Strontium Enhances Osteogenic Differentiation of Mesenchymal Stem Cells and In Vivo Bone Formation by Activating Wnt/Catenin Signaling

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ABSTRACT

Strontium ranelate is a newly approved drug that can reduce the risk of vertebral fracture, which is attributed to its dual function in increasing the bone formation and decreasing the bone resorption. Strontium-containing hydroxyapatite was also demonstrated to stimulate the osteoblast activity and inhibit the osteoclast activity. However, the molecular mechanisms of strontium underlying such beneficial effects were still not fully understood. In this study, we investigated the effects of strontium on the osteogenic differentiation of human mesenchymal stem cells (MSCs) and its related mechanism; its osteogenic potential was also evaluated using a calvarial defect model in rats. We found that strontium could enhance the osteogenic differentiation of the MSCs, with upregulated extracellular matrix (ECM) gene expression and activated Wnt/β-catenin pathway. After transplanting the collagen-strontium-substituted hydroxyapatite scaffold into the bone defect region, histology and computed tomography scanning revealed that in vivo bone formation was significantly enhanced; the quantity of mature and remodeled bone substantially increased and ECM accumulated. Interestingly, strontium induced an increase of β-catenin expression in newly formed bone area. In this study, we showed for the first time that strontium could stimulate the β-catenin expression in vitro and in vivo, which might contribute to the enhanced osteogenic differentiation of MSCs and in vivo bone formation. STEM CELLS 2011;29:981–991

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Strontium ranelate is a newly available drug that has been shown to reduce the risk of vertebral fracture in postmenopausal women [1]. Strontium-containing bone cement was also demonstrated to have good bioactivity and possess good bone binding strength [2]. The beneficial effects of strontium on promoting bone formation are closely related to its capability to increase bone formation and decrease bone resorption [3, 4]. The molecular factors involved include calcium sensing receptor (CaR), which is an important receptor involved in the strontium-induced replication of the osteoblasts [5] and the apoptosis of the osteoclasts [6]. However, the CaR-independent pathway may also be involved in the osteogenic process [7]; a full understanding of the exact role of strontium on osteoblastic differentiation and related molecular mechanisms will substantiate the use of strontium in tissue engineering.

Mesenchymal stem cells (MSCs), which possess the self-renewal capacity and the capability to differentiate into osteoblasts [8], have been used as a model to study the strontium-induced osteogenic process. Previous studies have shown that strontium could induce prostaglandin production and cyclooxygenase expression to increase osteoblastic differentiation of MSCs [9]. Peng et al. [10] demonstrated that strontium could increase in vivo osteoblastic differentiation of rat bone marrow-derived MSCs; Ras/mitogen-activated protein kinase (MAPK) signaling might play a role in the in vitro differentiation process of MSC [11]. However, how strontium influences important signaling pathways to regulate osteogenic
osteoblastic differentiation of human MSC and promote in vivo bone formation remained unknown.

Wnt/β-catenin signaling is involved in almost every aspect in embryonic development [12] and plays a central role in bone development and homeostasis [13]. β-Catenin signaling also plays an important role in regulating the commitment of the differentiation of pluripotent stem cell into osteoblast lineage during fracture healing [14]. The canonical Wnt signaling could regulate osteogenesis of MSCs [15] and improve the efficiency of bone tissue engineering [16]. Recently, Fromigué et al. [17] also showed that the Wnt signaling pathway was involved in strontium-induced proliferation and differentiation of the murine osteoblasts.

Based on the important role of Wnt/catenin signaling in osteogenesis, here we hypothesized that strontium could promote osteoblastic differentiation of MSC and in vivo bone formation through Wnt/catenin activation. To test the hypothesis, we first investigated the effects of strontium on in vitro osteogenic differentiation of MSCs, then we established a calvarial defect rat model for transplanting the strontium-containing scaffold. We found that both the osteogenic differentiation of MSCs and in vivo bone formation was enhanced by strontium. Expression of β-catenin was upregulated; extracellular matrix (ECM) accumulated in the osteogenic differentiation of MSCs and in vivo bone formation. Our study has provided new insights into the mechanism by which strontium regulates osteoblastic differentiation of MSC and promotes in vivo bone formation through the Wnt/β-catenin signaling pathway.

