Biodegradable magnesium implants: a potential scaffold for bone tumor patients

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ABSTRACT Relapse and metastasis of tumor may occur for osteosarcoma (OS) patients after clinical resection. Conventional metallic scaffolds provide sufficient mechanical support to the defected bone but fail to eradicate recurring tumors. Here we report that biodegradable magnesium (Mg) wire-based implant can inhibit OS growth. In brief, the Mg wires release Mg ions to activate the transport of zinc finger protein Snail1 from cytoplasm to cell nucleus, which induces apoptosis and inhibits proliferation of OS cells through a parallel anti-tumor signaling pathway of miRNA-181d-5p/TIMP3 and miRNA-181c-5p/NLK downstream. Simultaneously, the hydrogen gas evolution from Mg wires eliminates intracellular excessive reactive oxygen species, by which the growth of bone tumor cells is suppressed. The subcutaneous tumor-bearing experiment of OS cells in nude mice further confirms that Mg wires can effectively inhibit the growth of tumors and prolong the survival of tumor-bearing mice. In addition, Mg wires have no toxicity to normal cells and tissues. These results suggest that Mg implant is a potential anti-tumor scaffold for OS patients.

Keywords: magnesium wire, osteosarcoma inhibition, hydrogen evolution, Snail1, miRNA-181

INTRODUCTION

Osteosarcoma (OS) is a common malignant bone tumor derived from bone-forming mesenchymal cells in a pre-mature state [1]. In the USA, about 3450 new cases of

bone sarcoma are diagnosed in 2018 [2]. Clinically, OS is often treated by removing through surgery combined with additional chemotherapy [3]. Nevertheless, incomplete surgical resection of the tumor tissue induces the metastasis of tumor cells, and the 5-year survival rate of these tumor patients after operation is only 60%–70% [4]. A promising solution for the tumor disease is to refill the bone defects with an antitumor and regenerative scaffold, which provides additional mechanical support to the bone and simultaneously inhibits the recurring of bone tumors [5].

Among various candidate materials, Mg and its alloys have been employed to promote the regeneration of fractured bone or bone loss [6,7], due to their biodegradability, excellent biocompatibility, and mechanical robustness for bone rehabilitation [8–10]. Implantation of Mg scaffold is a promising solution for the treatment of OS due to the following reasons: (1) the released Mg²⁺ promotes proliferation of osteoblast *in vitro* and accelerates the repair of premature bone *in vivo* [11,12]; (2) degradation products of Mg exhibit anti-tumor properties *in vitro* [13], and these degradation products are well-tolerated *in vivo*; (3) the *in vivo* degradation cycle of Mg can be tuned from six months to several years, which matches the long-term treatment cycle of OS [14–16]. Previous studies demonstrated that the degradation of Mg releases Mg(OH)₂ and elevates the alkalinity of the cell culture medium [17], by which the tumor cells were killed

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[18,19]. However, it remains unknown if the increased alkalinity can be maintained *in vivo*, where the pH buffer system in the body may dampen the antitumor effect.

In this work, we exploited high-purity Mg wires with a good mechanical property and stable degradation performance, which dramatically inhibit the growth of OS cells *in vitro* and *in vivo* through a unique pathway. Different from previous studies, we revealed the alkalinity-induced damage to tumor cells; instead, the antitumor effect of Mg ions or H_2 was scrutinized separately. Our animal experiment also confirmed that implanted Mg wires successfully prolong the survival time and suppress the bone tumors in mice.

EXPERIMENTAL SECTION

Material preparation and sterilization

The as-casted Mg with a high purity of 99.98% was extruded to a rod and then pulled into a thin wire with a final diameter of 0.77 mm provided by Suzhou Origin Medical Technology Co. Ltd., China. The sample was ultrasonically cleaned with acetone (for 10 min) and ethanol (for 15 min) to reduce organic substances on the surface. Ultraviolet (UV) radiation was used for specimen sterilization for 20 min before corrosion and cell culture tests.

Mechanical and *in vitro* corrosion tests

The mechanical properties of Mg wires were tested and

shown in Fig. 1. The corrosion test for high-purity Mg wires was carried out in the modified simulated body fluid (m-SBF) for two weeks. The ratio of the surface area of Mg to the volume of m-SBF was maintained at $1\text{ cm}^2/30\text{ mL}$. The hydrogen released from the Mg sample was dissolved in solution and tested by a dissolved hydrogen analyzer (ENH-1000, Truslex, Japan). Changes in the pH value of the m-SBF were recorded during the immersion test. After 1, 4, 7, and 14 days, the concentration of Mg^{2+} released into m-SBF was quantified with an inductively coupled plasma optical emission spectrometer (ICP-OES, ICAP 6300, Thermo Scientific, USA). The corroded samples were sequentially rinsed in a solution containing $180\text{ g L}^{-1}\text{ CrO}_3$ and $10\text{ g L}^{-1}\text{ AgNO}_3$, and distilled water to remove $Mg(OH)_2$ adsorbed. The surface morphology of the Mg wire after the immersion test was observed using scanning electron microscopy (FE-SEM, Sirion 200, FEI, USA). The weight loss of Mg wire before and after the immersion test was calculated.

