

# <sup>99</sup>Tc-MDP-induced human osteoblast proliferation, differentiation and expression of osteoprotegerin

JIE CHEN<sup>1,2</sup>, YOUYU LAN<sup>2</sup>, YUE HE<sup>3</sup>, CHENGSONG HE<sup>2</sup>, FEN XU<sup>2</sup>, YUGAO ZHANG<sup>2</sup>, YI ZHAO<sup>1</sup> and YI LIU<sup>1</sup>

<sup>1</sup>Department of Rheumatology and Immunology, West China Hospital of Sichuan University, Chengdu, Sichuan 610041;  
Departments of <sup>2</sup>Rheumatology and Immunology and <sup>3</sup>Ophthalmology,  
The First Affiliated Hospital of Sichuan Medical University, Luzhou, Sichuan 646000, P.R. China

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**Abstract.** The aim of the present study was to examine the influence of technetium methylenediphosphonate (<sup>99</sup>Tc-MDP) on the proliferation and differentiation of human osteoblasts. Human iliac cancellous bone was isolated and cultured with either <sup>99</sup>Tc-MDP,  $\beta$  fibroblast growth factor (as a positive control) or medium only (as a negative control). Proliferation was assessed by direct cell counting, CCK-8 assay and bromodeoxyuridine staining. The cell cycle and rate of apoptosis was assessed by propidium iodide staining and flow cytometry. Alkaline phosphatase (ALP) activity was assessed by the p-nitrophenyl phosphate method and mineralized nodules were stained with Alizarin Red. Expression of osteocalcin (OCN) and bone morphogenetic protein-2 (BMP-2) was assessed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and expression levels of osteoprotegerin (OPG) and receptor activator of NF- $\kappa$ B ligand (RANKL) were assessed by RT-qPCR and ELISA. Isolated human osteoblasts stained positively for ALP and developed mineralized nodules. Treatment with  $10^{-5}$ - $10^{-10}$  M <sup>99</sup>Tc-MDP enhanced proliferation and 48 h incubation with  $10^{-8}$  M <sup>99</sup>Tc-MDP increased the proportion of cells in S-phase, decreased the proportion in G<sub>0</sub>/G<sub>1</sub> phase, and increased the cell proliferation index. The rate of apoptosis also increased, but the increase was not significant. Cells incubated with  $10^{-6}$ - $10^{-9}$  M <sup>99</sup>Tc-MDP for 3-9 days exhibited increased ALP activity and mineralized nodule development.  $10^{-8}$  M <sup>99</sup>Tc-MDP increased BMP-2 and OPG expression levels and OPG secretion, but OCN mRNA expression levels and RANKL secretion were not significantly altered at 72 h. <sup>99</sup>Tc-MDP treatment induced osteoblast proliferation and differentiation without affecting apoptosis.

These findings provide proof of concept for the future use of <sup>99</sup>Tc-MDP in the treatment of bone-destructive diseases.

## Introduction

The metabolism of bone tissues, also known as bone remodeling, is a cyclic process involving constant resorption of old bone by osteoclasts and formation of new bone by osteoblasts. Overactive bone resorption or dysfunction of bone formation perturbs the balance between bone resorption and formation, damaging the structural integrity and strength of bone, resulting in the loss of structural integrity and bone mass, manifesting as osteoporosis (1). An imbalance between osteoclast and osteoblast activity is also involved in the pathogenesis of common rheumatic diseases, including rheumatoid arthritis (RA), ankylosing spondylitis (AS), osteoarthritis and osteoporosis (1). Thus, one of the targets for treating these diseases is to restore this balance and prevent bone destruction by inhibiting osteoclast or inducing osteoblast functions.

Osteoblasts differentiate from bone marrow stromal cells. The process of osteoblast differentiation consists of 3 steps: Cell proliferation, extracellular matrix formation and maturation, and bone matrix mineralization (2). Each differentiation period involves the expression and regulation of distinct genes. During osteoblast proliferation, the expression of type I collagen peaks, facilitating bone matrix maturation and mineralization. During bone matrix maturation, osteoblast proliferation is reduced and expression of alkaline phosphatase (ALP) increases. The peak of ALP activity marks the maturation of the bone matrix. During bone matrix mineralization, ALP activity decreases while osteocalcin (OCN) expression increases, and the formation of bone nodules indicates bone matrix mineralization (3). Bone morphogenetic protein-2 (BMP-2) is the only cytokine that can independently induce ectopic bone formation. BMP-2 has been reported to be involved in the induction of pluripotent stem cell differentiation into bone cells, and is associated with multiple signal pathways that promote osteogenesis (4).

During bone formation, cytokines synthesized and secreted by osteoblasts regulate the differentiation and maturation of osteoclasts (5). The osteoprotegerin (OPG)/receptor activator of nuclear factor- $\kappa$ B (NF- $\kappa$ B; RANK)/receptor activator of NF- $\kappa$ B ligand (RANKL) system has been reported to regulate

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*Correspondence to:* Dr Yi Liu, Department of Rheumatology and Immunology, West China Hospital of Sichuan University, 37 Guo Xue Xiang, Chengdu, Sichuan 610041, P.R. China  
E-mail: yi2006liu@163.com

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osteoclast function and bone remodeling (6). RANKL secreted by osteoblasts and bone marrow stromal cells binds to RANK expressed on the surface of osteoclast precursor cells or osteoclasts, inducing osteoclast differentiation and increasing bone resorption. OPG secreted by osteoblasts and bone marrow stromal cells competitively binds to RANKL, inhibiting RANKL and RANK binding. Thus, the balance of OPG and RANKL expression regulates osteoclast differentiation, activation and function (6). RANKL expression and soluble RANKL (sRANKL) release induce bone resorption and destruction (7). OPG and RANKL expression regulates osteoclast activation, and this further influences bone remodeling (8).

The major component of technetium methylenediphosphonate ( $^{99}\text{Tc}$ -MDP) injection is the chelate of stannous chloride-reduced technetium (agent A) with methylene diphosphonate (agent B).  $^{99}\text{Tc}$ -MDP is widely used in the treatment of many diseases, including RA, AS, osteonecrosis, osteoarthritis, osteoporosis, Graves' eye disease, psoriatic arthritis, bone metastases and multiple myeloma (9-12). However, previous studies have also reported additional pharmacological effects of  $^{99}\text{Tc}$ -MDP, including inhibition of inflammatory reactions, immune regulation and regulation of bone metabolism (11,13-15). As the synovial membrane is semi-permeable, generic drugs cannot penetrate it, but MDP is able to carry  $^{99}\text{Tc}$  into the joint cavity and facilitate its function close to the lesion. When  $^{99}\text{Tc}$ -MDP enters the joint cavity and reaches an area of synovitis or abnormal bone, it binds immature collagen or is absorbed by hydroxyapatite crystals, thereby persisting and exerting a long-lasting therapeutic effect.