**Materials and Methods**

**In Vitro Culture and Osteogenic Differentiation of MSCs**

The experiments were approved by Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences. The MSCs isolated from human umbilical cord were kindly donated by Chinese Eastern Union Stem cell & Gene Engineering Company (Huzhou, China, www.huadongstemcell.com). The isolation and culture of human MSCs was performed as described previously, with the consent of the patients [18]. Briefly the tissue pieces were digested with 0.01% collagenase II (Sigma-Aldrich, St. Louis, MO, www.sigmalldrich.com) and 0.25% trypsin (Gibco, Invitrogen, Carlsbad, CA, www.invitrogen.com) for 20 minutes. The supernatant was plated in nonadherent flasks, and the nonadherent cells were removed. The passage one cells were plated in 75 cm² flasks and cultured with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 1% antibiotics mixture (10,000 U of penicillin and streptomycin) for 7 days. The culture medium was refreshed every 3 days before the cells reached confluence [19]. Phase contrast images were taken using a microscope (IX71, Olympus, Center Valley, PA, www.olympusflouview.com).

For osteogenic differentiation, the cells were plated in six-well plates at a concentration of 4 × 10⁵ cells per square centimeter and divided into three groups: control group was cultured in DMEM only; dexamethasone (Dex) group was cultured in osteogenic medium; and strontium (Sr) group was cultured in osteogenic medium containing 2 mM strontium chloride. The osteogenic medium was composed of DMEM supplemented with 0.1 μM dexamethasone (Sigma), 50 μg/ml ascorbic acid (Sigma), and 10 mM glycerol 2-phosphate (Sigma). The medium in each group was changed three times every week. Osteogenic differentiation was examined by alkaline phosphatase (ALP) staining, Von Kossa staining, and immunostaining with specific antibodies [11].

**ALP Staining**

ALP staining was carried out at day 10 of the osteogenic differentiation, using BCIP/NBT ALP kit according to the manufacturer’s instructions (Beyotime, Shanghai, China, www.beyotime.com). Briefly after a 30-minute fixation with 4% buffered neutral formalin, the cell was incubated in a mixture of nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate for 1 hour. The resulting blue, insoluble, granular dye deposit indicated sites of ALP activity. The percentage of ALP-positive cells in each high magnification field of each group was calculated and compared among groups.

**Von Kossa Staining**

At day 28 of differentiation, the cells were fixed with 4% formalin for 30 minutes at room temperature and rinsed three times with phosphate buffered saline (PBS). Silver nitrate (5%) was then added and the plates were positioned under ultraviolet light for 1.5 hours. The cells were rinsed three times with tap water and treated with 5% sodium thiosulphate to remove the background staining. After washing three times, the dishes were finally air dried before microscopy. The percentage of bone nodules in each high magnification field of control, Dex, and Sr groups was calculated and compared among groups.

**Gene Expression Analysis Using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)**

At 2 weeks after the osteogenic differentiation, total RNAs were extracted from cells in control, Dex, and Sr groups using Trizol reagent. RT-PCR was performed using PrimeScript One Step RT-PCR Kit (Takara, Shiga, Japan, www.takara-bio.com) using 1 μg of purified RNA. Sequence of primers, annealing temperature, and product size are shown in Supporting Information Table 1. In brief, RNAs were transcribed at 50°C for 30 minutes, and then the reactions were performed by denaturation at 94°C for 30 seconds, annealing at the specific annealing temperature (Supporting Information Table 1) for 30 seconds, and extension at 72°C for 30 seconds for a total of 30 cycles. Products were fractioned using agarose gel and analyzed under UV light. The gels were scanned under UV light with a densitograph system (Dolphin dopclus, Wealtec Bioscience, Taipei, Taiwan, www.wealtec.com). The band density was semiquantified and normalized to the β-actin gene using the Image J software (version, 1.36, NIH) [20].

**Fluorescence-Activated Cell Sorting**

Human umbilical cord-derived MSCs (HUMSCs) at passage three were washed once with PBS and resuspended in 0.1 ml of ice cold PBS. The suspension was incubated for 10 minutes at room temperature. Then the cells were washed in PBS for 5 minutes and incubated for 30 minutes with phycoerythrin (PE) or fluorescein isothiocyanate-conjugated mouse monoclonal antibodies. The antibodies used included CD34, CD45, CD73, and CD105 (BD Biosciences, Franklin Lakes, NJ, www.bdbiosciences.com). Nonspecific staining was excluded using a mouse isotype immunoglobulin control. The cells were passed through a flow cytometer (Cell lab Quanta, Beckman Coulter, Brea, CA, www.beckmancoulter.com); particles of incorrect forward and 90° light scatter were rejected as debris.