Preparation of culture medium

To test the effect of Mg^{2+} on the cell viability, the culture medium was supplemented with additional Mg^{2+} concentrations by adding magnesium chloride ($MgCl_2$). The osmolality of the medium was measured using a vapor pressure osmometer (Osmo210, YASH, UK). The final concentrations of Mg^{2+} were maintained at 5, 10, 20 and 40 mmol L^{-1} .

To evaluate the effect of alkaline on the cell viability,

Figure 1 Microstructure characterization and mechanical properties of Mg wires with different diameters. (a) Metallographic images of Mg wires with different diameters. Scale bar: 50 μm . (b) Stress-strain curves, and (c) mechanical properties of Mg wires with different diameters, $n = 3$. UTS: ultimate tensile strength; EL: elongation.

the Minimum Essential Medium (MEM) and Roswell Park Memorial Institute (RPMI-1640) culture medium of tumor cells were adjusted with different pH values by a sterilized sodium hydroxide (NaOH) solution. The final pH values of the media were adjusted to 7.4, 7.7 and 8.0.

Setups for hydrogen exposure during cell culture

Mg wires ($\Phi 0.26 \times 40$ mm) were laid into a 6-well microplate filled with 1 mL phosphate-buffered saline (PBS) in each well. Plate inserts fitting the 6-well microplates were coated with polycarbonate membrane and seeded with tumor cells. To control the level of hydrogen exposed to cells, a number of Mg wires (up to 4 pieces) were immersed into each well and labeled accordingly, e.g., a label of “MW2” denotes 2 pieces of Mg wires that were used for the cell test in each well.

Cytotoxicity measurement of Mg wire and degradation products

The human OS cells, MG63 and U2-OS, and normal human bone cells, C28/I2 cells, were seeded and cultured in 96-well plates. After overnight culture, the medium was replaced by (1) the complete medium with sterilized Mg wires ($\Phi 0.26 \times 5$ mm), or (2) medium supplemented with different concentrations of MgCl_2 , or (3) media with adjusted pH values. The control group was used without any supplement or Mg wire. After incubation at 37°C in 5% CO_2 for cell proliferation, 10 μL Cell Counting Kit-8 (CCK-8) solution (DOJINDO, Japan) was added into each well, and the cells were incubated for another 4 h. Thereafter, the absorbance of each well (optical density at 450 nm (OD450)) was tested using a microplate reader (ELX800, BioTek, USA). In the H_2 exposure test, tumor cells were seeded on the upper chamber with a density of 2×10^5 cells per well and Mg wires were placed in the lower-chamber for two days. Cells in the up-chamber were treated with 100 μL CCK-8 solution and transferred into 96-well plates for further proliferation test.

Apoptosis analysis

The MG63 and U2-OS cells were incubated in medium with different concentrations of Mg^{2+} (0, 5, 10, 20 mmol L^{-1}) or adjusted pH values (7.4, 7.7 and 8.0) for 48 h and were harvested for apoptosis analysis. The levels of apoptotic cells were measured by FITC Annexin V and propidium iodide solution (Sony Biotechnology, Japan), according to the manufacturer's instructions. The mixture solution was analyzed by a flow cytometer (Cytometer FLEX S, Beckman Coulter, USA).

Intracellular ROS detection

The level of intracellular reactive oxygen species (ROS) was detected by the ROS assay kit (Mlbio, Shanghai, China) according to the manufacturer's instructions.

Effect of Mg^{2+} on the half-life and nucleocytoplasmic ratio change of Snail protein in OS cells

After 48-h treatment with Mg^{2+} (20 mmol L^{-1}), MG63 cells were suspended in MEM with 10% fetal bovine serum (FBS), seeded to 6-well plates at a density of 2×10^5 cells per well. After overnight culture, the medium was replaced with serum-free MEM containing either 50 $\mu\text{mol L}^{-1}$ MG132 or 100 $\mu\text{g mL}^{-1}$ cycloheximide (CHX, sigma, USA). After being further cultured for 0.5, 1, 2, 4 and 8 h, cells were collected and lysed. The total protein was extracted and the expression of Snail1 was detected by western blotting. At the same time, MG63 cells were treated with a gradient of Mg^{2+} (0, 5, 10 and 20 mmol L^{-1}) for 48 h, and the protein of Snail1 was located by immunofluorescence staining (1:200, Abcam, UK). The nucleus was separated and the nucleoprotein was extracted for nucleocytoplasmic ratio change assay of Snail1 by western blotting.

In vivo antitumor effect on tumor-bearing mouse model

Our animal experiment was approved by the Experiment Animal Ethics Committee of the Second Military Medical University. MG63 cells ($1 \times 10^6 \mu\text{L}^{-1}$) were suspended in 50 μL medium, which was injected subcutaneously into the flank regions of 30 female athymic nude mice. Three weeks after inoculation, tumors were grown into a diameter of 2.5 mm. These mice were randomly divided into five groups (six mice in each group), including a model group without any treatment, a stainless steel (SS) group, an Mg-implanted group, an Mg-implanted group combined with miRNA-181c/d-5p expression, as well as the Mg-implanted group with negative control (NC). For mice to be implanted with Mg, Mg wires with an average diameter of 0.77 mm and an average length of 2.5 mm were inserted into tumors. The SS wires of the same size were inserted as a control. The miRNA-181c/d-5p + Mg represents overexpressed exogenous miRNA-181c/d-5p by lentivirus with inserted Mg wires. The NC + Mg group represents an overexpressed control sequence of the miRNA-181c/d-5p and inserted Mg wires. Starting from the 2nd week, each animal in all intervention groups was injected with 50 μL lentivirus (5×10^7 IFU) twice a week, lasting for four weeks. The control model group were injected with the same volume of saline instead. For the Mg-implanted animals, the Mg wires were sterilized be-