However, the mechanism by which  $^{99}\text{Tc}$ -MDP influences bone metabolism has not been elucidated, and to the best of our knowledge, no study has reported the effect of  $^{99}\text{Tc}$ -MDP on the proliferation and function of osteoblasts cultured *in vitro*. The aim of the present study was to investigate the effect of  $^{99}\text{Tc}$ -MDP on *in vitro*-cultured osteoblasts, and to explore the mechanisms responsible for its inhibition of bone destruction, providing a theoretical basis for the use of  $^{99}\text{Tc}$ -MDP in the treatment of bone-destructive diseases.

## Materials and methods

**Isolation and culture of human osteoblasts.** Iliac cancellous bone was sampled from 8 patients aged between 30 and 50 years, presenting with no metabolic bone diseases but receiving autologous bone transplantation to repair fracture in the West China Hospital of Sichuan University (Chengdu, China). The present study was approved by the ethics committee of the West China Hospital of Sichuan University (Chengdu, China) and all patients gave their informed consent.

Iliac cancellous bone samples were stripped of periosteum and soft tissues, washed with sterilized phosphate buffered saline (PBS) 3 times, cut into 1 mm<sup>2</sup> sections, washed in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and PBS and incubated at 37°C for 8 min with erythrocyte lysate (840 mg NaHCO<sub>3</sub>, 37.2 mg EDTA, 8.023 g ammonia chloride, 1000 ml distilled water; pH 7.2). The suspension was subsequently centrifuged at 500 x g for 10 min at room temperature, and the precipitate was rinsed until it appeared white. The precipitate was pre-digested in 0.25% EDTA containing trypsin (HyClone;

GE Healthcare Life Sciences, Chalfont, UK) for 20 min at 37°C. Digestion was terminated with fetal bovine serum (FBS; Yuanheng Jinma Biotechnology Co., Ltd., Beijing, China), and the supernatant was discarded. Type I collagenase (0.1%; BD Biosciences, Franklin Lakes, NJ, USA) was added at a volume ratio of 1:8, and bone cells were digested for 1 h at 37°C. Bone cells were then centrifuged at 500 x g for 5 min at room temperature and rinsed in PBS twice, then resuspended in high-glucose DMEM containing 10% FBS and antibiotics (penicillin 100 IU/ml, streptomycin 100 mg/ml) (Gibco; Thermo Fisher Scientific, Inc.) and passed through a 200-mesh stainless steel screen (75 μm pore size) to remove non-osteoblasts and impurities. The filtered suspension was further digested by type I collagenase 3 times. The cell suspension was counted using a hemocytometer and trypan blue staining. Cells were ≥95% viable and cultured at 5x10<sup>4</sup> cells/ml in a sterile Petri dish at 37°C with 5% CO<sub>2</sub> and 95% humidity. The medium was changed every 48 h and cells were passaged with a 1:2 division using 0.25% trypsin/0.02% EDTA.

**Preparation of  $^{99}\text{Tc}$ -MDP and  $\beta$  fibroblast growth factor ( $\beta$ -FGF) working solutions.**  $^{99}\text{Tc}$ -MDP (Chengdu Yunke Pharmaceutical Co., Ltd., Chengdu, China) was prepared according to the manufacturer's protocol. Agent A (5 ml, containing 0.05 μg of  $^{99}\text{Tc}$ ) and B (containing 5 mg MDP and 0.5 mg stannous chloride) were mixed at room temperature for 5 min. The stock solution was then adjusted to 1 ml/mg MDP. The working solutions were freshly prepared prior to use.  $\beta$ -FGF (PeproTech, Inc., Rocky Hill, NJ, USA) was diluted in complete medium (high-glucose DMEM supplemented with 10% FBS and antibiotics) to 10 ng/ml.

**CCK-8 measurement of cell proliferation.** Passage 2 osteoblasts were cultured at 2.5x10<sup>3</sup> cells/well in a 96-well plate for 24 h. Osteoblasts were subsequently synchronized by serum starvation (24 h in DMEM containing 0.1% FBS), then incubated with medium supplemented with 10<sup>-4</sup>-10<sup>-12</sup> M  $^{99}\text{Tc}$ -MDP, 10 ng/ml  $\beta$ -FGF or medium alone. Each condition was replicated five times. Proliferation was measured at 24, 48 and 72 h with a CCK-8 kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's protocol.

**Bromodeoxyuridine (BrdU) measurement of cell proliferation.** Passage 2 osteoblasts were cultured at 1x10<sup>4</sup> cells/well in a 24-well plate pre-covered with coverslips for 24 h. Osteoblasts were subsequently synchronized as previously described, then incubated with 10<sup>-8</sup> M  $^{99}\text{Tc}$ -MDP, 10 ng/ml  $\beta$ -FGF or medium (high-glucose DMEM supplemented with 10% FBS and antibiotics) alone for 72 h. Each condition was replicated three times. The medium was then aspirated and 10 μg/ml BrdU (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) was added for 4 h. The slides were then washed with PBS, fixed in 4% paraformaldehyde at 4°C for 30 min, then washed with 0.1 M PBS containing 1% Triton X-100 (Amresco, Inc., Framingham, MA, USA), incubated in 1 M ice-cold HCl for 10 min, then 2 M HCl for 10 min at room temperature and incubated at 37°C for 20 min. Slides were then washed with PBS, incubated in 0.1 M sodium borate (pH=8.6) at room temperature for 12 min, washed in 0.1 M PBS containing 1% Triton X-100 and blocked in 5% FBS containing 1 M glycine for 1 h.