**Immunostaining**

HUMSCs at passage three were fixed with paraformaldehyde, then incubated with mouse antibodies to CD73, CD105, CD34, and CD45 (BD Bioscience) at 5 μg/ml to characterize the surface markers. After washing three times, the slides were incubated with PE-conjugated secondary antibody.
Finally, the sections were counterstained with 4',6-diamidino-2-phenylindole to stain the nuclei and then mounted.

For tissue sections, the sections were deparaffinized, rehydrated, and blocked by goat serum. Then the sections were incubated with primary rabbit anti-rat collagen I antibody (Beijing Biosynthesis Biotechnology, Beijing, China, www.bioss.com.cn) at 5 μg/ml, rabbit anti-rat osteopontin (OPN) antibody (Abzoom, Dallas, TX, www.abzoom.com) and anti-human β-catenin antibody (Cell Signaling, Beverly, MA, www.cellsignal.com) at 10 μg/ml for 60 minutes. The sections were then washed with PBS for three times. Labeling and detection of secondary antibody were accomplished using the Vectastain Elite ABC kit (Vector, Burlingame, CA, www.vectorlabs.com) with diaminobenzidine as the chromogen. Then the sections were counterstained with hematoxylin and mounted.

**Preparation of Collagen-Sr-HA Scaffold**

To make three-dimensional (3D) scaffold (mixture of collagen and hydroxyapatite), 0.8 ml of rat tail collagen type I (Gibco, 5 mg/ml) was slowly pipetted into a tube in an ice-bath, and 0.1 ml of sterile 10× PBS was added, then gently pipetted up and down to mix the solution well. After adding 0.02 ml of sterile 1 M NaOH and 0.08 ml of dH2O in the tube, 0.2 g of strontium-substituted hydroxyapatite (Sr-HA, 100% strontium substitution) [21] or HA were added and mixed. The solutions were adjusted into pH range of 7.2–7.5. Then the mixture was incubated in a 37°C, 95% humidity incubator for 30 minutes until a firm gel was formed. The gel solutions were put into the 5 mm diameter dishes and then frozen in a refrigerator at –20°C for 12 hours. After the mix gel was formed, we moved the mix gel to –80°C for 1 hour. The composites were dried with a freeze dryer, leaving a scaffold of 5 mm diameter (Fig. 4A, 4B).

**Establishment of Rat Calvarial Defect Model and Transplantation of the Scaffold**

All experimental protocols were approved by Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences. Eighteen female 8-week-old Sprague-Dawley rats (250–300g) were evenly divided into three groups: (a) control group; (b) HA group; and (c) Sr group. For establishing the calvarial defect model, the rats were intraperitoneally anesthetized with phenobarbitol sodium (100 mg/kg) and the dorsal cranium was exposed. A 5-mm diameter defect was created with a high-speed drill with a trephine bur, avoiding perforation of dura mater. Each defect was flushed with saline to remove bone debris and then implanted with scaffolds. Collagen, collagen-

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**Figure 1.** Characterization of human umbilical cord-derived mesenchymal stem cells (HUMSCs). (A, B): HUMSCs were cultured in Dulbecco’s modified Eagle’s medium at passage three: the cells observed in the bright field, which grew into confluence (A), were spindle shaped and uniform in size under high magnification (B). Scale bar = 100 μm (A), 20 μm (B). (C): Immunofluorescence of CD73, CD105, CD34, and CD45 on the HUMSC. HUMSCs expressed CD73 and CD105, whereas they did not express CD34 and CD45. Scale bar = 50 μm. (D): Fluorescence-activated cell sorting analysis of the cultured HUMSC using fluorescein isothiocyanate- or phycoerythrin-labeled antibodies. The percentage of cells that expressed CD73, CD105, CD34, and CD45, respectively, are shown in small panels. Abbreviations: FL1, fluorescence 1 (fluorescein isothiocyanate); FL2, fluorescence 2 (phycoerythrin).