fore implantation. To plot the growth curve of the tumor, the diameter of the tumor was measured weekly since the 2nd week. The volume of the tumor, V , was roughly estimated by $V = ab^2/2$, in which a and b represent the length of the major and minor axis, respectively. Each group at different treatments was euthanatized on the 28th day after administration. Hematoxylin & eosin (H&E) and Ki67 assay were performed using tissues from subcutaneous tumorigenesis of MG63 cells. Images were collected with an inverted fluorescence microscope. The expressions of NLK and TIMP3, and phosphorylation of Snail1 in subcutaneous tumors were quantified by western blotting, levels of miRNA-181c/d-5p were measured by real-time quantitative PCR (RT-qPCR).

RT-qPCR

The PCR system includes Takara SYBR Premix Ex Tap (10 μ L), forward and reverse primers (20 μ mol L⁻¹, 0.2 μ L each), and 2 μ L of cDNA supplemented with deionized H₂O to 20 μ L. The cycling steps include 40 cycles, and each cycle contains denaturation at 95°C for 10 s, annealing at 60°C for 20 s, and elongation at 72°C for 20 s. U6 snRNA was used as a reference to normalize the miRNA-181a/b/c/d-5p level using the 2^{- $\Delta\Delta C_t$} method. Each RNA sample was run in triplicate. The primers' sequences are shown in Table S1.

Immunoblot analysis

The total protein was extracted from the cells using the M-PER mammalian protein extraction reagent or from tissues using the T-PER tissue protein extraction reagent (Pierce, USA). An equal amount of protein (20 μ g per lane) estimated by the bicinchoninic acid protein assay kit (Pierce) was loaded onto (11%) SDS-PAGE gels and transferred onto nitrocellulose membranes. The blots were probed with a monoclonal antibody against human Snail1 (1:200), NLK (1:500), TIMP3 (1:600) and β -actin (1:1200, Abcam), followed by a secondary HRP-conjugated anti-rabbit antibody (Abcam) staining. After washing, the bands were detected by chemiluminescence and imaged with X-ray films. β -actin was used as an endogenous reference for normalization.

Molecular docking analysis

To analyze the inhibitory action of Mg by computational docking studies, a computational ligand-target docking approach was used to analyze structural complexes of the Snail1 (target) with Mg²⁺ (ligand) to understand the structural basis of this protein target specificity. And the docking was carried out by the PyMOL and Autodock

software.

Statistical analyses

All statistical analyses were performed using SPSS 20.0 (SPSS, Chicago, IL, USA). Data were expressed as mean $\pm$ standard deviation (SD). Differences among groups were analyzed by one-way analysis of variance (ANOVA) followed by Least square difference's post hoc test. $p < 0.05$ was considered a significant statistic difference.

RESULTS

Microstructure characterization, mechanical properties and degradable properties of different Mg wires

The microstructure and mechanical property of Mg wires were studied as shown in Fig. 1. During the whole drawing process, the diameter of Mg wire decreased gradually from 1.22 to 0.77 mm. In this process, the deformation twin could be found in samples ϕ 1.22, ϕ 1.0 and ϕ 0.9 (as marked arrow in Fig. 1a), except ϕ 0.77, indicating the recrystallization in ϕ 0.77 is more sufficient than that in other samples. Thus, grain refinement of ϕ 0.77 was the most remarkable in all Mg wires. With the increase in drawing reduction, the grain size of Mg wires reduced. Fig. 1b shows the stress-strain curves during the tensile test of these Mg wires. The ultimate tensile strength and elongation are shown in Fig. 1c. According to the Hall-Petch relation, decreasing the grain size can harden both metals and alloys [20]. Therefore, both of strength and elongation increase with the process of drawing (decrease in grain size). Finally, the sample ϕ 0.77 which is used *in vivo* study of present work achieves good mechanical properties (UTS (175 $\pm$ 5.6) MPa, elongation (4.8 $\pm$ 0.5)%).

The corrosion behavior of Mg wires was tested in an m-SBF solution for 14 days. As time proceeded, we observed an increasing pH value of the medium, release of H₂ bubbles and accumulation of Mg²⁺ in the m-SBF, in agreement with previous studies (Fig. S1a–c) [21]. The degradation rate declines with the proceeding of degradation until a stable degradation rate reached on the 4th day. The corresponding release rate of hydrogen is about 250 μ g L⁻¹. With an extension of soaking time, the concentration of Mg²⁺ in the solution increases accordingly, and the slope of the Mg²⁺ release curve is observed to decline as a result of decreased corrosion rate. SEM observation reveals that a uniform Ca-P corrosion layer formed on the surface of Mg retards the corrosion, and Mg wire maintains integrity after 14-day immersion (Fig. S2). The corrosion rate of Mg was about 1 mm per year

(Fig. S1d).