Slides were incubated with mouse anti-BrdU immunoglobulin G (IgG; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; catalog no. sc-70443; 1:100) and RNase (Ambion; Thermo Fisher Scientific, Inc.) at 4°C overnight, then washed in 0.1 M PBS containing 1% Triton X-100, and incubated with Cy3 goat-anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA; catalog no. 115-165-205; 1:200) at 37°C or room temperature for 1 h, then washed in PBS and mounted with antifade medium (Thermo Fisher Scientific, Inc. P36934). The sections were preserved at 4°C in the dark, and the proportion of BrdU-positive cells was calculated by counting 10 randomly selected fields under a fluorescence microscope, at x200 magnification.

*Flow cytometric analysis of cell cycle and apoptosis.* Passage 2 osteoblasts were cultured at  $4 \times 10^5$  cells/dish in 100 mm dishes for 24 h. Osteoblasts were then synchronized and incubated with  $10^{-8}$  M  $^{99}\text{Tc}$ -MDP, 10 ng/ml  $\beta$ -FGF or medium (high-glucose DMEM supplemented with 10% FBS and antibiotics) alone for 48 h. Each condition was replicated three times. Cells were then digested with trypsin, washed with PBS and collected by centrifuging at  $500 \times g$  for 5 min at room temperature. Pre-cooled ethanol (70%, 1 ml) was added to the precipitate and the cell suspension was fixed at 4°C overnight. Following centrifugation at  $500 \times g$  for 5 min at room temperature, the precipitate was resuspended in 3 ml PBS, passed through a 400-mesh screen and stained with 1 ml propidium iodide (PI) at 4°C for 30 min in the dark. The cell cycle was assessed by flow cytometry, and  $3 \times 10^4$  cells were collected for each sample. Modfit software Version 4.0 (Verity Software House, Inc., Topsham, ME, USA) was used for data acquisition, processing, calculation of apoptotic cell proportion, and the determination of cell cycle distribution. The S-phase fraction (SPF) and proliferation index [proliferation index =  $(S + G_2/M)/(G_0/G_1 + S + G_2/M) \times 100\%$ ] were used to evaluate the speed of proliferation.

*Determination of alkaline phosphatase activity.* Passage 2 osteoblasts were cultured at  $5 \times 10^4$  cells/well in 12-well plates for 24 h. Osteoblasts were then synchronized and incubated with  $10^{-4}$ - $10^{-12}$  M  $^{99}\text{Tc}$ -MDP, 10 ng/ml  $\beta$ -FGF or medium (high-glucose DMEM supplemented with 10% FBS and antibiotics) alone. Each condition was replicated three times. Medium was changed every 48 h, and cells were collected 3, 6 and 9 days later, washed in PBS and treated with 80  $\mu$ l of lysate (50 mM Tris-HCL; pH 7.4; 150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS; stored in the dark at 4°C) at 4°C for 30 min, then centrifuged at  $5,000 \times g$  at 4°C for 30 min. The supernatant was collected and 30  $\mu$ l was added to each well of a 96-well plate. ALP activity was determined using the p-nitrophenyl phosphate (pNPP) method, with Alkaline Phosphatase Assay Kit (Beyotime Institute of Biotechnology, Haimen, China; P0321), according to the manufacturer's protocol; optical density at 520 nm was measured using a BioTek Synergy micro-plate reader (Thermo Fisher Scientific, Inc.). Protein content was quantified using the Thermo Scientific™ Pierce™ Coomassie Plus™ (Bradford) Protein Assay (Thermo Fisher Scientific, Inc.) (16).

*Mineralized nodule counts.* Passage 2 osteoblasts were cultured at  $1 \times 10^4$  cells/well in 24-well plates for 24 h.

Osteoblasts were then synchronized and incubated with  $10^{-8}$  M  $^{99}\text{Tc}$ -MDP, 10 ng/ml  $\beta$ -FGF or medium (high-glucose DMEM supplemented with 10% FBS and antibiotics) alone. Each condition was replicated three times. Medium was changed every 48 h, and at 14 days complete medium containing 10 mM  $\beta$ -glycerophosphate and 50 mg/l ascorbic acid was added. Cells were subsequently washed in PBS, fixed with 2.5% glutaraldehyde at room temperature for 15 min, rinsed in PBS (pH 4.2), incubated with 2% Alizarin Red at 37°C for 10 min, and washed in PBS (pH 4.2). Using a polyester film with 0.2 mm<sup>2</sup> grid marks to assist counting, red mineralized nodules  $>200 \mu\text{m}$  with a clear boundary were counted in 10 randomly selected high power fields under a light microscope (x40 magnification) by two independent investigators blinded to the grouping.

*Reverse transcription-quantitative polymerase chain reaction (RT-PCR).* Passage 2 osteoblasts were cultured at  $1 \times 10^5$  cells/well in a 6-well plate for 24 h. Osteoblasts were subsequently synchronized and incubated with  $10^{-8}$  M  $^{99}\text{Tc}$ -MDP, 10 ng/ml  $\beta$ -FGF or medium (high-glucose DMEM supplemented with 10% FBS and antibiotics) alone for 72 h. Each condition was replicated three times. Total RNA extraction was performed using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (1  $\mu$ g) was then reverse transcribed into cDNA (20  $\mu$ l reaction volume) using First Strand cDNA Synthesis Kit (Fermentas; Thermo Fisher Scientific, Inc.) as previously described (17). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. Forward and reverse primers of OCN, BMP-2, OPG, RANKL and GAPDH are listed in Table I. Primers were designed by Primer Premier 5.0 (PREMIER Biosoft, Palo Alto, CA, USA) and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). SYBR Green I (Takara Bio, Inc., Otsu, Japan) was used for fluorescent detection according to the manufacturer's protocol. The thermocycler conditions used were as follows: 94°C pre-denaturation for 120 sec, 94°C denaturation for 20 sec, annealing at 58, 54, 54, 54 and 50°C for OCN, BMP-2, OPG, RANKL and GAPDH, respectively, for 20 sec, 72°C elongation for 40 sec, and 45 cycles. The melting curve was plotted to determine the specificity of amplified products. Relative OCN, BMP-2, OPG, RANKL and GAPDH mRNA expression levels were determined using the  $2^{-\Delta\Delta C_q}$  method (18).

*ELISA.* Passage 2 osteoblasts were cultured at  $1 \times 10^5$  cells/well in a 6-well plate for 24 h. Osteoblasts were then synchronized and incubated with  $10^{-8}$  M  $^{99}\text{Tc}$ -MDP, 10 ng/ml  $\beta$ -FGF or medium (high-glucose DMEM supplemented with 10% FBS and antibiotics) alone for 72 h. Each condition was replicated three times. The OPG and RANKL contents in supernatants were assessed by specific ELISA kits (Cusabio Biotech Co., Ltd., College Park, MD, USA; catalog nos. CSB-E04692 h and CSB-E05125 h, respectively) was performed according to the manufacturer's protocol.

