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Original Research

Peptide 11R-VIVIT stimulates osteoblastogenesis through regulating the expression of nuclear factor of activated T cells cytoplasmic 1

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Abstract: Osteoporosis is characterized by the imbalance of two relatively independent processes-osteoblastogenesis and osteoclastogenesis. Calcineurin (Cn)/nuclear factor of activated T cells (NFAT)(Cn/NFAT) signaling pathway is involved in these two processes in bone metabolism, but its potential as a target to treat osteoporosis needs to be defined. The aim of this study is to investigate the inhibition of polypeptide 11R-VIVIT onCn/NFAT signaling pathway. Rat calvaria (RC) cells were prepared from experimental model of osteoporosis in rat.11R-VIVIT wasused to treat cultured RC cells from wide type (WT) rat or from osteoporosis (OP) rat, we then measured the expressions of NFATc1, osteopontin (OPN), osteocalcin (OC), cytokines, NFκB subunit p65 by real time PCR, western blot or immunofluorescence. Then ALP expression and staining, and alizarin red S (ARS) staining were employed to study the osteoblastodifferentiation. 11R-VIVIT regulates the expression of NFATc1, and some other molecules in Cn/NFAT signaling pathway, such as OPN and OC, at transcriptional level and protein level. Further, it can regulate the expression of inflammatory cytokine like IL-1beta, IL-10 and TNF-alpha and NFκB activity. Further, 11R-VIVIT can accelerate osteoblastodifferentiation of RC cells demonstrated by ALP and ARS staining.11R-VIVIT can stimulate the bone formation by decreasing NFATc1 expression and regulating the expression of inflammation related molecules.

Key words: 11R-VIVIT; Osteoporosis; Cytokines; Differentiation.

Introduction

Osteoporosis is a disease with reduced bone mass and deteriorated bone micro-architecture, clinically characterized by increased skeletal fragility and bone ach. As a multifactorial and incurable disease, osteoporosis roughly comes from the imbalance of two relatively independent processes-osteoblastogenesis and osteoclastogenesis. Around 200 million people are at risk of osteoporosis world wide, which make it a very serious health concern. Bisphosphonates (1) are widely used for the therapy of osteoporosiswith robust efficacy in preventing fractures, rare but serious adverse events associated with bisphosphonates, such as atypical femur fractures, osteonecrosis of the jaw, and esophageal cancer, have been encountered. Therefore, alternative therapeuticsis to be defined.

Marrowmesenchymal stem cells (MSCs) can differentiate into osteoblasts, fat cells, chondrocytes, muscle cells and stromal cells, and play a dominant role in pathogenesis of osteoporosis (2). AbnormalMSCsare attributable, in a great extent, to compromised osteoblastogenesis, resulting indelayed fracture healing anddecreased callus quality in osteoporosis (3), which implies that theinhibition of osteoporotic differentiationbe the center for developing drugs against osteoporosis. Fetal rat calvaria cell line (RC) mimics the functions of MSC in many different ways, such as osteoblastodifferentiation, and RC cell line is easy to be prepared and cultured (4). Thus, RC is widely used to study the pathogenesis and treatment of bone metabolism disorders like osteoporosis.

Lately, calcineurin/nuclear factor of activating T cell (Cn/NFAT) signaling pathway was found to play a critical role in bone metabolism, such as in process of osteogenesis, for example, molecule NFAT cytoplasmic 1 (NFATc1) stimulated in Cn/NFAT signaling pathway inhibitsfos related antigen (Fra-2) (5), by thus, to inhibit the formation of osteoprogenitor cell. In another study performed in MSCs, CyclosporinA (CsA) can promote osteogenesis process mediated by blocking theCn/NFAT signaling pathways (6, 7). It should be pointed out that CsA and FK506 (7, 8), two classic Cn/NFAT inhibitors, not only inhibit molecule NFAT, but the expression of Cn, which may cause progressive renal failure, hyperglycemia, and nervous lesion and increase the risk of malignancy (9). To avoid these adverse effects, Aramburu (10) constructed VIVIT polypeptide (I: ileucine, V: valine, T: threonine) based on the Cn/NFAT interaction region to block specifically Cn/NFAT signaling pathway. Later, Noguchi (11) upgraded and synthesized cell permeable NFAT inhibitor-11R-VIVIT polypeptide (R, Arginine). 11R-VIVIT can only specifically block the expression of relevant molecules downstream of Cn/NFAT signaling pathway, no effect on the expression of Cn, which may avoid the adverse effect seen in conventional inhibitors. At present, studies on 11R-VIVIT are focused on only cardiovascular diseases, neurologic disorders and so forth (12, 13). The effect of inhibiting Cn/NFAT signaling pathway by IIR-VIVIT on fracture

healing and osteoblastogenesis in osteoporosis needs to be defined.