Mg wires inhibit the viability of OS tumor cells

To test whether the degradable Mg wires affect the viability of bone tumor cells, Mg wires were separately cultured with two kinds of common tumor cells, MG63 and U2-OS. When four pieces of Mg wires were co-cultured with cells for 24 h, the proliferation inhibition rate of MG63 and U2-OS cells were about 99% and 56%, respectively (Fig. 2b, d). The more remarkable anti-tumor effect on MG63 cells is attributed to more degradation of Mg wires, and severe pitting corrosion is observed in the MG63 culture medium, but not in the U2-OS culture medium (Fig. 2a, c). The severe pitting corrosion in the MG63 culture medium is attributed to the presence of a higher concentration of Cl^- in MEM than that in U2-OS (RPMI-1640) culture medium. Aggressive Cl^- have been shown to react with the intermediate corrosion product of Mg and thus penetrate through the passivated corrosion layer, by which corrosion pits are formed.

Effect of Mg corrosion products on the viability and apoptosis of cells

To quantitatively understand how the degradation of Mg

affects the viability of tumor cells, three independent degradation products of Mg on the cellular viability were separately studied.

The effect of pH value

Similar to the alkalization in m-SBF, degradation of Mg in the cell culture medium causes an increased pH from 7.4 to 8.0 during the first 24 h, as shown in Fig. S3. To test the effect of pH while excluding the influence of Mg^{2+} or H_2 exposure on the viability of cells, the initial pH values of the MEM or RPMI-1640 medium were adjusted to 7.4, 7.7 and 8.0. The increased alkalinity was not shown to impair the viability of the MG63 and U2-OS cells (Fig. S4) or change their apoptosis rate (Fig. S5).

The effect of H_2

To control the level of hydrogen exposed to cells, a number of Mg wires (up to four pieces) were immersed into each well (Fig. 3a). To rule out the effects of the other two degradation products (Mg^{2+} and pH) on tumor cells, we tested the concentration of Mg^{2+} in the culture medium. We found there was no significant difference in the concentration of Mg^{2+} in the upper-medium of the three groups (Fig. 3b). It indicates that the upper chamber cell

Figure 2 Mg wires inhibit the viability of tumor cells. (a, c) SEM images showing the surface morphology of Mg wires immersed in the culture medium of MG63 cells (a) and U2-OS cells (c) for 24 h; (b, d) cell viability of MG63 (b) and U2-OS (d) cells after co-culture with different numbers of Mg wires (marked as MW 1, 2, 3 and 4). * $p < 0.05$; ** $p < 0.01$.

Figure 3 The effect of H₂ on OS cells. (a) Schematic diagram of hydrogen release device; (b) concentration of Mg ions in the different culture medium of upper chamber; (c) cell viability of MG63 and U2-OS cells after exposure to H₂; (d) levels of intracellular ROS of MG63 and U2-OS cells after exposure to H₂. Data are expressed as mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$.

culture and the lower chamber Mg degradation environment are not connected, only the H₂ acts on the cells through upward diffusion. We next tested the effect of H₂ on the viability of OS cells. As an important corrosion product of Mg, hydrogen gas has been reported to affect the activity of tumor cells [22]. Hydrogen gas released from degraded Mg could pass through the cell membrane and partially eradicate the endogenous free radicals from tumor cells, evidenced by measuring the ROS released from lysed cells, as shown in Fig. 3c. With the sustained exposure to a higher dose of hydrogen, the proliferation of OS cells was inhibited (Fig. 3d) [23].

The effect of Mg²⁺

To test the influence of Mg²⁺ on the viability of tumor cells, Mg²⁺ was supplemented into the culture medium through the addition of MgCl₂. As the addition of excessive MgCl₂ may increase the osmolality of the cell culture medium and thereby causes cell death through osmotic dehydration [24]. To rule out the effect of high osmolality on OS cells, we tested the osmolality of MEM with different concentrations of Mg²⁺. As shown in Fig. 4a, osmotic pressure has a linear relationship with Mg²⁺ concentration. The susceptibility of normal human bone cells C28/I2 to the excessive Mg²⁺ was tested. When the concentration of Mg²⁺ is no more than 20 mmol L⁻¹, no significant statistical difference in the viability of normal bone cells was seen (Fig. 4b). In comparison, when MG63 and U2-OS cells were cultured in Mg²⁺-rich culture

medium (10–20 mmol L⁻¹), the proliferation of tumor cells was significantly restrained (Fig. 4c, d). Particularly, the apoptosis rate of the OS cells was increased with the concentration of Mg²⁺. The apoptosis rate of MG63 cells treated with 20 mmol L⁻¹ Mg²⁺ was up to 53.89% compared with 10.27% without Mg²⁺ treatment, and the apoptotic rate of U2-OS increased from 6.45% to 67.55% with the 20 mmol L⁻¹ Mg²⁺ treatment (Fig. 4e, f).

Combining the degradable properties of Mg wires, we found that Mg²⁺ and H₂ could inhibit the growth of OS cells. The excessive Mg²⁺ exhibited pronounced antitumor activity, hence we next investigated the mechanism of Mg wires on tumor cells by regulating the Mg²⁺.