*Statistical analysis.* Data are expressed as the mean  $\pm$  standard deviation. Statistical analysis was performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). Multiple sets of data were compared by one-way analysis of variance (ANOVA). If

Table I. Reverse transcription-quantitative polymerase chain reaction primer sequences.

Genes	Sequence	Target sequence length (bp)
OCN	F: 5'-GTGCAGCCTTTGTGTCCAAG-3' R: 5'-GTCAGCCAACCTCGTCACAGT-3'	158
BMP-2	F: 5'-CGGTCTCCTAAAGGTGACCAT-3' R: 5'-CGAACTTCCTGCGGCCAGCT-3'	120
OPG	F: 5'-TCAGAAAGGAAATGCAACACA-3' R: 5'-CCGTTTTATCCTCTCTACT-3'	205
RANKL	F: 5'-GCCTCCCGTCCATGTTC-3' R: 5'-TTAGGATCCATCTGCGCTC-3'	101
GAPDH	F: 5'-TGACATCAAGAAGGTGGTGA-3' R: 5'-TCATACCAGGAAATGAGCTT-3'	177

OCN, osteocalcin; BMP-2, bone morphogenetic protein-2; OPG, osteoprotegerin; RANKL, receptor activator of NF- $\kappa$ B ligand; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; F, forward; R, reverse.

$H_0$  was refused, the Least Significant Difference-*t* test was used for multiple comparisons. Different conditions were compared by ANOVA with Dunnett's *t*-test and comparisons of level data were made by the rank sum test. Test level  $\alpha$  (bilateral) was defined at 0.05, and  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Osteoblast culturing.** Primary human osteoblasts were isolated from iliac cancellous bone and cultured *ex vivo*. Following isolation, cells were spherical, evenly distributed, and surrounded by translucent membranes. Osteoblasts began to attach to the dish 12-24 h post inoculation, and cells swelled and formed a triangle shape with a visibly enlarged nucleus. Between 24-120 h most cells were attached and cell shape varied from polygonal to spindle, fusiform and triangle (Fig. 1A and B). Projections increased and enlarged with extended culture. The nuclei were large, clear, round or oval-shaped and contained abundant cytoplasm and 1-3 nucleoli. Cell secretions also gradually increased with time, and by day 7-12 osteoblasts were spindle or cord-shaped and grew to ~100% confluence, forming a single cell layer of clustered and fused cells, with unclear cell boundaries (Fig. 1C).

**Impact of <sup>99</sup>Tc-MDP on osteoblast cell proliferation.** When incubated with  $10^{-4}$  M <sup>99</sup>Tc-MDP, osteoblast proliferation was significantly inhibited at 24, 48 and 72 h compared with controls ( $P=0.007$ ,  $P=0.007$  and  $P=0.014$ , respectively; Table II). However, in the presence of lower concentrations of <sup>99</sup>Tc-MDP ( $10^{-5}$ - $10^{-9}$  M), osteoblast proliferation was significantly increased at 24, 48 and 72 h compared with controls ( $P < 0.05$ ; Table II), and in the presence of  $10^{-5}$ - $10^{-12}$  M <sup>99</sup>Tc-MDP osteoblast proliferation was significantly enhanced at 72 h compared with controls ( $P < 0.05$ ; Table II). However, the result was not significantly associated with time of intervention ( $P > 0.05$ ; Table II).

The proportion of cells in the proliferative phase was assessed by BrdU assay. The fraction of cells in the proliferative phase was  $16.78 \pm 3.00\%$  higher in cultures incubated with

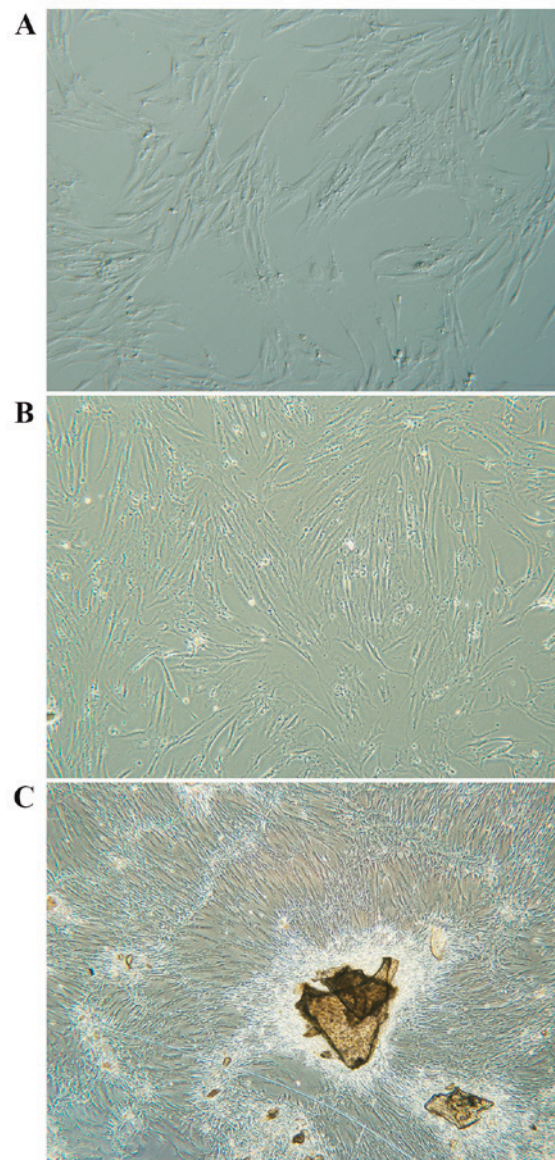


Figure 1. Morphology of passage 2 human osteoblasts. (A) Light microscopic image of human osteoblasts at day 3 (x100 magnification). (B) Image of human osteoblasts at day 6 (x100 magnification) and (C) Human osteoblasts at day 12 at 100% confluence (x40 magnification).

Table II. Detection of osteoblast proliferation using the CCK-8 assay.