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hydroxyapatite and collagen-strontium-substituted hydroxyapatite were transplanted into the control, HA, and Sr groups, respectively. After implantation of the biomaterials, the soft tissues were closed with skin staples and the rats were allowed free activity in the cages. The rats were sacrificed by cervical dislocation at specific time points (1 month and 3 months after the transplantation) for tissue processing.

**Computed Tomography (CT) Scanning**

The animals were scanned by CT (Aquilion 16, Toshiba, Tochigi-ken, Japan, www.toshiba-medical.co.jp), and the whole calvaria was scanned with slice thickness of 0.5 mm. For tomographic imaging, the following conditions were used: energy 120 kV, current 10 mA, and slice thickness 0.5 mm. For radiographic density quantification in control, HA, and Sr groups, the density of the 5 mm bone defect region of each animal was measured repeatedly at specific time points using the analytic system (Vitrea 2, version 3.4, Toshiba, Japan), and the average radiographic density was calculated and compared among groups. The 3D anatomic parameter of the calvarial defect area was obtained by 3D reconstruction.

**Histological Staining and Histomorphometry**

The rat calvaria from three groups were isolated at 1 month and 3 months after the transplantation. Tissue processing and sectioning were carried out as previously described [22]. In brief, the tissue samples were fixed in 4% phosphate-buffered formalin for 48 hours at 4°C and then decalcified in 4% EDTA for 30 days. Following decalcification, the calvaria was embedded in paraffin and sectioned at 5 μm thickness. H&E and Masson staining were performed separately on consecutive tissue sections, and images were taken using a microscope (ECLIPSE 50i, Nikon, Melville, NY, www.nikoninstruments.com). For histomorphometry, three mid sagittal Masson’s trichrome-stained sections were selected from control, HA, and Sr groups, respectively. Red stain represented the remodeled or lamellar bone, and blue stain represented the immature bone or collagen fibers [23]. The areas of the blue and red regions were semiquantitatively determined using Image Pro Plus software (Media Cybernetics, Bethesda, MD, www.mediacy.com). The area mean of total bone (red + blue) and remodeled bone (red) content was normalized to control group and compared among three groups.

**Statistical Analysis**

The significance of differences among the number of bone nodules, ALP-positive cells, and CT bone density in different groups was tested by analysis of variance (ANOVA), and Bonferroni’s multiple comparison test was used as a post hoc test. The gene expression level among different groups was tested by one-way ANOVA, and Dunnett’s multiple comparison test was used as a post hoc test. Mann-Whitney U test was used to analyze the

![Figure 2.](image)

**Figure 2.** Strontium enhanced the osteogenic differentiation of human umbilical cord-derived mesenchymal stem cells (HUMSCs). (A–C): Alkaline phosphatase (ALP) staining of the cultured HUMSC in control, dexamethasone (Dex), and strontium (Sr) groups: few positive cells were observed in control group (A), while positive cells were observed in the Dex (B) and Sr groups (C). (D): The percentage of ALP-positive cells in control, Dex, and Sr groups, n = 10. (E–G): Immunostaining of type 1 collagen in control (E), Dex (F, arrow), and Sr groups (G, arrow): the signals in the Sr group increased when compared with that of the control and Dex groups. (H–J): Immunostaining of osteopontin in control (H), Dex (I, arrow), and Sr (J, arrow) groups: the signals in the Sr group increased when compared with that of the control and Dex groups. (K–M): Von Kossa staining of the bone nodules formed in control (K), Dex (L, arrow), and Sr (M, arrow) groups: more bone nodules are formed in the Sr group when compared with that of the Dex group. (N) The number of bone nodules per high magnification field formed in control, Dex, and Sr groups, n = 12. Scale bar = 20 μm. *p < .05. Abbreviations: ALP, alkaline phosphatase; Dex, dexamethasone; Sr, strontium.
scores of mature or total bone formation areas in the three groups. All the statistical tests were performed using the Statview (Version 10.0, SPSS, Chicago, IL, www.spss.com) program package. A p value < .05 was taken as statistical significance.

**Results**

Morphology and Characterization of In Vitro HUMSC Culture

In our study, the freshly harvested HUMSC had variable size and a heterogeneous population; after three passages, the cells grew to confluence (Fig. 1A), and cell size and shape appeared uniform (Fig. 1B). To characterize the surface markers of HUMSC, immunofluorescence was performed on the cultured cells. Most of the cells expressed the standard MSC markers such as CD73 and CD105, whereas they did not express the hematopoietic stem markers CD34 and CD45 (Fig. 1C). Flow cytometric analysis was further performed to confirm the expression profile of HUMSCs: more than 95% of HUMSCs were positive for CD73 and CD105, while less than 1% of HUMSCs expressed detectable levels of CD34 and CD45 (Fig. 1D). Immunofluorescence and flow cytometry data together showed that our cultured HUMSCs could express standard surface markers of MSCs and could be used for experiments described below.