Mg²⁺-mediated signaling pathway for inhibition of OS

To further clarify the molecular mechanism supporting the anti-OS effect of Mg wires, we explored the Mg²⁺-involving signaling pathway for suppression of tumor cells. Snail1 is a transcription factor that plays vital roles in various physiological processes of OS, and the statistics of the clinical case have shown that Snail1 is highly expressed in tumor cells (Fig. S6) [25–27]. However, it remains unknown whether Mg²⁺ modulates the activation of Snail1. Hence, computational docking analysis was performed to analyze the interaction between Mg²⁺ and the target protein Snail1. According to the results, we found Mg²⁺ had contacts with Cys and His residues of the protein (Fig. 5a). Therefore, Snail1 is proposed as a target protein in the downstream of the

Figure 4 The effect of Mg^{2+} on normal human bone cells (C28/I2) and OS cells (MG63 and U2-OS). (a) The osmolality of MEM supplemented with different concentrations of Mg^{2+} ; (b) cell proliferation of normal human bone cells (C28/I2) in medium of different concentrations of Mg^{2+} for 12, 24, 48 and 72 h, respectively; (c, d) cell viability of MG63 and U2-OS in medium with Mg^{2+} concentrations of 0, 5, 10 and 20 mmol L^{-1} ; (e, f) apoptosis rates of MG63 and U2-OS with different concentrations of Mg^{2+} in medium. Data are expressed as mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$.

Mg^{2+} -activated pathway for inhibition of OS cells.

Mg^{2+} promotes nuclear import and phosphorylation of Snail1 protein

Snail1 has been reported to modulate the epithelial-to-mesenchymal transition by suppressing E-cadherin dur-

ing the metastasis of cancer [28]. The cytological function of Snail1 is regulated by the level of phosphorylation and its subcellular localization. Different from healthy bone cells, Snail1 protein mainly distributes in the cytosol of OS cells, as shown in Fig. 5b. After MG63 cells were cultured in Mg^{2+} -enriched medium for 48 h, the majority

Figure 5 Effect of Mg^{2+} on the expression and distribution of Snail1 protein. (a) The contacts of Mg^{2+} and Snail1 protein by protein docking; (b) immunofluorescence staining of Snail1 in MG63 cells for 48 h. Scale bar: 50 μ m. (c) Distribution of Snail1 in cell nucleus relative to the total protein (left) and phosphorylation of Snail1 (right) after treatment with 0, 5, 10, 20 mmol L⁻¹ Mg^{2+} ; (d) cellular synthesis (left) and half-life time (right) of Snail1 after treatment with Mg^{2+} in the indicated concentrations.

of the Snail1 protein was observed to translocate into the nucleus, as shown by the immunofluorescence staining in Fig. 5b. Western blotting data further confirmed that the phosphorylated Snail1 in MG63 cells were imported back to the nucleus with the increasing Mg^{2+} concentration in the culture medium. Correspondingly, the transcriptional activity of Snail1 in the nucleus was increased (Fig. 5c). By separately inhibiting the pathways of intracellular protein synthesis and degradation using 50 μ mol L⁻¹ of CHX and 10 μ mol L⁻¹ of MG132, we found that the half-life of Snail1 became shortened after treatment with excessive Mg^{2+} , which may be attributed from the accelerated ubiquitination and degradation of Snail1 inside the nucleus (Fig. 5d).

Effect of Mg^{2+} on the expressions of miRNA-181c/d-5p and their target proteins

NLK and TIMP3 proteins are key regulators for proliferation, migration, and invasion of tumor cells [29,30]. In the previous work, miRNA-181-5p has been shown to directly target the antitumor proteins of NLK and TIMP3 [31]. To elucidate whether Mg^{2+} regulates the expressions of NLK and TIMP3 through the miRNA-regulated post-transcription, changes in the miRNAs were determined through RT-qPCR and the expression levels of the target

proteins were tested by western blotting. We found that the NLK and TIMP3 proteins were overexpressed after Mg^{2+} treatment, as shown in Fig. S7. The expressions of miRNA-181c-5p and miRNA-181d-5p in OS cells were down-regulated with increasing Mg^{2+} (Fig. S8). Therefore, Mg^{2+} promotes the death of OS cells by increasing the NLK and TIMP3 expressions through the miRNA-regulated post-transcription.

Analysis of two parallel pathways of Mg^{2+} on anti-OS effect

In order to investigate the inhibitory pathway of OS cells activated by Mg^{2+} , a lentiviral approach was used for the expressions of Snail1 and miRNA-181c/d-5p, and the silence of NLK and TIMP3 proteins. The gene delivery efficiency (evaluated by calculating the percentage of the cells expressing GFP relative to the total cells) was over 90%, indicating a success of lentivirus transfection, as shown in Fig. S9. Our results show that the expression of Snail1 was not affected by the overexpressed miRNA-181c/d-5p or by deleting the NLK/TIMP3 proteins. The overexpression of Snail1 in the cell nucleus significantly reduced the level of miRNA-181c/d-5p, and the expressions of NLK and TIMP3 proteins were promoted. In addition, an elevated level of miRNA-181c-5p can deactivate the NLK protein system, and the increasing level of

miRNA-181d-5p deactivated the expression of TIMP3 protein (Figs S10 and S11). These observations indicate that Snail1 activates the expressions of NLK and TIMP3 by down-regulating the expressions of miRNA-181c-5p and miRNA-181d-5p.

