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(Degree)

博士 (医学)

(Date of Degree)

2007-02-14

(Date of Publication)

2013-03-18

(Resource Type)

doctoral thesis

(Report Number)

乙2922

(URL)

<https://hdl.handle.net/20.500.14094/D2002922>

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Osteoblast Differentiation and Bone Formation Gene Expression in Strontium-inducing Bone Marrow Mesenchymal Stem Cell

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Received 30 June 2006/ Accepted 11 September 2006

Key words: bone formation, mesenchymal stem cell, strontium, osteoblasts, Cbfa1 (Runx2), osteocalcin

Osteoblastic differentiation from human mesenchymal stem cell (hMSCs) is an important step of bone formation. We studied the *in vitro* induction of hMSCs by using strontium ranelate, a natural trace amount in water, food and human skeleton. The mRNA synthesis of various osteoblast specific genes was assessed by means of reverse transcription polymerase chain reaction (RT-PCR). In the hMSCs culture, strontium ranelate could enhance the induction of hMSCs to differentiate into osteoblasts. Cbfa1 gene was earlier expressed on day 4 of cell culture (the control group, on day 14) and osteonectin on day 11 (control, on day 21). The early Cbfa1 expression indicates that strontium could enhance osteoblastic differentiation. The detection of osteonectin using strontium induction indicates the role of strontium in enhancing bone remodeling, bone structure stabilization of hydroxyapatite molecule and collagen fibril organization. The cultured hMSCs in the presence of strontium expressed genes of bone extracellular matrix: collagen type I, bone sialoprotein and osteocalcin on the same days as control (same medium with no strontium). Concentration of strontium ranelate has been recommended to be optimized in between 0.2107 - 21.07 µg/ml whereas the high concentration up to 210.7 µg/ml have delayed effect on osteoblastic differentiation with delayed expression on Cbfa1 and osteonectin, and inhibitory effect on bone sialoprotein expression. In addition, strontium could help cell expansion by maintaining cell proliferation rate of hMSCs and osteoblast lineage. We recommend that the strontium is an important factor for inducing mesenchymal stem cells to differentiate into osteoblasts with further enhancement on bone formation. This model might provide a useful cell source for tissue engineering and bone repair.

Osteogenesis is a complex process that involves the differentiation of mesenchymal cells into pre-osteoblasts and osteoblasts that ultimately leads to the synthesis and deposition of bone matrix proteins [1-4]. Strontium is a naturally occurring mineral found in water and food. Human skeleton contains trace amounts of strontium. Strontium has an affinity for bone and is taken up at the bone matrix crystal surface. The influence of strontium on bone metabolism has been researched since the 1950's [5]. Recent studies show that strontium positively affects bone metabolism to promote bone formation and decrease bone resorption, leading to normalized bone density [6-7]. Strontium-ranelate is now being used as an effective treatment of osteoporosis in elderly patients and postmenopausal women [8-10]. The clinical trials has confirmed that strontium supplements decrease bone resorption and also stimulate bone-building osteoblasts activity and new bone formation in women with osteoporosis [11]. Although previous clinical studies have shown positive effects of strontium supplements on bone formation, the effect of strontium on human bone marrow mesenchymal stem cells (hMSCs) and osteoblast-derived hMSCs has not been elaborated. This paper has studied the effect of strontium on hMSCs and osteoblastic lineage. We have shown that strontium not only enhances osteogenic differentiation, but also shows strong evidence of bone structure stabilization by expressing genes related to bone formation at early day of cell differentiation. We also recommend that strontium at appropriate concentration is an important mineral for osteogenic induction from mesenchymal stem cells. For the field of bone tissue engineering, strontium may be effectively used for hMSCs induction for more rapid bone development.

MATERIALS AND METHODS

Cell culture. Human bone marrow mesenchymal stem cells (hMSC) were purchased from Poietics company. The hMSCs were expanded in MSCGM culture medium (Cambrex Bio Science Walkerville) at 37°C in a humidified atmosphere containing 95% air 5% CO₂. The confluent cells were subcultured to next passage by using 0.05% trypsin – EDTA for up to passage 4.