In the current study, with RC cell (mimic of MSCs) as cellular model *in vitro*, we found that 11R-VIVIT regulatesthe expression of genes in Cn/NFATc1 signaling pathway, thus plays a role in osteoblastogenesis, which shape itself as a potential drug against osteoporosis.

Materials and Methods

Rat model of osteoporosis

Construct of rat model of osteoporosis was based on the method described previously (14).Wistar rat were obtained from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences (Shanghai, China). All of theanimal related experiments were approved by The Institutional Animal Care and Use Committee of Hangzhou Normal University.

Fetal rat calvaria (RC) digestion and preparation of calvaria cells

RC cell line, one mimic of MSC models, was prepared and cultured as described previously (15). Briefly, calvariae from 24-hour-old new born healthy rats or rats with osteoporosis were collected, minced, and digested in a collagenase mixture (300 mg/ml collagenase, 4.5 U/ml elastase, 9.7 U/ml DNAse, 0.12 mM chondroitin sulfate, 100 mM sorbitol, 111.2 mMKCl, 1.3 mM $MgCl_2$, 13 mM glucose, 21.3 mMTris-HCl, pH 7.4, 0.5 mM $ZnCl_2$) at 37°C. The cell mixture were plated and grown in modified essential medium (MEM) containing 15% fetal calf serum (FCS) and antibiotics (100 µg/ml penicillin G; Sigma, St. Louis, MO, USA), 50µg/ml gentamycin (Sigma, St. Louis, MO, USA), 300ng/ml fungizone (Flow Laboratories, Mississauga, Ontario, Canada). RC cells werethen harvested with trypsin next day, counted and seeded as the density of 5000/cm^2 supplemental with vitamin C (50 pg/ml) and sodium glycerophosphate-beta (10 mmol/L). The cultured cells were then treated with 11R-VIVIT peptide (5uM),MEM medium only treated RC cells functions as a control.

Preparation of RC cell nuclear protein

The isolation and preparation of RC cell nuclear protein was performed according to standard protocol. Briefly, RC cells grown in large petri dish were lysed with lysis buffer 1 (20 mMTris pH 7.5–8.0 100 mMNaCl 300 mM sucrose 3 mM MgCl2) supplemented with protease inhibitors (Roche Diagnostics Indianapolis, IN, USA) and subjected to centrifuge. The pellet was resuspended in lysis buffer 2 (20 mMTris pH 8.0 300 mMNaCl 2 mM EDTA pH 8.0) followed by another centrifugation. Aliquot supernatant were stored at -70°C for use.

Western Blot Analysis

Western blots were performed as described previously (16). Briefly, cells were lysed at 4°C in RIPA buffer (150 mMNaCl, 0.5%. sodium deoxycholate, 50 mMTris-HCl, pH 8.0, 0.1% SDS, 0.1% NP40) supplemented with protease inhibitors (Roche Diagnostics Indianapolis, IN, USA). The homogenates were centrifuged, the supernatants of whole cell lysates and nuclear protein from previous preparationwere collected for western blots probed withpolyclonal NFATc1 antibody (1:2000 dilution, Santa Cruz Biotech, CA, USA), monoclonal NFkB subunit p65 antibody (1:3000 dilution, Santa Cruz Biotech, CA, USA), OC antibody (1:2000 dilution, Santa Cruz Biotech, CA, USA), OPN (1:2000 dilution, Santa Cruz Biotech, CA, USA) or Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibody (1:5000 dilution, Santa Cruz Biotech, CA, USA), followed withacorresponding horseradish peroxidase-conjugated secondary antibody (1:1000; Amersham, Piscataway, NJ, USA), visualized using enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham Bioscience, Piscataway, NJ, USA). Here, GAPDH functions as internal control for equal loading.