Strontium Enhanced the Osteogenic Differentiation of the HUMSC

To investigate the effects of strontium on the osteogenic differentiation process of HUMSC, ALP staining was first used to examine the osteoblastic differentiation status of HUMSC. After 10 days of induced differentiation, there were few positive purple cells in the control group (Fig. 2A); some positive cells that contain intracellular purple granular dye could be observed in the Dex group (Fig. 2B); while in Sr group, we observed more ALP-positive cells (Fig. 2C) compared with Dex group. Quantitative analysis showed that the percentage of ALP-positive cells in control, HA, and Sr groups was 1.83 ± 0.23%, 32.1 ± 1.31%, and 39.48 ± 1.48%, respectively; the percent of ALP-positive cells in the Sr group was significantly higher than that in the Dex group (p < .05) (Fig. 2D).

Next, we investigated the effects of strontium on the expression of collagen I and OPN [24]. Immunostaining showed that in the control group, little brown signal could be observed around the cells (Fig. 2E, 2H); in the Dex group, there were some collagen I (Fig. 2F) and OPN signals (Fig. 2I) around some cells; however, in the Sr group, strong collagen I and OPN signals could be observed around the cells (Fig. 2G, 2J).

At 4 weeks of osteogenic differentiation, Von Kossa staining showed that normal DMEM induced formation of few bone nodules in the control group (Fig. 2K); in the Dex group, there was also a few bone nodules observed (Fig. 2L); while in the Sr group, more mineralized bone nodules could be observed (Fig. 2M). The number of mineralized calcium nodules in the control, HA, and Sr groups was 0.8 ± 0.2, 2.5 ± 0.3, and 3.3 ± 0.3 per high-magnification field, respectively. The nodule number in the Sr group was significantly higher compared with control: 12.2 ± 4.6 vs. 47.4 ± 5.8, p < .05 (Fig. 2N).

Strontium Upregulated the Osteogenesis-Related Gene Expression and Activated the Wnt Signaling Pathway

RT-PCR was used to analyze the gene expression (Alp, Colla1, and Opn) changes at 2 weeks of the osteogenic differentiation in three groups. Our data showed that the normalized expressions of Alp, Colla1, and Opn in the Sr group were 716.9 ± 19.1%, 237 ± 4.3%, and 155.4 ± 1.2%, respectively, which were significantly higher than those of the Dex group (Alp: 416.5 ± 55.8%; Colla1: 168.9 ± 11.1%; Opn: 114.8 ± 1.7%, p < .01 for all) (Fig. 3A), suggesting that the expression of osteogenesis-related genes was upregulated by strontium.

Then, we used RT-PCR to analyze the gene expression changes of key molecules (β-catenin, frizzled 8 [FZD8], and protein phosphatase 2A [PP2A]) in the Wnt signaling pathway [25]. We found that the normalized expressions of β-catenin and FZD8 in Sr group were 469.9 ± 48.4% and 1435 ± 198%, respectively, which were significantly higher than those of the Dex group (β-catenin: 234.4 ± 28.5% and FZD8: 285.4 ± 57.6%, p < .05 for both) (Fig. 3B); however, the normalized expression of PP2A (the inhibitor of the Wnt signaling [26]) in the Sr group was significantly lower when compared with that of the Dex group (interpreted as fold increase compared with control: 12.2 ± 4.6 vs. 47.4 ± 5.8, p < .05) (Fig. 3B).

Synthesis and Transplantation of the Collagen-Sr-HA Scaffold into Rat Calvarial Defect Model

To investigate the effects of strontium on bone formation in vivo, we synthesized the strontium-containing scaffold; then we used a scanning electronic microscope (SEM) to examine the porous structure and surface topography of strontium-substituted hydroxyapatite-collagen scaffold. SEM showed that the crystal grains of strontium-containing scaffold were...
regularly distributed (Fig. 4C), forming a porous and intercon
nected structure (Fig. 4D).