Osteogenic induction. Approximately 1×10^4 cells hMSCs were placed in a 35 mm culture dish (Corning). The cells were maintained in control medium consisting of DMEM-high glucose with 10% FBS (Hyclone) and 100-100 µg/ml penicillin-streptomycin (Gibco). The hMSCs were cultured in five experimental conditions. The first was osteogenic medium composing of DMEM-high glucose, 10% FBS (Hyclone), 100-100 µg/ml penicillin-streptomycin (Gibco), 50 µg/ml L-ascorbic acid-2-phosphate (Sigma), L-glutamine (Sigma), 10^{-7} M dexamethasone (Sigma-Aldrich), and 10 mM β-glycerophosphate (Sigma). The second, third, fourth and fifth types were osteogenic medium with addition of strontium at 0.2107 µg/ml, 2.107 µg/ml, 21.07 µg/ml and 210.7 µg/ml concentrations respectively. The medium of the cultured cells was replaced every 2-3 days. Identification of osteoblast markers and gene expression (RT-PCR) were done on days 4, 7, 11, 14, 18, 21, 25 and 28. Histochemical staining and cell proliferation assay (XTT assay) were studied.

Alkaline phosphatase and Alizarin red S histochemical staining. Alkaline phosphatase histochemistry was performed on days 4, 7, 11, 14, 18, 21, 25 and 28. On those days, the medium was removed, and the cell layers were rinsed with PBS 3 times and fixed in cold 10% neutral buffer formalin (NFB) for 1 hour at 4°C. After 1 hour at 4°C, the cell layers were washed with deionized water and allowed air dry. Then, the fixed cells were incubated with buffer containing 0.1 mg/ml naphthol AS-MX phosphate disodium salt (Sigma) and 0.6 mg/ml Fast Red TR salt (Sigma). After 1 hour at 37°C, the

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cell layers were washed with deionized water and observed both grossly and with the light microscope.

Using Alizarin red S histochemistry, the cultured cells were stained on days 4, 7, 11, 14, 18, 21, 25 and 28 for assessing the mineralized matrix. The medium was removed, and the cell layers were rinsed with PBS 3 times and fixed in cold 70% ethanol for 1 hour at 4°C. Then, the cell layers were washed with deionized water and allowed to air dry. The fixed cells were stained with 2% Alizarin red S pH 7.2 (Sigma). After 1 hour at 37°C, the cell layers were washed with deionized water and observed both grossly and with the light microscope.

Proliferation assay. Approximately 2.5×10^3 cells hMSCs were placed in a 96-well microplate (Greiner). Cell proliferation assay was done by using XTT Cell Viability Assay Kit on days 4, 7, 11, 14, 18, 21, 25 and 28. The medium was discarded and the cells in each well were added with the 100 μ l medium and 25 μ l of the activated XTT solution. The plate was then put into the incubator. After 5 hours, proliferating cells in each well were estimated by measuring the degree of increasing at wavelength of 450-500 nm. The reference absorbance for non-specific readings was done at 630-690 nm wavelength.

Alkaline phosphatase activity. Alkaline phosphatase activity was performed on days 0, 4, 7, 11, and 14. On those days, the medium was removed, and the cell layers were rinsed with PBS 3 times and lysed by lysis buffer containing 0.1% Triton X – 100 (Sigma). Twenty microliters of cell lysate was mixed with 100 μ l Tris – glycine buffer pH 10.3 (50 mM Tris – HCl, 100 mM glycine) (BioRad, Hercules, CA USA) and 2 mM $MgCl_2$ (Sigma) and 100 μ l of p-nitrophenyl phosphate (Sigma). The reaction mixture was incubated at 37 °C for 30 minutes and the reaction was stop by adding 50 μ l of 3 M NaOH. Absorbance was read at 405 nm in a microplate reader. Enzymatic activity was normalized to total protein concentration using bovine serum albumin (BSA; Roche, Basel, Switzerland). The measurement of protein was done by using the standard method of Bradford (Sigma). The ALP activity was expressed as μ M / mg protein / assay time. The comparison was done by plotting OD intensity.

