0 and 5.0 mM Mg^{2+}.

(A) After 96 h western blot was performed with antibodies against MagT1. Anti-β-actin antibodies were used as control of equal loading.

(B) SaOS-2 cells were silenced for *MagT1* with specific siRNAs. Scrambled, non-silencing sequences (NS) were used as controls. Western blot was performed on protein lysates using antibodies against MagT1. β-actin was used as control of loading. The histogram shows the quantitative evaluation of MagT1 vs β-actin by densitometry on three separate experiments ± standard deviation (*p ≤ 0.05).

(C) SaOS-2 cells were counted. The bars are the mean ± standard deviation of four separate experiments (*p ≤ 0.05).

(D) Nitric oxide release was measured with the Griess method. Data are expressed as the mean ± standard deviation of three separate assays (**p ≤ 0.01, ***p ≤ 0.001).

Figure 4

SaOS-2 cultured in different extracellular Mg^{2+} concentrations were silenced for *MagT1* with specific siRNAs or NS and treated with L-NIL (A) or L-NAME (B). After 96 h the cells were counted. The bars are the mean ± standard deviation of four separate experiments (*p ≤ 0.05, **p ≤ 0.01).