After establishing the calvarial defect model (Fig. 4F), we transplanted the collagen scaffold, collagen-hydroxyapatite scaffold, and the collagen-Sr-HA scaffold into the control, HA, and Sr groups, respectively (Fig. 4G). The schematic representation of transplantation is shown in Figure 4E.

**Strontium Promoted the In Vivo Bone Formation in the Calvarial Defect Model**

At 1 month after the transplantation, the bone defect region with low radiographic density in the control group was evident in the coronal plane (Fig. 5A-a) as well as in the transverse and sagittal planes of 3D-reconstructed CT images (Supporting Information Fig. 1A). In the HA group, the CT bone density in the defect region was increased compared with that of the control group (Fig. 5A-b); in the Sr group, the bone density in the bone defect region was increased further, and the defect region area was reduced, compared with that of the HA group (Fig. 5A-c). Quantification showed that the radiographic density in control, HA, and Sr groups was $15.7 \pm 3.3$, $121 \pm 14.2$, and $219.4 \pm 37.1$ (Hounsfield unit [HU]), respectively; the radiographic density in the Sr group was significantly higher than that of the control group ($p < .001$) or HA group ($p < .05$) (Fig. 5A-d). Histological staining revealed that, in the control group, the fibrous tissues were formed adjacent to the original bone nodules (Fig. 5B-a). Masson staining showed that the fibrous tissue was mainly composed of newly formed collagen fibers which were stained blue (Fig. 5C-a). In the HA group, the fibrous tissue surrounding the calvarial defect was more regularly aligned (Fig. 5B-b); Masson staining showed that there were both newly formed blue-stained collagen tissues and some mature red-stained collagen fibers (Fig. 5C-b) at the margin of the bone defect. In the Sr group, H&E staining revealed that more abundant bone structures were formed in the middle of the bone defect region (Fig. 5B-c), while Masson staining showed that more mature and remodeled bone structures were formed in the abundant osteoid ECM to fill the bone defect region (Fig. 5C-c).

At 3 months after the transplantation, the bone defect region with low radiographic density was still evident in the control group (Fig. 6A-a); in the HA group, the area of the bone defect region was reduced compared with that of the control group, although the radiographic density in the defect region was still lower compared with that of the surrounding region (Fig. 6A-b). In the Sr group, there was no significant bone density difference between the defect area and the surrounding region (Fig. 6A-c), and 3D reconstruction showed no evident bone defect (Supporting Information Fig. 1B). The radiographic density (HU) in the control, HA, and Sr groups was $59.4 \pm 8.5$, $249.2 \pm 20.5$, and $381.7 \pm 25.3$, respectively; the radiographic density in the Sr group was significantly higher than that of the control group ($p < .001$) or HA group ($p < .001$) (Fig. 6A-d). Histological analysis showed that in the control group, the original blue-stained collagen tissues were replaced by loose connective tissues in the
central bone defect region (Fig. 6B-a) adjacent to the defect margin (Fig. 6C-a); in the HA group, H&E staining revealed the formation of mature bone structure (Fig. 6B-b) integrating into the bone defect region: these bone structures contain both the mature lamellar bone and the newly formed woven bone (Fig. 6C-b). In the Sr group, the defect area with low radiographic density decreased (c, arrow). The radiographic density in the defect region of the Sr group significantly increased when compared with that of the control and HA groups (d). (B): Representative histological analysis (H&E staining) of bone formation at 1 month after the transplantation in the control (a), HA (b), and Sr groups (c). In the control group, fibrous tissues are formed adjacent to the original bone nodules (a, arrow); in the HA group, the regularly patterned fibrous tissues (b, arrow) are formed in the defect region; in the Sr group, the bone defect region was filled by solid formed bone tissue (c, arrow). (C): Representative histological analysis (Masson staining) in the control group (a), HA group (b) and Sr group (c). In the control group, the fibrous tissues were stained blue by Masson staining (a, arrow); in the HA group, some remodeled and matured collagen components (b, arrow) were formed; in the Sr group, the formed bone contained both newly formed (c, arrowhead) and remodeled bone structure (c, arrow). Scale bar = 800 μm (B, C, low magnification), 75 μm (B, C, high magnification). *, p < .05; ***, p < .001. Abbreviations: CT, computed tomography; HA, hydroxyapatite; HU, Hounsfield unit; Sr, strontium.