The effect of the pathway on the proliferation, apoptosis, invasion and migration of OS cells

As shown in Fig. S12, an up-regulated level of miRNA-181c-5p or miRNA-181d-5p promotes the proliferation of MG63 cells, while excessive Mg^{2+} (20 mmol L^{-1}) can inhibit the growth of OS cells by down-regulating the expression of miRNA-181c/d-5p. We also carried out an invasion and wound healing assays to separately track the invasion and migration of MG63 cells transfected by lentivirus. Results show that excessive Mg^{2+} successfully reduced the invasion and migration of MG63 cells, compared with the control medium supplemented with a normal concentration of Mg^{2+} (Figs S13 and S14). Ad-

ditionally, the TUNEL assay showed the apoptosis of MG63 cells was enhanced in the presence of excessive Mg^{2+} . In contrast, the Mg^{2+} -induced apoptosis was greatly diminished after the cells were transfected with miRNA-181c-5p and miRNA-181d-5p (Fig. S15). These observations confirm that Mg^{2+} can effectively suppress the expression of miRNA-181c/d-5p, by which the proliferation, invasion, migration of OS cells were strongly inhibited.

The anti-tumor effect of Mg wires *in vivo*

In our attempt to further use Mg scaffold as a new anti-OS therapy, animal experiments were carried out by transplanting MG63 tumor xenograft on nude mice. Changes in the volume of the tumors were recorded for four weeks (Fig. 6a). Compared with the mice in the model group without implantation of Mg wires, the implantation of Mg wires could significantly inhibit the growth of subcutaneous tumors, while the rapid growth

Figure 6 Anti-tumor effect of Mg *in vivo*. (a) The volume of tumors in MG63 tumor-bearing mice after different treatments ($n = 6$); (b) survival rate of mice with different treatments for 15 weeks ($n = 12$); (c, d) quantitative measurement of miRNA-181c-5p and miRNA-181d-5p in tumors with different treatments; (e) the expressions of Snail1, pSnail1, NLK and TIMP3 in tumors after different treatments. β -actin treatment was used as a reference. (f) H&E and Ki67 staining of tumor sections from each group, scale bar: 50 μm . Images were collected on the 28th day. Data are expressed as mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$.

of tumors was observed in the miRNA-181c/d-5p over-expressed group. It should be noted that the suppressed growth of tumors in mice was not elicited by the operative injury. When SS wires were implanted into the same position of the nude mice, the growth of the tumor was not suppressed. The survival rate of the Mg-implanted animal group was 100% at the 10th week, which declined to 66.67% at the 15th week, as shown in Fig. 6b. In comparison, a drastic death rate was noticed since the 4th week for the Mg-free animal group and all mice died in the 14th week. The death rate of miRNA-181c/d-5p + Mg group drops even faster than that of the model group. In addition, the biosafety of Mg implantation was evaluated by H&E staining of major organs. As depicted in Fig. S16, no obvious pathological changes were found in the heart, liver, spleen, lung, and kidney after different treatments, reflecting no side effect of Mg wires *in vivo*. An analysis of the homogenized tumor tissues revealed that Mg wire can promote the activity and degradation of Snail1 protein. Subsequently, it down-regulates the expressions of miRNA-181c-5p and miRNA-181d-5p, by which the expressions of NLK and TIMP3 were elevated (Fig. 6c–e). This observation is consistent with the *in vitro* observation that Mg^{2+} induces the death of tumor cells by the parallel pathway. In the H&E staining study, a lower density of tumor cell nuclei was observed (violet) and the shape of cells is relatively homogeneous in the Mg group. In contrast, the tumor cells in the model group have hyperchromatic nuclei, scant cytoplasm, and polygonal cellular shape, as depicted in Fig. 6f. Furthermore, more tumor cells are arrested in the division stage (labeled in black circle) in the model group compared with the Mg group. In addition, the proliferating cell nuclear antigen (Ki67) measurement also indicates that Ki67-positive (brown) tumor cells decline quickly after treatment with Mg wires, i.e., the tumor cell proliferation was significantly inhibited. However, the overexpression of miRNA-181c/d-5p by lentivirus promoted the division and growth of the OS tumor cells (Fig. 6f), yielding increased mortality in nude mice.

DISCUSSION

Previous studies have reported that Mg-based biodegradable materials can significantly promote the regeneration of bone in bone defect and thereby they have been widely employed as bone screws and scaffolds for orthopedic implantation [32]. Despite their general biosafety and biocompatibility, the clinical effectiveness of this anti-tumor material to patients with fatal diseases, such as bone cancers, needs further investigation

[10,33,34]. Qiao *et al.* [35] reported that Mg inhibited the growth of ovarian cancer cells *in vitro* and *in vivo*. Yang *et al.* [36] found that Mg alloy killed murine breast cancer cells and rabbit hepatocellular carcinoma cells using magnetic hyperthermia therapy. Herein, we explored whether the Mg implantation could inhibit the relapse of bone tumor cells while exploited as a scaffold after the surgery. First, we prepared Mg wires of different diameters and found that the $\phi 0.77$ Mg wire showed an excellent mechanical property and a stable corrosion rate of approximately 1 mm per year. (Fig. 1 and Fig. S1). SEM images also showed unbroken Mg wires after long time immersion. These results indicate the robustness of using Mg implants for providing long-term mechanical support to bone rehabilitation and cancer therapy.