	Concentration	Percent of controls (%)		
		24 h	48 h	72 h
Control		100.00±3.69	100.00±3.91	100.00±2.68
β-FGF (ng/ml)	10	111.77±3.25 <sup>a</sup>	125.58±5.24 <sup>a</sup>	123.05±7.99 <sup>a</sup>
<sup>99</sup> Tc-MDP (M)	10 <sup>-4</sup>	92.72±2.40 <sup>a</sup>	73.80±8.27 <sup>a</sup>	69.79±5.26 <sup>a</sup>
	10 <sup>-5</sup>	114.91±2.86 <sup>a</sup>	118.83±8.74 <sup>a</sup>	104.81±2.60 <sup>a</sup>
	10 <sup>-6</sup>	109.62±2.05 <sup>a</sup>	120.03±8.24 <sup>a</sup>	105.61±5.03 <sup>a</sup>
	10 <sup>-7</sup>	115.05±4.78 <sup>a</sup>	129.86±7.27 <sup>a</sup>	103.91±2.05 <sup>a</sup>
	10 <sup>-8</sup>	124.70±3.12 <sup>a</sup>	127.95±8.13 <sup>a</sup>	130.85±8.10 <sup>a</sup>
	10 <sup>-9</sup>	113.85±4.17 <sup>a</sup>	115.80±3.65 <sup>a</sup>	124.18±6.80 <sup>a</sup>
	10 <sup>-10</sup>	119.41±5.51 <sup>a</sup>	111.51±2.52	121.36±7.89 <sup>a</sup>
	10 <sup>-11</sup>	105.26±3.21	117.92±7.51	113.52±8.00 <sup>a</sup>
	10 <sup>-12</sup>	103.61±2.82	116.68±7.94 <sup>a</sup>	117.20±8.41 <sup>a</sup>

<sup>a</sup>P<0.05 vs. control. Data are expressed as the mean ± standard deviation; n=5. β-FGF, β fibroblast growth factor; <sup>99</sup>Tc-MDP, technetium methylenediphosphonate.

Table III. Determination of osteoblast proliferation using bromodeoxyuridine staining.

	Concentration	Percent of control (%)
Control		100.00±2.56
β-FGF (ng/ml)	10	115.87±1.83 <sup>a</sup>
<sup>99</sup> Tc-MDP (M)	10 <sup>-8</sup>	116.78±3.00 <sup>a</sup>

<sup>a</sup>P<0.05 vs. control. Data are expressed as the mean ± standard deviation; n=3. β-FGF, β fibroblast growth factor; <sup>99</sup>Tc-MDP, technetium methylenediphosphonate.

10<sup>-8</sup> M <sup>99</sup>Tc-MDP for 24 h compared with cultures incubated in medium alone (P=0.026; Fig. 2 and Table III).

**Influence of <sup>99</sup>Tc-MDP on the osteoblast cell cycle.** Flow cytometry and PI staining was used to assess the osteoblast cell cycle stage. Following 48 h incubation with 10<sup>-8</sup> M <sup>99</sup>Tc-MDP, the fraction of cells in the S-phase (6.62±1.18%) significantly increased compared with the control (27.47±2.38%; P=0.004; Fig. 3 and Table IV). The fraction of cells in G<sub>2</sub>/M phase was not significantly altered (P>0.05; Fig. 3 and Table IV), but the fraction of cells in the G<sub>0</sub>/G<sub>1</sub> phase was significantly reduced from 90.30±0.69 to 69.17±2.32% compared with the control (P=0.001; Fig. 3 and Table IV). The proliferation index of cells incubated with 10<sup>-8</sup> M <sup>99</sup>Tc-MDP was also significantly increased from 9.7±0.69 to 30.83±2.32 compared with controls (P=0.006; Table IV). The rate of apoptosis in osteoblasts incubated with 10<sup>-8</sup> M <sup>99</sup>Tc-MDP also increased, but the difference was not statistically significant (P>0.05; Fig. 4).

**Influence of <sup>99</sup>Tc-MDP on alkaline phosphatase activity.** As an early marker of osteoblast differentiation, ALP activity reflects the differentiation and functional status of osteoblasts. Higher ALP activity indicates more significant differentiation

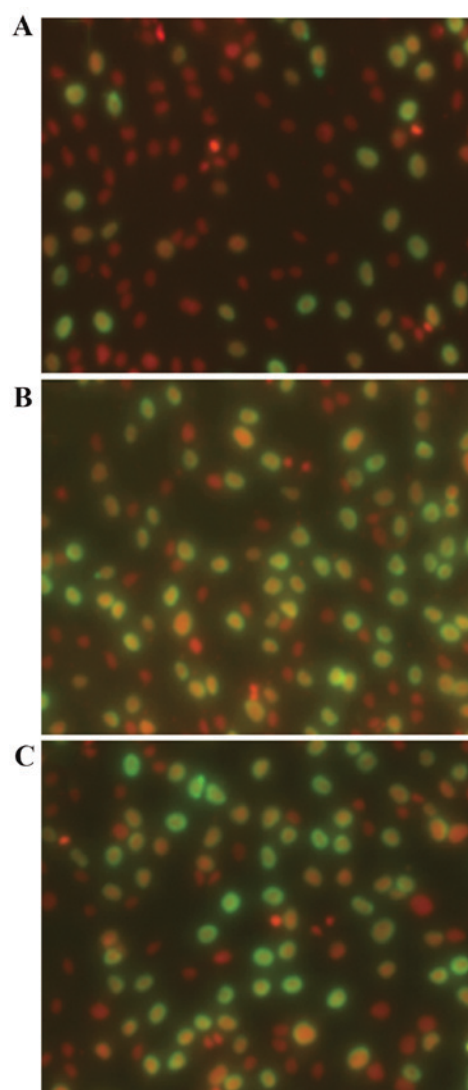


Figure 2. Detection of osteoblast proliferation using bromodeoxyuridine staining 72 h following treatment (x200 magnification). (A) control, (B) β fibroblast growth factor and (C) technetium methylenediphosphonate.

Table IV. Determination of osteoblast proliferation using flow cytometry.