Reverse transcriptase-PCR (RT-PCR). Osteoblasts differentiated from hMSCs were isolated to obtain total RNA by using TRIzol reagent (Invitrogen). Then 1 μ g of total RNA was reversibly transcribed to cDNA using 25 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 50 mM KCl, 2 mM DTT, 1 mM dNTP each, 40 μ g/ml primer dT15 and 200 U/ml AMV reverse transcriptase in final volume of 25 μ l. Then the suspension was incubated for 40 min at 42°C. Reverse transcription was terminated by heating at 95°C for 5 min and 5% of cDNA was used as the template for PCR. These reactions were performed in 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM each dNTP, 0.5 mM each primer, and 1.25 U of Taq polymerase in final reaction volume of 50 μ l. The details of primers were summarized in Table 1. The PCR reaction was done at 94°C for 45 second, 60°C for 45 second, and 72°C for 1 min for 30 cycles. The PCR products were analyzed on 1 % agarose gel and stained by ethidium bromide.

TABLE 1. Description of the primers and probes.

Gene	GenBank no.	Sense primer	Antisense primer
Osterix	AF477981	TAATGGGCTCCTTTC ACCTG	CACTGGGCAGACAGT CAGAA
Cbfa1	AF001450	GATGACACTGCCACC TCTGA	GACTGGCGGGGTGTA AGT AA
Osteocalcin	NM199173 NM000711	ATGAGAGCCCTCACA CTCCTC	CGTAGAAGCGCCGAT AGGC
Collagen Type I	BC036531	GATGGATTCCAGTTC GAGTAT G	GTTTGGGTGCTTGTCTG TTTG
Osteonectin	BC072457 BC004974	AGTAGGGCCTGGATC TTC TT	CTGCTTCTCAGTCAG AAGGT
Bone Sialoprotein	J05213	TCAGCATTTTGGGAA TGGCC	GAGGTGTTGTCTTC GAGGT
GAPDH	NM002046	GTCAGTGGTGGACCT GACCT	AGGGGAGATTCACTG TGG TG

Gene	Reference	PCR Product (bp)
Osterix	Gao,Y., et.al (2004)	160
Cbfa1	Albright,S., et.al (1997)	171
Osteocalcin	Kuliskiewicz- et.al (2005)	291
Collagen Type I	Strausberg,R.L.,et.al. (1997)	479
Osteonectin	Strausberg,R.L.,et.al. (1997)	575
Bone Sialoprotein	McBride,O.W.,et.al (1990)	666
GAPDH	Valenti,M.T.et.al (2006)	391

RESULTS

Figure 1. shows expression of bone related genes in the experiments of hMSCs culture under osteogenic induction using osteogenic medium with addition of strontium at varying concentrations (0.2107, 2.107, 21.07 and 210.7 $\mu\text{g/ml}$. Strontium ranelate (concentration 0.2107 $\mu\text{g/ml}$ and 21.07 $\mu\text{g/ml}$) could induce early expression of Cbfa1 gene on day 4 of hMSCs culture whereas other concentrations had the same effect as osteogenic medium. For bone matrix genes, all experiments both in osteogenic medium and in varying concentrations of strontium showed the same pattern of gene expression on day 11 for collagen type I, day 14 for bone sialoprotein and day 14 on osteocalcin. However, the high concentration of strontium (210.7 $\mu\text{g/ml}$) has shown an inhibitory effect on bone sialoprotein expression. For osteonectin gene expression, cell in osteogenic medium had delayed expression which started on day 21 of cell culture. By contrast, cells in the presence of strontium at all concentrations started osteonectin gene expression on day 11. High concentration of strontium (210.7 $\mu\text{g/ml}$) had slightly delay on osteonectin gene expression which started on day 14. All experiments were controlled by the presence of GAPDH expression. All experiments were started at the same number of cell (1×10^4 cells / plate) on day 0. The cell numbers were comparable on the following days for all experiments as shown by relatively increased cell proliferation at comparable rate in Figure 2. The significant finding was the detection of osteonectin since day 11 using strontium 0.2107 $\mu\text{g/ml}$, 2.107 $\mu\text{g/ml}$, and 21.07 $\mu\text{g/ml}$. Figure 3 shows alkaline phosphatase