Subsequently, the Mg wires were co-cultured with the OS cells for 24 h, and dramatically damaged the growth of the cells. Impressively, the inhibiting rate of MG63 cells reaches up to 99%. Based on the prominent anti-tumor effect, we separately studied the effects of three Mg degradation products (pH, H_2 and Mg^{2+}) on MG63 and U2-OS cells to further investigate the mechanism of Mg wires against the bone tumor. In recent studies, Mg and Mg alloys exhibited anti-tumor properties after immersed in extracts of cell culture medium [18]. The antitumor effect of Mg has been mainly ascribed to the rapid alkalization of the culture solution. The statement is well-established when the volume of the culture solution is relatively small compared with the exposed surface area of Mg *in vitro*. However, the rapid alkalization is less reproducible *in vivo* due to the pH buffer system in the body. Accordingly, our study suggests that a mild pH increase from 7.4 to 8.0 did not affect the proliferation and apoptosis of OS cells (Figs S4 and S5). Increased generation of ROS has long been observed in tumor cells, and Ohsawa *et al.* [37] found that H_2 can selectively eliminate the hydroxyl radical which is the most cytotoxic of ROS in tumor cells, while not affecting the role of ROS in normal cells. Here, we designed a unique device that releases hydrogen from Mg and found H_2 can interfere with the growth of the OS cells by eradicating the overproduced ROS in MG63 and U2-OS cells, but not efficiently kill them (Fig. 3) [18,19,38].

The discrepancy between the limited tumor death induced by alkalinity/ H_2 *in vitro* and the pronounced anti-tumor activity of Mg in mice suggest a potential anti-tumor activity of excessive Mg^{2+} . Excessive Mg ions were generated through the decomposition of $Mg(OH)_2$ and their accumulative concentration increased with the degradation of Mg over time. Within the tested range of

Mg^{2+} concentrations from 5 to 20 mmol L^{-1} , faster growth of human osteoblast cells has been reported, and the rehabilitation of bone fracture is also accelerated *in vivo* [11,39]. Similarly, we found that 5–20 mmol L^{-1} of Mg^{2+} was not toxic to normal bone cells. When the concentration of Mg^{2+} was elevated to 10–20 mmol L^{-1} , we demonstrated that the proliferation of MG63 and U2-OS cells were suppressed and their apoptosis rates were increased. The osmolality of culture medium supplemented with 20 mmol L^{-1} MgCl_2 is about 344 mOsm kg^{-1} (Fig. 4a), which is not harmful to normal cells. However, an even higher dose of Mg^{2+} ($\sim 40 \text{ mmol L}^{-1}$) will affect the viability of bone cells, attributed to the high osmolality of culture medium. An optimized medium supplemented with about 20 mmol L^{-1} MgCl_2 inhibited the proliferation of MG63 and U2-OS cells by promoting their apoptosis.

The anti-tumor effect of excessive Mg^{2+} could be explained within the framework of competitive transportation of Mg^{2+} and Ca^{2+} through the TRPM7 channel [40], a ubiquitous ion channel controlling the transportation of divalent cations and activation of Snail1 protein [41]. In patients with colon neoplasia, the homeostasis of Mg^{2+} is frequently impaired and the cellular absorption ratio of $\text{Ca}^{2+}/\text{Mg}^{2+}$ is elevated. The deficient cellular uptake of Mg will activate the Snail1 pathways and promote the epithelia-to-mesenchymal transition (EMT). In a typical process, more Snail1 protein was observed to transport from the nucleus to cytoplasm, which clinically

correlates to the formation of malignant carcinoma and increased mortality in patients with cancers (Fig. S6) [42]. Interestingly, the increasing Mg intake has been shown to negatively correlate to the risk of colorectal adenoma, indicating that excessive Mg may revert the viability of these cancer cells.

As Mg^{2+} competes with Ca^{2+} for the same transporters, in our study, the presence of excessive Mg^{2+} (a natural antagonist of calcium ion) may suppress the cellular absorption ratio of $\text{Ca}^{2+}/\text{Mg}^{2+}$, thus further restricting the transportation of Snail1 proteins from nucleus to cytoplasm. According to the protein docking analysis, Mg^{2+} may bind to multiple sites to activate Snail1 channel (Fig. 5a). Once activated, Snail1 may transport from the cytoplasm back to the nucleus, where ubiquitylation and degradation of the Snail1 occur (Fig. 5c, d) [42,43]. As a hematopoietic lineage modulator, the miRNA-181 family is involved in the Snail1-mediated EMT process [44]. When Snail1 ubiquitinates in the nucleus, it down-regulates the expressions of miRNA-181c-5p and miRNA-181d-5p in OS cells (Fig. S8). The change of miRNA-181-5p then modulates downstream target protein, such as NLK and TIMP3. It has been reported that NLK protein is a key suppressor against the proliferation and migration of tumor cells [29,43], and TIMP3 protein performs as a key regulator to apoptotic tumor cells [30,45]. Herein, we report a new mechanism about Mg wire-induced death of OS cells, as depicted in Scheme 1. In brief, we found that Mg wires sustainedly degraded to produce

Scheme 1 A schematic diagram showing how the biodegradable magnesium stimulates the molecular pathway for inhibition of OS cells. Mg^{2+} released from the Mg wires promotes the phosphorylation and nuclear import of protein Snail1. The increase in the transcriptional activity of Snail1 down-regulates miRNA-181c-5p and miRNA-181d-5p, which enhances the expressions of proteins of NLK and TIMP3. At the same time, H_2 released by Mg quenches excessive $\cdot\text{OH}$ inside cells, reduces the ROS level and modulates cell proliferation.