	Concentration	G <sub>0</sub> /G <sub>1</sub> (%)	S (%)	G <sub>2</sub> /M (%)	Proliferation index
Control		90.30±0.69	6.62±1.18	3.07±1.52	9.7±0.69
β-FGF (ng/ml)	10	68.74±2.87 <sup>a</sup>	27.97±3.00 <sup>a</sup>	3.29±1.21	31.26±2.87 <sup>a</sup>
$^{99}\text{Tc}$ -MDP (M)	10 <sup>-8</sup>	69.17±2.32 <sup>a</sup>	27.47±2.38 <sup>a</sup>	3.36±1.14	30.83±2.32 <sup>a</sup>

<sup>a</sup>P<0.05 vs. control. Data are expressed as the mean ± standard deviation; n=3. β-FGF, β fibroblast growth factor;  $^{99}\text{Tc}$ -MDP, technetium methylenediphosphonate.

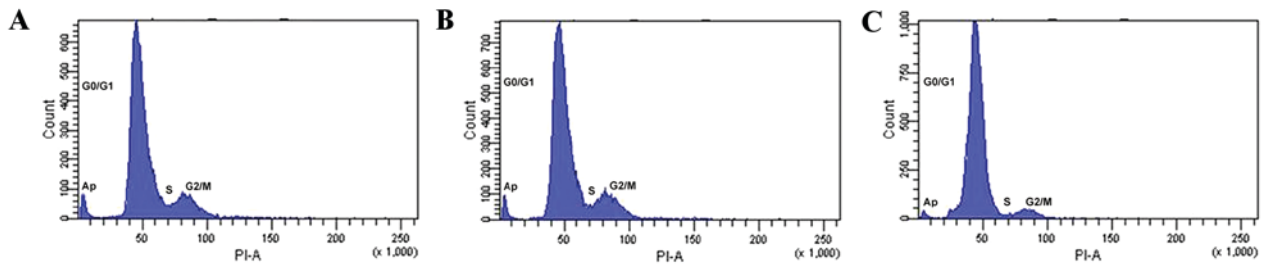


Figure 3. Influence of  $^{99}\text{Tc}$ -MDP on the osteoblast cell cycle 48 h following treatment. (A):  $^{99}\text{Tc}$ -MDP, (B): β fibroblast growth factor and (C): control.  $^{99}\text{Tc}$ -MDP, technetium methylenediphosphonate.

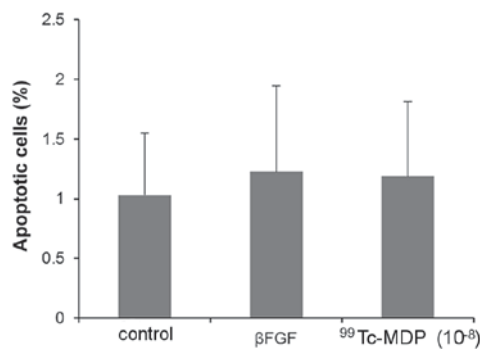


Figure 4. Evaluating the influence of  $^{99}\text{Tc}$ -MDP (10<sup>-8</sup> M) on osteoblast apoptosis using propidium iodide staining, detected by flow cytometry 48 h following treatment. Data are presented as the mean + standard deviation; n=3; P>0.05 vs. control.  $^{99}\text{Tc}$ -MDP, technetium methylenediphosphonate; βFGF, β fibroblast growth factor.

of pre-osteoblasts into mature osteoblasts (19). The level of ALP activity in osteoblasts was assessed by the pNPP method following 3, 6 and 9 days of incubation with 10<sup>-5</sup> to 10<sup>-12</sup> M  $^{99}\text{Tc}$ -MDP. Significantly enhanced phosphatase activity was observed following 3, 6 and 9 days of incubation with 10<sup>-6</sup> to 10<sup>-9</sup> M  $^{99}\text{Tc}$ -MDP compared with controls (P<0.05; Table V), peaking at 44.80±4.66% over control following incubation with 10<sup>-9</sup>  $^{99}\text{Tc}$ -MDP for 3 days (Table V). The effect of  $^{99}\text{Tc}$ -MDP was not significantly associated with the time of intervention (P>0.05; Table V).

**Influence of  $^{99}\text{Tc}$ -MDP on mineralized nodules.** The formation of mineralized nodules marks the mature differentiation of osteoblasts, and represents a morphological manifestation of osteoblast function. The influence of  $^{99}\text{Tc}$ -MDP on the development of mineralized nodules in osteoblasts was assessed by light microscopy. Following 14 days of incubation with 10<sup>-8</sup>

$^{99}\text{Tc}$ -MDP, Alizarin Red staining revealed the level of mineralized nodules to be significantly increased compared with controls (P<0.001; Fig. 5).

**Influence of  $^{99}\text{Tc}$ -MDP on OCN, BMP-2, OPG/RANKL mRNA expression levels.** RT-qPCR revealed that incubation with 10<sup>-8</sup> M  $^{99}\text{Tc}$ -MDP for 72 h did not significantly affect OCN mRNA expression levels (Fig. 6A) but BMP-2 and OPG/RANKL mRNA expression levels were significantly increased compared with controls (P<0.001 and P<0.001, respectively; Fig. 6B and C).

**Influence of  $^{99}\text{Tc}$ -MDP on OPG/RANKL secretion.** ELISA revealed that secretion of OPG was significantly increased, by 10.15±5.32%, in osteoblasts incubated with 10<sup>-8</sup> M  $^{99}\text{Tc}$ -MDP for 72 h (P=0.002; Table VI). Although 10<sup>-8</sup> M  $^{99}\text{Tc}$ -MDP did not significantly affect the secretion of RANKL, the ratio of OPG to RANKL secretion was increased by 13.41±3.24% (Table VI).

## Discussion

$^{99}\text{Tc}$ -MDP has been effectively used in the treatment of many autoimmune and bone-destructive diseases (9-15). The primary aims of the present study were to investigate the influence of  $^{99}\text{Tc}$ -MDP on osteoblasts cultured *in vitro*, and to explore the mechanisms responsible for its depression of bone destruction. Primary human osteoblasts were isolated from iliac cancellous bone and cultured *ex vivo*.

A CCK-8 kit was used to measure proliferation of passage 2 osteoblasts. Whilst high concentrations of  $^{99}\text{Tc}$ -MDP (10<sup>-4</sup> M and above) inhibited osteoblast proliferation, at lower concentrations (10<sup>-5</sup>-10<sup>-12</sup> M) proliferation was enhanced. BrdU staining and flow cytometry were used to examine cell cycle, thereby reflecting cell proliferation. BrdU staining has

Table V. Determination of alkaline phosphatase activity using the p-nitrophenyl phosphate method.