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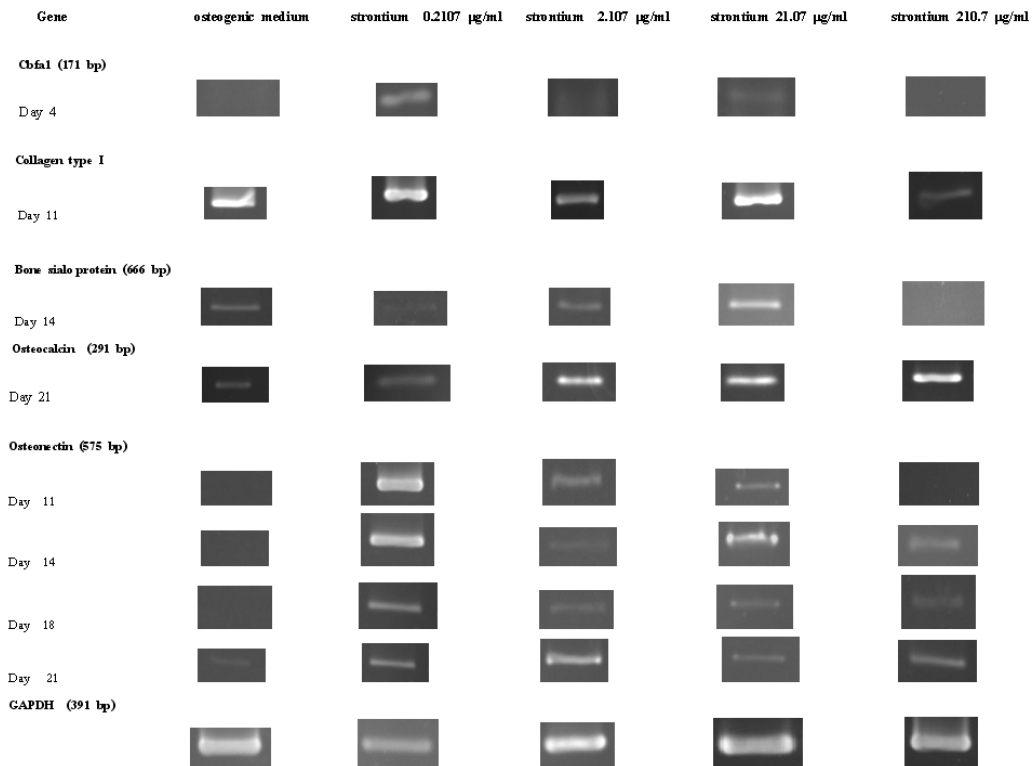


FIG 1. Gene expression of RT-PCR bands detected in hMSCs and osteoblastic cells. The hMSCs from bone marrow were cultured in osteogenic medium without and with addition of strontium at varying concentrations. The RT-PCR bands of each genes were shown in the figure only on the firstly detected day of cell culture. Early detection of Cbfa1 was demonstrated in the experiments using strontium 0.2107 $\mu\text{g/ml}$ and 21.07 $\mu\text{g/ml}$ on day 4. Collagen type I was detected on day 11, bone sialoprotein on day 14, osteocalcin on day 21, and osteonectin on day 11. High concentration of strontium (210.7 mg/ml) had delayed expression of Cbfa1 (day 14, data not shown) and osteonectin (day 14) and totally inhibited in bone sialoprotein. With no strontium, osteonectin expression was delayed until day 21. The expression of GAPDH, the house keeping gene, was qualitatively detected by the presence of bands in all experiments.