Mg²⁺ which could induce apoptosis of OS cells without causing damage to normal cells. To understand this anti-tumor phenomenon, we tried and established the regulation theory and mechanism of Mg²⁺ on inhibiting the progression of OS tumors with the nuclear transcription factor Snail1, a key protein in regulating EMT and tumor progression as the core [46]. We found that a suitable concentration of Mg²⁺ can shorten the half-life of Snail1 in tumor cells by changing the nuclear to cytoplasmic ratio, resulting in a decrease of Snail1 expression. Our mechanism study showed that Mg²⁺ could inhibit the proliferation and induce apoptosis of OS tumor cells through the two parallel pathways: Snail1/miRNA-181c-5p/NLK and Snail1/miRNA-181d-5p/TIMP3, which is consistent with the previous meta-analysis of the anti-tumor effect of Mg²⁺ [47]. In the meantime, Mg wires perform a unique anti-tumor effect by exposing molecular H₂ to disturb the physiological redox homeostasis in bone tumor cells.

In view of other metallic orthopedic implants in clinical trials, titanium (Ti) alloys and SS are frequently used in OS operation. However, residual OS cells often cause OS recurring from long-term clinical observations [6]. Compared with traditional implanted materials, biodegradable Mg exhibited a unique anti-tumor effect ascribed to its degraded products. Compared with the model group, Mg wires inserted into the tumor effectively restricted the size of tumors and prolonged the lifetime of mice. In addition, the implanted Mg wires avoided damage to normal tissue, which is safe for clinical use. RT-qPCR and western blotting revealed Mg wires activated Snail1 to degrade and the expressions of TIMP3 and NLK protein were up-regulated. In the meantime, Mg wires down-regulated the expression of miRNA-181c/d-5p. The parallel pathway of Mg wires inhibits OS cell growth, in agreement with the observations of *in vitro* test.

So far, a list of metal/metal oxide has been proved to be useful in promoting apoptosis of cancer cells, e.g., zinc acts as a tumor suppressor on the malignant cells by interacting with our immune system [48,49]; iron oxide, a degradation product of Fe implants, induces antitumor activity in tumor-associated macrophages [50]. Compared with other anti-tumor metals, the magnesium (and its degradation products) is a well-tolerant macroelement in our body, and the use of Mg-based implants is expected to develop a safe anti-tumor strategy.

CONCLUSIONS

In summary, the specialized Mg wires are considered as the desired scaffold after bone tumor surgery. The mo-

lecular biology underlying the anti-tumor effect of Mg wires was reported for the first time. Mg wire produces H₂ that alerts redox status in bone tumor cells and inhibits their proliferation. In addition, we showed that excessive Mg²⁺ from Mg wires can inhibit the proliferation and induce apoptosis of OS cells through Snail1/miRNA-181c-5p/NLK and Snail1/miRNA-181d-5p/TIMP3 pathway. In consistent with *in vitro* observations, our animal tests also highlight the importance of Mg wire-associated inhibition pathway *in vivo*. Compared with the SS, the implanted Mg wires effectively restrict the size of tumors and prolong the lifetime of mice with OS. Therefore, the safety and efficient Mg wires have a great promise in the future application for bone tumors in clinical surgery.

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Author contributions Zan R and Ji W designed the study and performed the cell experiments. Ni J, Wang W and Zhang S assisted in the animal experiment. Qiao S and Wu H prepared the Mg material. Zhang

X and Yang B analyzed the data. Song Y and Ji T revised the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

Supplementary information Experimental details and supporting data are available in the online version of the paper.

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生物可降解镁植入物——骨肿瘤患者的潜在支架

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摘要 骨癌患者切除术后可能发生复发和转移. 传统的金属支架可以对骨缺损部位提供力学支撑, 但无法有效清除复发的肿瘤细胞. 本文中, 我们介绍了一种可以抑制骨肉瘤生长的生物可降解镁丝植入物. 简而言之, 镁丝释放镁离子激活锌指蛋白Snail1从胞浆到细胞核的转运, 通过下游的miRNA-181d-5p/TIMP3和miRNA-181c-5p/NLK两条平行的抗肿瘤信号通路诱导骨肉瘤细胞凋亡, 抑制骨肉瘤细胞增殖. 同时, 镁丝释放出的氢气消除了细胞内过多的活性氧, 从而抑制了骨肿瘤细胞的生长. 裸鼠骨肉瘤细胞皮下荷瘤实验进一步证实镁丝能有效抑制肿瘤生长, 延长荷瘤小鼠生存期. 此外, 镁丝对正常细胞和组织无毒性, 揭示了镁植入物是骨肉瘤患者潜在的抗肿瘤支架材料.