Concentration	Percent of controls (%)		
	3 d	6 d	9 d
Control	100.00±3.25	100.00±4.01	100.00±2.79
β-FGF (ng/ml)	74.28±5.71 <sup>a</sup>	60.93±4.52 <sup>a</sup>	50.07±3.71 <sup>a</sup>
<sup>99</sup> Tc-MDP(M)	109.98±2.96	92.78±4.03	91.45±5.22
10 <sup>-4</sup>	113.75±2.83	108.92±3.58	103.24±2.54
10 <sup>-5</sup>	117.84±3.65 <sup>a</sup>	125.85±4.93 <sup>a</sup>	118.01±3.23 <sup>a</sup>
10 <sup>-6</sup>	125.04±3.53 <sup>a</sup>	129.09±5.73 <sup>a</sup>	123.65±5.26 <sup>a</sup>
10 <sup>-7</sup>	151.68±4.06 <sup>a</sup>	131.48±4.04 <sup>a</sup>	128.01±5.47 <sup>a</sup>
10 <sup>-8</sup>	144.80±4.66 <sup>a</sup>	116.34±3.47 <sup>a</sup>	118.74±4.77 <sup>a</sup>
10 <sup>-9</sup>	114.21±2.63 <sup>a</sup>	107.07±3.13	115.33±5.13 <sup>a</sup>
10 <sup>-10</sup>	125.68±4.55 <sup>a</sup>	104.13±2.41	111.05±5.83
10 <sup>-11</sup>	109.95±4.14	103.17±3.06	95.63±3.07
10 <sup>-12</sup>			

<sup>a</sup>P<0.05 vs. control. Data are expressed as the mean ± standard deviation; n=3. β-FGF, β fibroblast growth factor; <sup>99</sup>Tc-MDP, technetium methylenediphosphonate.

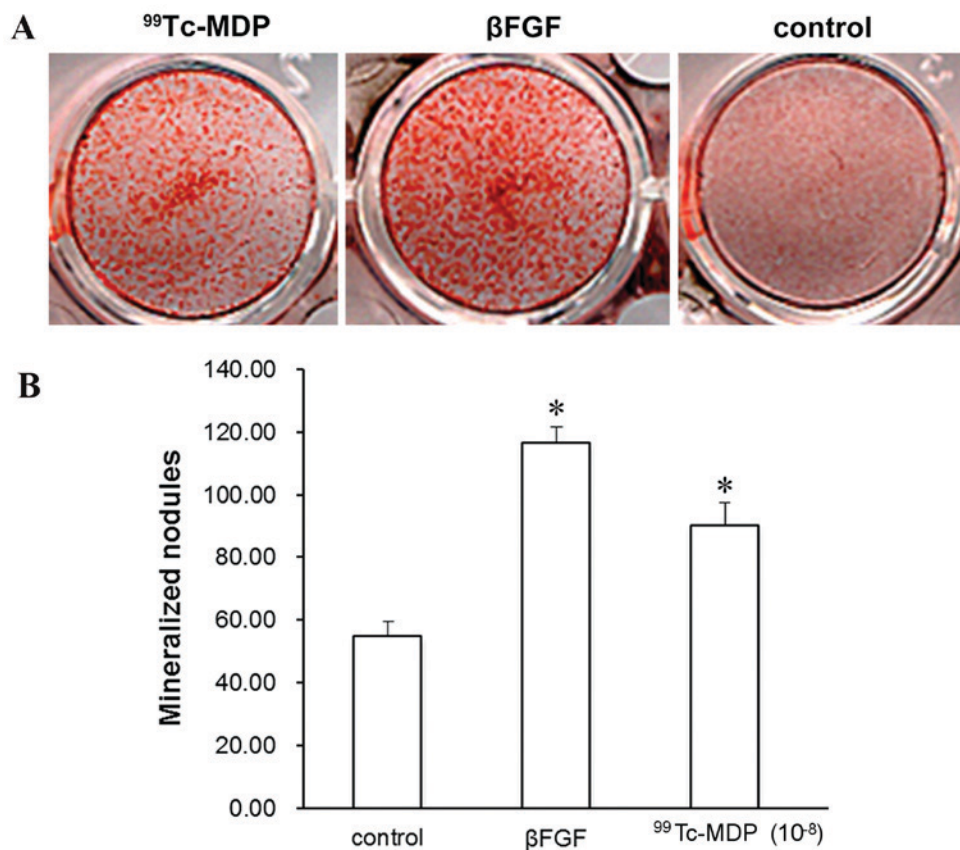


Figure 5. Influence of <sup>99</sup>Tc-MDP (10<sup>-8</sup> M) on mineralized nodule formation. (A) Representative images of mineralized nodules, stained with Alizarin Red 14 days following treatment. (B). Data are presented as the mean ± standard deviation of three independent experiments. \*P<0.05 vs. control. <sup>99</sup>Tc-MDP, technetium methylenediphosphonate; βFGF, β fibroblast growth factor.

been established as a highly sensitive method of measuring osteoblast differentiation (20,21). Treatment with 10<sup>-8</sup> M <sup>99</sup>Tc-MDP significantly increased SPF and proliferation index, further indicating that <sup>99</sup>Tc-MDP promoted osteoblast proliferation. However, <sup>99</sup>Tc-MDP did not significantly affect the rate

of apoptosis, indicating that <sup>99</sup>Tc-MDP-induced cell proliferation was not achieved by inhibiting osteoblast apoptosis.

BMP-2, the most active member of the transforming growth factor-β family, induces ectopic osteogenesis and is the only cytokine able to induce bone formation independently (22).

Table VI. Determination of OPG/RANKL protein expression using ELISA.

		Percent of controls (%)		
		OPG	RANKL	OPG/RANKL
Control		100.00±3.11	100.00±4.33	100.00±3.23
$\beta$ -FGF (ng/ml)	10	110.66±1.21 <sup>a</sup>	116.71±5.84 <sup>a</sup>	94.91±4.68
$^{99}\text{Tc}$ -MDP (M)	$10^{-8}$	110.15±5.32 <sup>a</sup>	97.12±3.08	113.41±3.24 <sup>a</sup>

<sup>a</sup>P<0.05 vs. control. Data are expressed as the mean ± standard deviation; n=3. OPG, osteoprotegerin; RANKL, receptor activator of NF- $\kappa$ B ligand;  $\beta$ -FGF,  $\beta$  fibroblast growth factor;  $^{99}\text{Tc}$ -MDP, technetium methylenediphosphonate.