Real-time PCR assay

Total RNA from RC cells was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), reverse transcribed using the Thermo-script™ RT-PCR System (Invitrogen, Carlsbad, CA, USA) and purified with RNeasy Mini Kit (Qiagen, Germantown, MD, USA). Real time PCRs were performed using iQ SYBR Green Supermix kit (Bio-Rad, Hercules, CA, USA) with the iCycler sequence detection system (Bio-Rad, Hercules, CA, USA) with specific primers for NFATc1, Alkaline phosphatase (ALP), Osteocalcin (OC), OPN, interleukin (IL)-1beta, tumor necrosis factor (TNF)-αa and Il-10and GAPDH as shown in Table 1. Here, GAPDH acts as internal control.

Immunofluorescence assay

To study the regulation of 11R-VIVIT on the expression of p65, one subunit of NFkB, immunostainings were performed according to the standard protocol with primary antibodie-p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and Alexa Fluor® 488 secondary antibody (Molecular Probes, Carlsbad, CA) as described previously (17). Samples were mounted in Prolong Gold Antifade Reagent and analyzed by ZEISS AXIOSKOP 2 PLUS Microscope (Carl Zeiss MicroImaging, Inc. Thornwood, NY).

Osteogenic differentiation induction

RC cells were plated in 2 mlMEM medium in a 6-well plate. After overnight incubation, osteogenic differentiation was induced performed by supplementing medium with 10 mM β-glycerophosphate and 250 µM ascorbic acid-2-phosphate (18). ALP expression and calcium deposit were used as early and late markers for osteogenesis and were detected by real time PCR, ALP and Alizarin Red Staining, respectively.

ALP staining

ALP staining was carried out based on the protocol described previously (19). Briefly, RC cells were washed with PBS and stained with 5-Bromo-4-chloro-3-indolyl phosphate/Nitrobluetetrazolium (BCIP/NBT, Sigma-Aldrich, ST. Louis, MO, USA) for 60 min in the dark. ALP positive cells were visualized by light microscope (dark purple color).To quantify the bone formation, analytical FCM was performed using the bright channel on FCM Canto™ (BD Biosciences, San Jose, CA, USA).

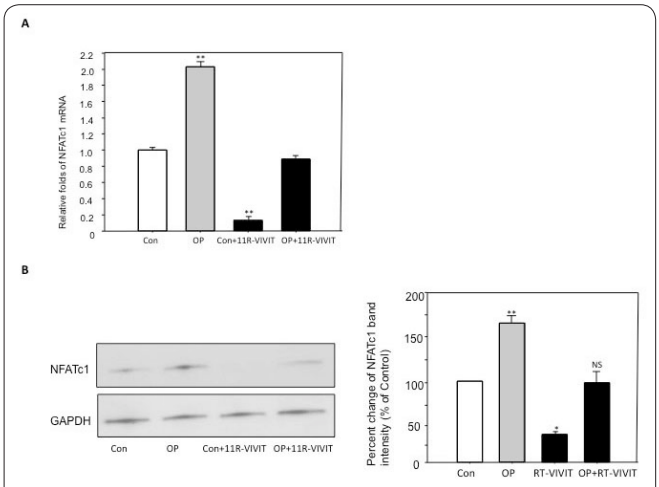

Figure 1. Polypeptide 11R-VIVIT regulates NFATc1 expression during OP condition. RC cells were isolated and prepared from rat with osteoporosis or wild type rat, and then subjected to 11R-VIVIT treatments. (**A**) In RC cells from rat with OP showed lower NFATc1 expression at transcriptional level compared to RC cell from WT rat, however, 11R-VIVIT stimulates significantly the mRNA level of NFATc1 in WT rat RC cells, further, 11R-VIVIT can rescue the compromised NFATc1 expression in OP rat RC cells to normal level. **(B)** By western blot, we found that there is significant increase of the expression of NFATc1 at protein level in comparison with that from WT rat. On the contrary, 11R-VIVIT can regulate positively the expression of NFATc1 compared to WT rat. Further, 11R-VIVIT can rescue the decreased expression of NFATc1 in OP. Western blot was quantified by scanning densitometry. Values are representative of three independent experiments, each bar represents mean ± S.E., *: p<0.05, **: *P<0.01*, NS: no significance.

Alizarin Red S (ARS) staining

ARS staining and analysis were performed according to the methods described previously (20). Briefly, ARS stock solution was prepared by dissolving 1mg of alizarin red S in 100ml of 1% KOH.After two weeks of culture in osteogenic induction medium, RC cells were washed, fixed then stained with 40mM freshly ARS solution (PH=4.2). Calcium deposits can be visualized by red color under light microscope. To quantify the staining, ARS Staining Quantification Assay (ARed-Q) method (ScienCell Research Laboratories, Carlsbad, CA, USA) was used based on the manufacturer instruction. Destained ARS dye was quantified by measuring the absorbance at 405 nm.