staining on the culture plates (gross observation) and microscopic observation. Cells from all experiments were slightly positive on day 4 and had relatively increased intensity of alkaline phosphatase stain on the following days of cell culture. Figure 2 demonstrates increased cell expansion upon cell culture. Strontium had better enhancing effect on cell proliferation as compared to those cultured in media with no strontium. It is noted that during the period of cell culture for the study of gene expression (up to day 18), there were no bone nodule formation. The Alizarin red staining, the stain for bone nodule formation was all negative in all culture experiments as shown in Figure 5. Investigation of alkaline phosphatase activity was done as shown in Figure 4. The control group had less activity significantly as compared to all treated experiments. Table 2 summarizes all experimental studies in relation to function of bone formation. This summarized table indicates that strontium has many positive effects on bone formation including osteoblast differentiation, formation of bone extracellular matrix, bone remodeling potential and cell expansion for

bone mass. The appropriated concentrations are 0.2107 – 210.7 $\mu\text{g/ml}$ of which 21.07 $\mu\text{g/ml}$ seems to be suggestive. High concentration (210.7 $\mu\text{g/ml}$) seems to loose some effects, particularly bone sialoprotein expression and the slightly delay on osteonectin expression.

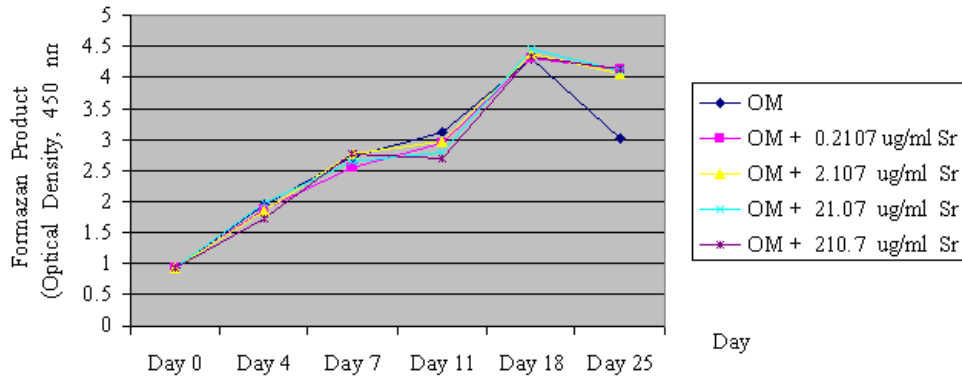


FIG 2. Cell proliferation assay in osteoblast-derived hMSCs. Cell proliferation was measured by using XTT Cell Viability Assay Kit on days 0, 4, 7, 11, 18, 25 and 28. Cell proliferation was related to the uptake of Formazan product into the increasing number of viable cells. All experiments using strontium (Sr) showed increasing uptake of Formazan product during period of cell culture. Triplicated measurements were done on each experiment. All experiments during day 0 until day 18 of cell culture had comparable increase in cell number as shown by Formazan uptake. However, significant decrease was shown on day 25 of the experiment using osteogenic medium with no strontium (OM) whereas those cells with varying strontium concentrations had the same proliferating rate with no significant difference when compared among various experiments on the same comparable days.