Strontium Enhanced the Accumulation of ECM in the Bone Defect Region

At 3 months after the transplantation, immunostaining using collagen I antibody (Fig. 7A, Supporting Information Fig. 2A) showed that in the control group, the collagen I was distributed sparsely in the fibrous tissues (Fig. 7A-b); however, in the Sr group, moderate collagen I signals were observed at the boundary of the newly formed woven bone (Fig. 7A-c); immunostaining using OPN antibody showed a similar distribution pattern (Fig. 7B, Supporting Information Fig. 2B). In the control group, the OPN signal was distributed sparsely (Fig. 7B-b); in the HA group, the OPN signal was
distributed evenly in the newly formed bone (Fig. 7B-c); while in the Sr group, strong OPN signals could be observed at the boundary of the newly formed woven bone (Fig. 7B-d).

**Strontium Enhanced the β-Catenin Expression In Vivo**

Then, we investigated whether strontium treatment could enhance the expression of β-catenin in the newly woven bone (Fig. 7C, Supporting Information Fig. 2C). At 1 month after the transplantation, there were few signals in the formed woven bone in the HA group (Fig. 7C-a); however, in the Sr group, β-catenin signals were observed on the cells located in the ECM (Fig. 7C-c). At 3 months, in the HA group, the β-catenin signals appeared on some cells at the boundary of the formed bone (Fig. 7C-b); in the Sr group, more intense and concentrated β-catenin signals were observed on the cells surrounding the newly formed ECM (Fig. 7C-d), suggesting that strontium could upregulate β-catenin expression in vivo.

**DISCUSSION**

In this study, we investigated the stimulatory effects and related mechanisms of strontium on the differentiation of MSCs in vitro, and then we established a rat calvarial defect model to explore the regulatory effects of strontium on the in vivo bone formation process. As hypothesized, our results showed that strontium could enhance the osteogenic...
differentiation of the MSCs and promote in vivo bone formation through activating the Wnt/β-catenin pathway.

In this study, HUMSCs were used to evaluate the effects of strontium on osteogenic differentiation. Possessing self-renewing and multipotent differentiation capacities [27], HUMSCs have greater expansion capability, superior osteogenic capacity [28], and better tolerance by the immune system compared with bone marrow-derived MSCs [29], and they are used widely in tissue engineering. During the process of osteogenic differentiation, strontium upregulated the expression of ALP, significantly increased the expression of type 1 collagen, and enhanced calcium deposition and bone nodules formation. Taken together, our data supported earlier findings that strontium could increase the gene expression and accumulation of ECM and accelerate the osteogenic differentiation of human MSCs [10].

Strontium-substituted hydroxyapatite could release strontium ions [21], which might contribute to the anabolic effects of strontium [30]. In this study, we synthesized collagen-Sr-HA scaffold to evaluate the in vivo effects of strontium on bone formation. At an early stage (1 month) after transplantation, collagen scaffold alone induced scarce mature and remodeled bone formation; HA scaffold only induced some mature collagen fibers at the boundary of the original bone defect; however, strontium-containing scaffold induced the formation of mature and remodeled bone both at the boundary and in the central bone defect, resulting in the relatively high bone density. The analysis of CT and histology at this stage suggested that strontium could promote new bone formation, and the newly formed bone contributed to the increased CT density. At the later stage (3 months) after the transplantation, the scanty mature bone formation in control group confirmed

![Figure 7.](image)