Statistical analysis

Values are expressed as mean ± SEM with unpaired two-tailed Student's *t* test by InStat v3.06 (GraphPad, San Diego, CA) software. $P< 0.05$ was considered statistically significant.

Results

11R-VIVIT inhibits the expression of NFATc1

To study the effect of 11R-VIVIT onosteoblastodifferentiation, we first treated the RC cells with 11R-VIVIT for 48 hr, we found that the expression of NFATc1 is increased in OP patients, but decreasedin 11R-VIVIT treated WT RC cells at mRNA level, further, 11R-VIVIT can restore the inhibition of NFATc1 expression in RC cells from OP patients (Figure 1A). To confirm our result, we did the western blots which came out with very similar results, OPincreases but 11R-VIVIT haunted the expression of NFATc1 at protein level, and 11R-VIVIT can resume the enhanced expression of NFATc1 in OP to normal level (figure 1B). The right panel in Figure 1B showed the percentage of density compared to control.

11R-VIVIT regulates the expression of down-stream molecules in Cn/NFAT signaling pathways

To further investigate the effect of inhibition of NFATc1 by IIR-VIVIT on the pathogenesis of osteoporosis, we then examined the expression of some molecules such as OC and OPN, which are important for osteroblastogenesis, by both real time PCR and western blot (Figure 2A and B). We found very opposite expression pattern of these molecules with NFATc1. Compared to control, 11R-VIVITincreases the expression of OC and OPN, but significant lower expressions were seen in OP patients. Further, 11R-VIVIT can restore their expression to normal level, even lower level at protein level.

11R-VIVITregulates the expression of cytokines related to osteoclastogenesis

Given the fact that cytokines play important roles in the onset and development of osteoblastogenesis and osteoclastogenesis, we examined the production of inflammatory cytokines at mRNA level by real time PCR (Figure 3). We found that 11R-VIVIT decreased the pro-inflammatory cytokines IL-1beta and TNF-alpha, can resume the enhanced expression of IL-1 and TNF-alpha in OP. Further, 11R-VIVIT can increase the production of anti-inflammatory cytokine IL-10 and restore its compromised expressionin OP. Our data indicates that 11R-VIVIT may be involved in the regulation of inflammatory cytokines.

NF-kB activity

To study further how 11R-VIVIT regulates the expression of NFATc1 and affects the the expression of

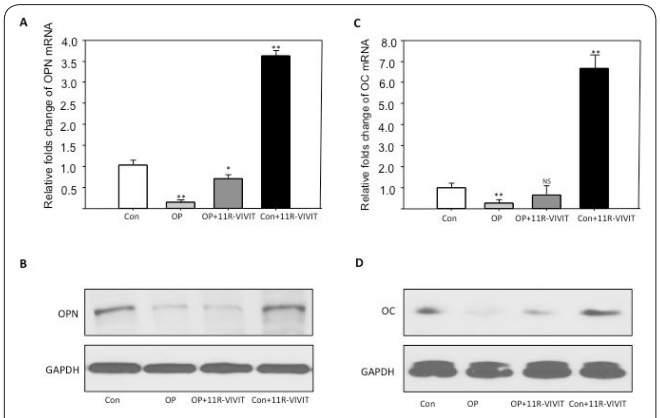

Figure 2. Polypeptide 11R-VIVIT regulates OC and OPN expression during OP condition. Real time PCR and western blot found decreased OC and OPN expression at mRNA level and protein level in RC cells from rat with OP compared to RC cell from WT rat, however, 11R-VIVIT stimulates significantly the mRNA level and protein level of OC and OPN in WT rat RC cells, further, 11R-VIVIT can rescue the compromised OC and OPN expression in OP rat RC cells to normal level. Values are representative of three independent experiments, each bar represents mean ± S.E., *: p<0.05, **: *P<0.01*, NS: no significance.