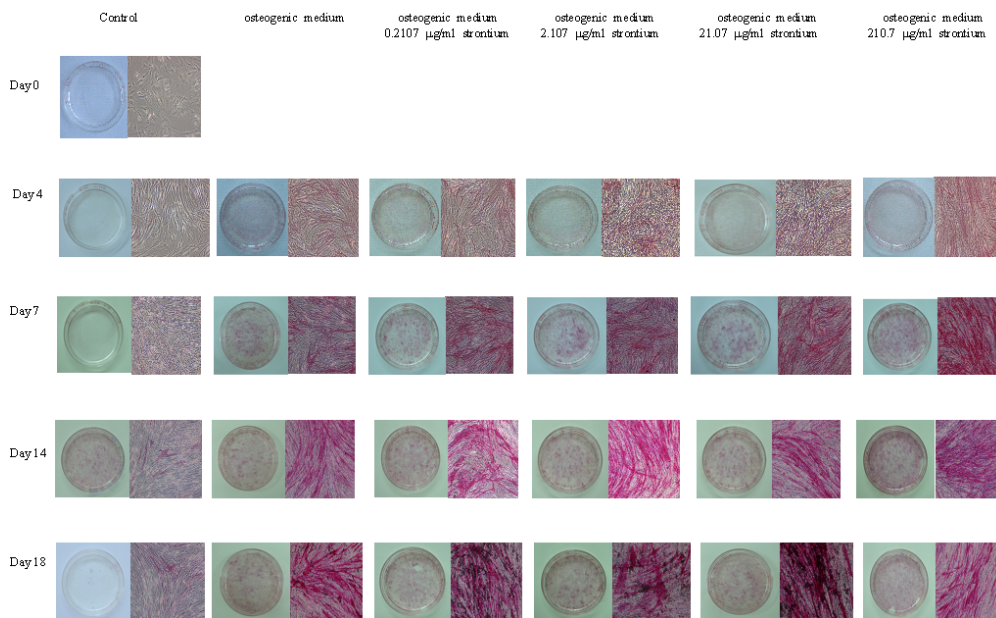


FIG 3. Alkaline phosphatase reaction appeared as gross and microscopic staining of osteoblasts differentiated from hMSCs. Positive reaction was observed as red staining in the cells.

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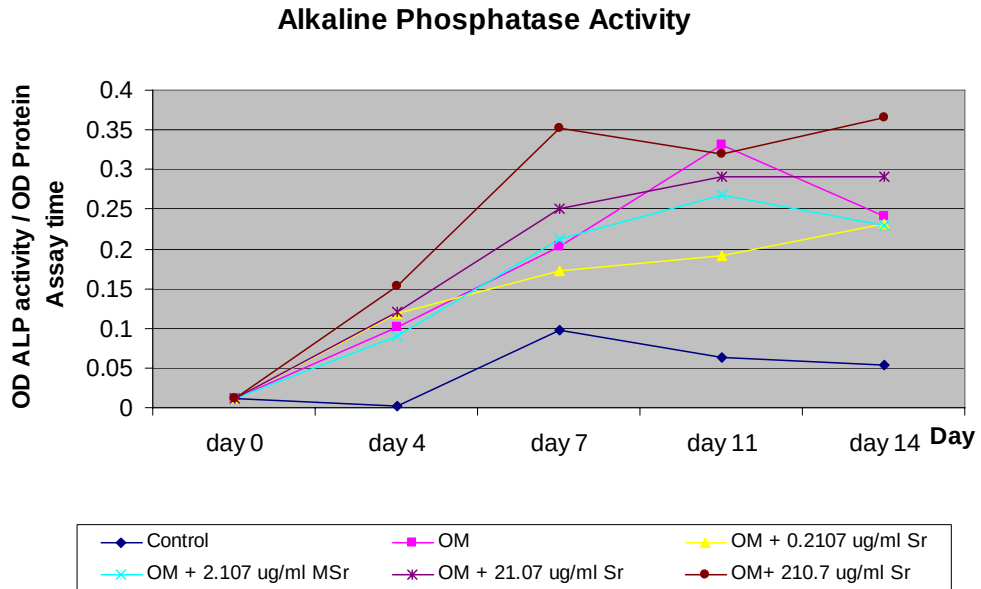


FIG 4. Comparison of alkaline phosphatase activity of various treatments of hMSCs upon varying period of culture. Each experiment was done on triplicated measurement. The control group had significantly decreased alkaline phosphatase activity as compared with treated cells.