**Figure 7.** Strontium promoted the accumulation of extracellular matrix and upregulated the expression of β-catenin. (A): Total bone formation area (a) and immunostaining of type I collagen in the control, hydroxyapatite (HA), and strontium (Sr) groups (b–d) at 3-month post-transplantation. The Sr group had significantly higher total bone formation (p < .05) when compared with that of the HA group, n = 3 (a); the collagen I staining in the Sr group (d, arrow) significantly increased when compared with that of HA (c, arrow) or control groups (b, arrow). (B): The mature bone formation area (a) and immunostaining of osteopontin in control, HA, and Sr groups (b–d) at 3 months post-transplantation. The Sr group had significantly higher remodeled bone formation (p < .01) when compared with that of the HA group, n = 3 (a); the staining in the Sr group (d, arrow) increased when compared with that of the control (b, arrow) and HA groups (c, arrow). (C): Immunostaining of β-catenin in HA and Sr groups at 1 month and 3 months post-transplantation: the expression of β-catenin in the Sr group (c, d, arrow) increased significantly when compared with that of the HA group (a, b, arrow) at both 1 month (a, c) and 3 months (b, d) after the transplantation. Scale bar = 40 μm. *, p < .05; **, p < .01. Abbreviations: HA, hydroxyapatite; Sr, strontium.
The low bone inductive capacity of collagen scaffold; HA scaffold induced limited new bone formation in a thin layer on the HA matrix surface, suggesting the limited bone inductive capacity of hydroxyapatite [31, 32]. In the Sr group, substantial increases of both the total bone and the matured bone were observed. Together, the series of histology and CT analysis demonstrated that transplantation of strontium-containing scaffold had stimulatory effects on the in vivo bone formation process. The significant effects of strontium on bone formation at both early and late stages suggested that strontium could not only promote the quantity of the newly formed bone but also enhance the maturation and remodeling of the newly formed bone, which was also supported by its higher density evaluated by CT.

The osteogenic differentiation of the stem cells and in vivo bone formation depended on the expression of ECM genes and accumulation of the osteoid matrix. The upregulated collagen and OPN expression in vivo in the Sr group suggested that strontium could stimulate the synthesis and accumulation of ECM during the in vivo osteogenesis, thus increased formation of both total and mature bone in the Sr group. The Wnt signaling pathway has been proven to play an important role in both regulating the in vivo osteogenic differentiation of stem cells and synthesis of ECM [12]. Our in vivo data demonstrated the expression of β-catenin was significantly increased by strontium. On one hand, strontium could increase the expression of β-catenin and Frizzed receptor, thus transducing signals that activated the downstream osteogenic transcriptional factors and enhancing osteoblastic differentiation; on the other hand, strontium could also inhibit the expression of Wnt pathway inhibitors, prevent the degradation of β-catenin, and promote osteogenic differentiation.

CaR is an important receptor involved in strontium-induced proliferation of the osteoblasts [7]; CaR is also reported to stimulate Wnt3a secretion in colon cancer cells to regulate epithelial homeostasis [33, 34], so in our study, strontium might activate the CaR of MSCs to stimulate Wnt secretion and upregulate the expression of β-catenin and FZD8 receptor, thus enhancing the osteogenesis process of HUMSC. Ras/MAPK signaling is another important pathway involved in strontium-stimulated differentiation of MSC [11]. On one hand, the MAPK signaling pathway could regulate canonical Wnt/β-catenin pathway by inactivation of glycogen synthase kinase 3 [35, 36]; on the other hand, Wnt3a could activate protein kinase p38 to upregulate ALP and matrix mineralization in mesenchymal cells [37], suggesting that there might be interactions between strontium-induced Wnt pathway and MAPK pathway. The detailed interaction between MAPK and Wnt pathway molecules should be investigated further.

The detailed mechanism by which strontium stimulates stem cell differentiation in vivo should be studied further. Previous studies have shown that endogenous MSCs played important roles in regulating the bone regeneration process [16, 38]; the endogenous MSCs could be mobilized and they exert reparative effects at the site of injury [38]. Strontium could modulate the in vivo differentiation of bone marrow-derived MSCs toward osteoblastic lineage [10]; so in our study, the enhanced new bone formation by strontium might be contributed by the increased osteogenic differentiation of endogenous MSCs. Further studies using double immunostaining or optogenetics [39, 40] are needed to investigate differentiation of MSCs regulated by strontium. Second, we used strontium ions to evaluate the strontium effects in vitro; the amount of strontium ions released from transplanted scaffold to induce the in vivo activation of Wnt/β-catenin requires further study. In addition, we incorporated strontium into collagen scaffold for transplantation; the mechanism by which strontium influences the structural changes of the scaffold to regulate the in vivo bone formation process also needs further study.

**CONCLUSION**

In conclusion, this experimental study reported that strontium was a bone anabolic agent that promoted the in vitro differentiation of MSCs and in vivo bone formation through Wnt/β-catenin signaling. The activation of the Wnt/β-catenin signaling pathway contributed to the accumulation of ECM, enhanced osteogenic process, and bone remodeling. Further understanding of the molecular mechanisms of strontium will potentially provide a possible application of strontium in bone tissue engineering in the future.

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**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicate no potential conflicts of interest.
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