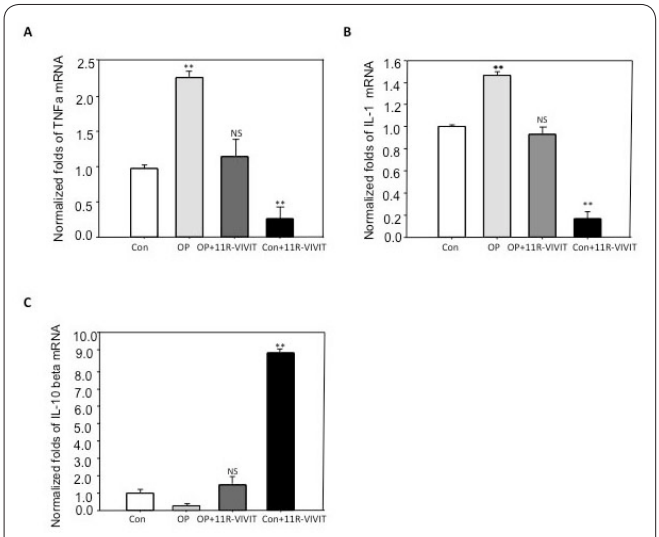

Figure 3. Polypeptide 11R-VIVIT regulates IL-1beta, TNF-a and IL-10 expression during OP condition. (A) and (B) Real time PCR and western blot found decreased **IL-1beta, TNF-a** expression at mRNA level and protein level in RC cells from rat with OP compared to RC cell from WT rat, however, 11R-VIVIT stimulates significantly the mRNA level and protein level of **IL-1beta, TNF-a** in WT rat RC cells, further, 11R-VIVIT can rescue the compromised **IL-1beta, TNF-a** expression in OP rat RC cells to normal level. However, (C) Real time PCR and western blot found decreased **IL-10** expression at mRNA level and protein level in RC cells from rat with OP compared to RC cell from WT rat, however, 11R-VIVIT stimulates significantly the mRNA level and protein level of **IL-10** in WT rat RC cells, further, 11R-VIVIT can rescue the compromised **IL-10** expression in OP rat RC cells to normal level. Values are representative of three independent experiments, each bar represents mean ± S.E., *: p<0.05, ***: P<0.01*, NS: no significance.

genes, cytokines and even osteoblastogenesis, we then investigated the status of NFkB by real time PCR, western blot and immunofluorescence (Figure 4A, 4B and 4C). We found that 11R-VIVIT cannot change the expression of p65 at both transcriptional level and protein level in all experimental groups. Western blot demonstrated that p65 is predominantly localized to the cytosolic pool, only basal level of p65 translocate to nucleus in RC cells. After 11R-VIVIT treatment, more p65 undergo translocation to nucleus demonstrated by western blot and immunostaining, indicating that NFkB is crucial for the regulation of 11R-VIVIT in osteoblastodifferentiation, even though that the expression of total NFKB is not changed.

11R-VIVIT accelerate bone formation

ALP is an early and sensitive marker for osteogenesis. By immunostaining, we found that 11R-VIVIT can increase significantly the quantity of ALP at transcriptional level (Figure 5A), indicating increased osteogenesis at early stage, further it can restore compromised ALP quantityin OP patients.ALP staining demonstrated that 11R-VIVIT stimulates the bone formation significantly in comparison with compromised bone in OP patients (Figure 5B). Figure 5C shows the percentage of area of bones. Further, calcium deposit is a late marker for osteogenesis. ARS staining, indicator of calcium deposit can reflect the extent of osteogenesis. In Figure 6, we can see that 11R-VIVIT can stimulate the osteogenesis dramatically in RC cells in comparison with control-treated counterpart. In opposite, there is compromised bone formation in OPpatients, but 11R-VIVIT can resume the osteogenic process in OP patients (Figure 6A). The percentage of the area of the bone was analyzed by software (Figure 6B).

Discussion

In the current study, we demonstrated that 11R-VIVIT decreases the expression of NFATc1, by which