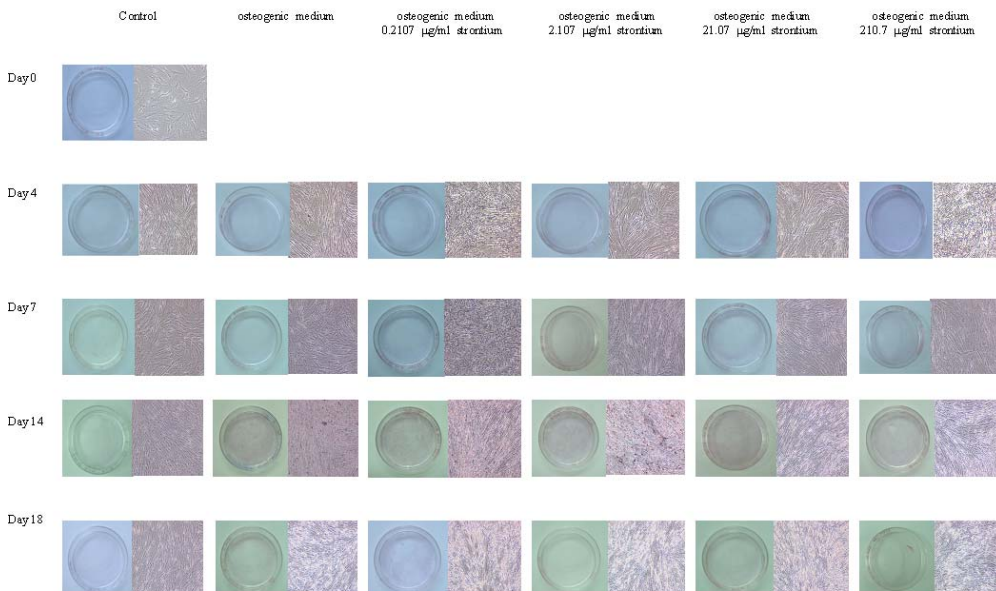


FIG 5. Alizarin red staining appeared as gross and microscopic observation of osteoblasts differentiated from hMSCs. All experiments (up to day 18) showed no positive staining. Positive staining with pink color indicated bone nodule formation which was not found in this experiment.

TABLE 2. Gene expression and other assays related to cell function, detected in hMSCs and osteoblastic cells. The data show comparison between the absence and the presence of strontium at varying concentrations.

Type of Medium	Osteoblast Differentiation		Bone Extracellular Matrix			Bone Remodeling	Cell Proliferation
	Cbfa1	Alkaline Phosphatase	Osteocalcin	Bone sialoprotein	Collagen type I	Osteonectin	Formazan Uptake
Osteogenic medium (No strontium)	+	+	+	+	+	Delay until day 21	Increase and drop on day 25
Strontium 0.2107 µg/ml	+ (early detected)	+	+	+	+	+ detected on day 11	Increase
Strontium 2.107 µg/ml	+	+	+	+	+	+ detected on day 11	Increase
Strontium 21.07 µg/ml	+ (early detected)	+	+	+	+ (early detected)	+ detected on day 11	Increase
Strontium 210.7 µg/ml	+	+	+	-	+ (early detected)	+ detected on day 14	Increase

DISCUSSION

Cbfa1 is essential for osteoblastic differentiation and bone formation. Overexpression of Cbfa1 induces nonosteogenic cells to express osteoblast-related genes *in vitro* [12]. The early detection of Cbfa1 in strontium-inducing hMSCs indicates that strontium play an enhancing role in osteoblastic differentiation and bone formation. Cbfa1 expression is also demonstrated in hMSCs cultured in osteogenic medium. Dexamethasone, L-ascorbic acid, β -glycerophosphate and L-glutamine are the essential components in osteogenic medium. We found that strontium (0.2107 µg/ml and 21.07 µg/ml) was another essential factor that could further enhance Cbfa1 expression in hMSCs. Cbfa1 has been known to be up regulated by Smads and other cytokine and hormones. The up-regulation of Cbfa1 may be started by cooperation of Shh, Ihh into BMPs which leads to BMPs-BMPRs interaction. Then, the BMPs-BMPRs interaction may up-regulate expression of Cbfa1 through Smads [12]. The mechanism of Cbfa1 up-regulation in the presence of strontium is not known. If strontium uses the SMAD pathway, it may have some unknown effects on Shh and Ihh cooperation into BMPs. There are two types of proteins in bone extracellular matrix: the collagens, mostly type I collagen, which account for 90% of the bone matrix proteins [13] and the noncollagenous proteins, including osteocalcin and bone sialoprotein [14-16]. Osteocalcin is a major noncollagenous protein component of bone extracellular matrix, synthesized and secreted exclusively by osteoblastic cells in the late stage of maturation, and is considered indicator of osteoblasts differentiation. Osteocalcin is believed to play positive role in controlling nucleation of hydroxyapatite crystals. Osteocalcin expression is modulated by parathyroid hormone (PTH) and a variety of other factors. The cAMP-dependent protein kinase pathway has been shown previously to have an essential role in PTH signaling and regulation of osteocalcin expression. Extracellular matrix proteins play an important role in the organization, architecture and differentiated function of bone [17-23]. Osteocalcin binds with high affinity to hydroxyapatite crystals, the key mineral component of bone and regulates bone crystal growth [24]. Bone sialoprotein is a highly sulfated, phosphorylated, and glycosylated protein that mediates cell attachment through a RGD motif to extracellular matrices [25]. Due to its highly negatively charged

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characteristics, bone sialoprotein can sequester calcium ions while conserving polyglutamate regions, which have hydroxyapatite crystal nucleation potential [26]. In the absence of osteocalcin, bone sialoprotein could contribute to an overall metabolic shift toward new bone formation [27–29]. Induction of osteoblastic differentiation by strontium has also lead to the expression of both noncollagenous proteins, (osteocalcin and bone sialoprotein) and collagen type I. These noncollagenous protein and collagen type I are major components for ECM deposition. In addition, we found earlier expression of osteonectin in our strontium experiments. Osteonectin, also known as SPARC, is a glycoprotein abundantly expressed in bone undergoing active remodeling. We found the consequence of bone formation evidence as shown by expression of collagen type I, bone sialoprotein, osteocalcin and early expression of osteonectin in these strontium-inducing cells. These genes are related to bone extracellular matrix formation. Osteonectin is synthesized by cells of the osteoblastic lineage; binds hydroxyapatite, calcium, and type I collagen; and inhibits mineralization in vitro [30-32]. Early gene expression of osteonectin may suggest that strontium could help active bone remodeling, bone structure stabilization of hydroxyapatite molecule and collagen fibril organization. The inhibition of in vitro mineralization was demonstrated in this report by the negative staining of Alizarin red staining. The study in this report has focused on the beginning period of cell differentiation to detect the gene expression. At this period there was no bone nodule formation as shown by negative staining of Alizarin red staining. Usually, nodule formation in the bone cell culture takes longer period up to 6 weeks of cell culture. Effect on increasing cell proliferation by strontium indicates the production of cell in osteoblastic lineage which are alkaline phosphatase positive. Maintenance of bone mass and normal remodeling by the effect of osteonectin expression [33] and increased cells in osteoblastic lineage in our strontium-inducing model are a good evidence of using strontium for enhancing bone formation. The early expression of Cbfa1 is also supporting evidence for positive effect strontium on enhancing rapid bone formation by inducing early osteoblast differentiation.

ACKNOWLEDGMENTS

The research work is supported by stem cell project grant of National Research Council of Thailand (NRCT). The project is the research collaboration between Mahidol University and Kobe University School of Medicine for Ronpaku program, Japan.

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