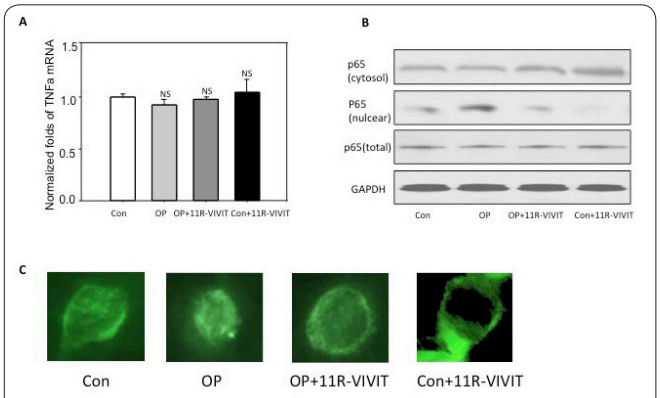

Figure 4. 11R-VIVIT blocks the function of NFkB. (A) real time PCR did not show significant different NFkB subunit p65 expression at mRNA level in all the groups tested. (B) whole lysates and nuclear protein were isolated and prepared as described in Materials & Methods, and subjected to western blots, there are no parent change of p35 in whole lysates of RC cells, and more p65 translocate to nucleus in OP rat RC cells, but 11R-VIVIT can block this translocation of p65 to nucleus and further haunt the translocation of p65 to nucleus in OP RC cells. (C) immunostaining showed in RC cells from WT rat, p65 is located at both cytosol and nucleus, but in OP condition, p65 undergoes translocation to nucleus dramatically, but 11R-VIVIT can inhibit this translocation, make the expression of p65 predominant in cytosol part, and can block significantly translocation of p65 stimulated by OP. Data are pooled from three independent experiments. *: P<0.05, **: P<0.01*, NS: no significance.

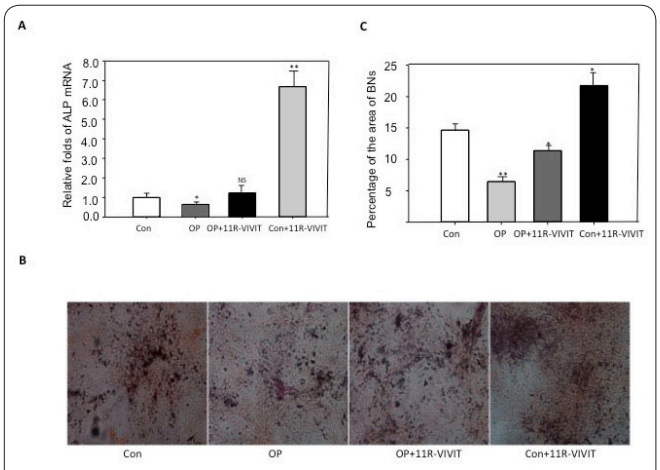

Figure 5. ALP staining and quantification of human mesenchymal stem cell (hMSC) osteogenesis. (A) Real time PCRs demonstrate that OP and 11R-VIVIT regulate the early differentiation marker ALP in an opposite way. In comparison with WT rat, OP rat RC cells showed compromised ALP transcription, but 11R-VIVIT increases the expression of ALP, further restore the compromised ALP to normal level. Data are pooled from three independent experiments. *: P<0.05, **: P<0.01*, NS: no significance.

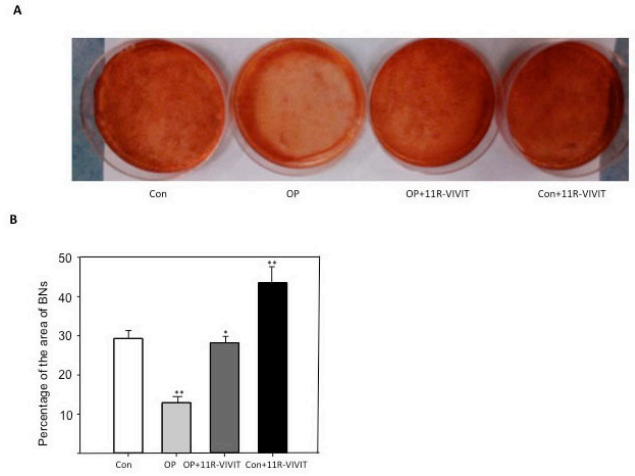

Figure 6. ARS staining and quantification of human mesenchymal stem cell (hMSC) osteogenesis Human MSCs were treated under 6 different conditions (sample 1 – 6) to induce osteogenesis for 18 days. ScienCell's Alizarin Red S staining quantification assay was applied to the treated samples and controls cultured in the hMSC growth medium without induction. **(A)** Typical ARS staining for controls (top) and treated samples (bottom); **(B)** ARS quantification using the assay. Data are pooled from three independent experiments. **: P<0.05, **: P<0.01*, NS: no significance.

regulates the expression and function of osteoblastodifferention related genes, inflammatory cytokines and NFkB, resulting in osteoblastic differentiation. This study shed some light on the mechanistic insight into the perspective of treatment of osteoporosis.

Bone formation and reabsorption are dynamic and elaborated processes resulted from coupled activation of bone-forming osteoblasts (21)and bone-resorbing osteoclasts (22). Osteoporosis is characterized by imbalanced processes, in which bone resorption dominates over bone formation(23). In process of bone metabolism, Cn/NFAT signaling pathway plays critical roles in osteoblast and osteoclast as anindispensable transcription factor.Treatment of receptor activator of nuclear factor-kappa B ligand (RANKL), one major stimulator of osteoclastogenesis and bone remodeling, fails in stimulating the differentiation ofNFATc1-deficient embryonic stem cells into osteoclasts (24). Also, Conditional NFATc1-knockout mice exhibit an osteopetrotic bone phenotype owing to a severe defect in the osteoclastogenesis process (25, 26). However, the ectopic expression of NFATc1 in MSCs induces osteoclast differentiation in these cells despite the absence of RANKL (24). All of these indicate that NFATc1 medicates the function of RANKL in osteoclastogenesis.

Opposite toincreased NFATc1, our data showed that the expressions of OPNand OC wereinhibitedsignificantly in OP patients, which are consistent with data reported previously (18, 27).OPN and OC are two osteoblast related genes, the expression of OPN is regulated by large number of factors such as cytokines (like TGFbeta, IL-1, TNFa, IL-10) which can affect its transcriptional rate, mRNA processing, stability and translation, and post-translational modifications (28, 29). Study showed that OPN can inhibit the expression of IL-10 (29), which is consistent with our data, in which enhanced OPN parallels with decreased IL-10. OC, another late marker of osteoblast differentiation, is the most abundant noncollagenous protein in bone (30). Studies demonstrated the expression of NFATc1 is proportional to the expression of OC by different mechanisms (18, 31).

Linkage has been established by epidemiological, clinical and experimental studies between osteoporosis and inflammatory conditions such as ankylosing spondylitis (32), rheumatoid arthritis (32),inflammatory bowel disease (33, 34) and systemic lupus erythematosus (35). In rheumatoid arthritis, bone loss is proportional to the release of proinflammatory cytokines and C-reactive protein (CRP), a sensitive marker of systemic inflammation (32). Many of inflammatory mediators in patients with inflammatory bowel disease like TNF-α, interleukins 1α and 1β (IL-1α, IL-1β), IL-6, IL-11, IL-17, IL-10, transforming growth factor, epidermal growth factor, and prostaglandin E2 are deregulated in patients with osteoporosis(36-40). The mechanisms underlying the involvement of inflammation in bone loss include interfering the bone remodeling process, favoring bone resorption activity.IL-1 plays important roles in the pathogenesis of various diseases including osteoporosis by stimulating the development of osteoclasts and excessive bone resorption (41, 42). Besides, IL-1 beta can also activate nuclear factor κB (NFκB) pathway to inhibit apoptosis of osteoclast (43).TNFa is involved in tumor-induced bone resorption and non-tumor-induced osteopenia (44-46). It stimulates formation of osteoclasts by including either proliferation of osteoclast-precursor cells or activation of differentiated osteoclasts (47). Linkage was established between TNFa and osteoporosis by sib-pair analysis (48) and single nucleotide polymorphism (SNP) assay (49). Receptor activator of NF-κB (RANK) pathway,a new TNF family pathway, plays important roles inbone metabolism. RANK binds to its ligand RANKL, leading to osteoclasts differentiating and maturing,increased bone loss.Interleukin 10 (IL-10) is another susceptibility gene associated with the pathogenesis of osteoporosis with polymorphisms (39). Interleukin-10 inhibits osteoclastogenesis by reducing NFATc1 expression and preventing its translocation to the nucleus (50). Interleukin-10 inhibits bone resorption: a potential therapeutic strategy in periodontitis and other bone loss diseases (40). Besides, some other cytokines were identified involved in the onset and development of osteoporosis. IL-6 promoted the differentiation of osteoclasts from its precursor (51); transforming growth factor β1 (TGF-β1) is linked to pathogenesis for its polymorphism (39). Study found increased IL-31 in osteoporosis (52).

In conclusion, 11R-VIVIT increase osteoblasto differentiation and restore the compromised bone metabolism in OP, evidenced by the facts that 11R-VIVIT regulates osteoblast related genes, inflammatory related cytokines and NFkB pathways.

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