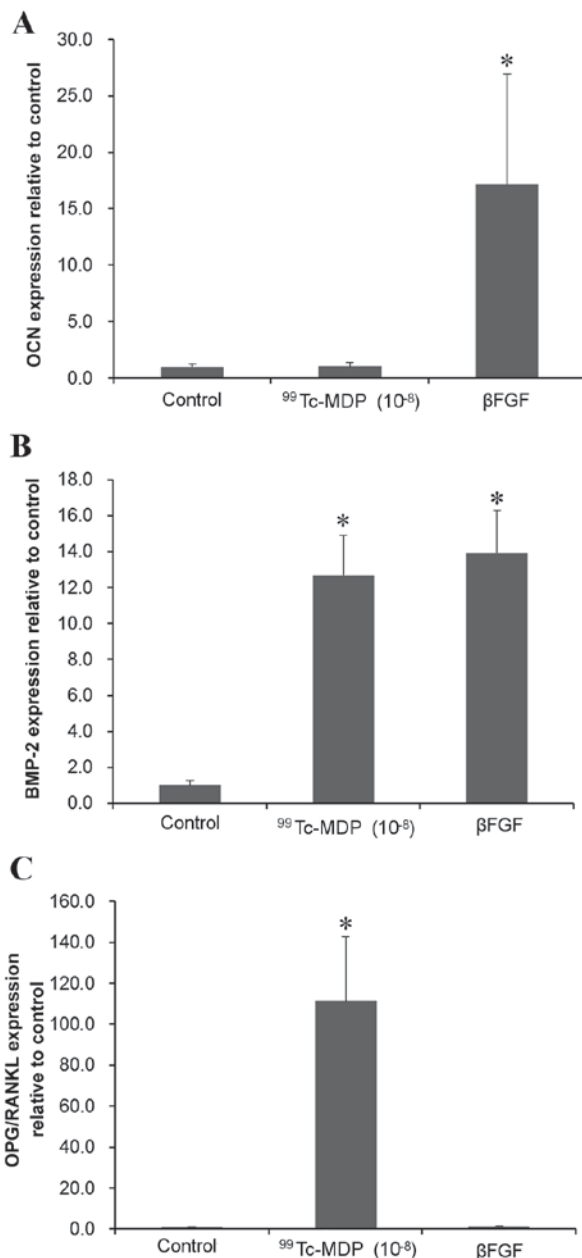


Figure 6. mRNA expression levels of (A) OCN, (B) BMP-2 and (C) OPG/RANKL in osteoblasts treated with  $10^{-8}$  M  $^{99}\text{Tc}$ -MDP, measured by reverse transcription-quantitative polymerase chain reaction following 72 h incubation. Data are expressed as the mean + standard deviation; n=3; \*P<0.05 vs. control. OCN, osteocalcin; BMP-2, bone morphogenetic protein-2; OPG/RANKL, osteoprotegerin/receptor activator of NF- $\kappa$ B ligand;  $^{99}\text{Tc}$ -MDP, technetium methylenediphosphonate;  $\beta$ FGF,  $\beta$  fibroblast growth factor.

BMP-2 also induces osteoblast differentiation, and has been reported to induce the differentiation of pluripotent cells isolated from mouse bone marrow into various mesoderm-derived cells, including muscle, fat and cartilage cells, while inhibiting the formation of fat and muscle cells (23). BMP-2 was also reported to induce differentiation of the MC3T3-E1 cell line into osteoblasts, stimulate OCN expression and enhance ALP activity (24). The present study demonstrated that  $10^{-8}$  M  $^{99}\text{Tc}$ -MDP significantly induced BMP-2 mRNA expression, enhanced ALP activity and induced formation of mineralized nodules, indicating that  $^{99}\text{Tc}$ -MDP induced osteoblast differentiation.

OCN is another marker of osteoblast maturation, and reflects osteogenic function (19). OCN mRNA expression levels were measured by RT-qPCR, and treatment with  $10^{-8}$  M  $^{99}\text{Tc}$ -MDP did not significantly affect OCN mRNA expression levels within 72 h. Potentially, OCN expression would reach a detectable level only later in the culture period, for example the period of bone matrix mineralization.

Decreased OPG/RANKL expression has been reported to be associated with bone destruction and loss of bone mass in several rheumatic diseases, including RA, AS and osteoporosis (25). In many patients with RA, inflammatory factors regulate the expression of OPG and RANKL and further influence bone metabolism, and the expression of OPG/RANKL can be used to predict the level of bone destruction in RA patients (25). Kim *et al* (26) demonstrated that in 75% of patients with AS, bone density declined with disease activity (26). sRANKL serum levels and the ratio of sRANKL to OPG were also increased in patients with RA and these changes were associated with bone density and radiological changes, indicating that the imbalance between RANKL and OPG might be associated with the development of AS and osteoporosis. The involvement of the OPG/RANKL signaling pathway in osteoporosis has been studied intensively, and OPG/RANKL are considered a target for osteoporosis treatment (27).  $^{99}\text{Tc}$  has also previously been reported to conjugate with methylene diphosphonate, and inhibit receptor activator of NF- $\kappa$ B ligand-induced osteoclastogenesis (28). In the present study, OPG mRNA expression levels were significantly increased in osteoblasts treated with  $^{99}\text{Tc}$ -MDP, while the protein expression levels were not significantly elevated. The level of RANKL mRNA was not significantly decreased, but the ratio of OPG mRNA to / RANKL mRNA was significantly elevated. These findings



indicate that  $^{99}\text{Tc}$ -MDP induced osteoblast expression of OPG and increased the ratio of OPG to RANKL, thereby inhibiting differentiation and activation of osteoclasts and osteoclast-mediated bone destruction.

In conclusion,  $^{99}\text{Tc}$ -MDP induced osteoblast proliferation and differentiation, enhanced osteoblast growth and matrix mineralization, specifically stimulated expression of BMP-2 and ALP and thus bone formation, and enhanced the osteogenic function of osteoblasts. Meanwhile,  $^{99}\text{Tc}$ -MDP induced osteoblasts to express OPG and increased the ratio between OPG/RANKL, thereby inhibiting the differentiation and activation of osteoclasts and osteoclast-mediated bone destruction. Overall, the findings of the present study suggested that  $^{99}\text{Tc}$ -MDP may be further assessed as a therapeutic agent for the treatment of bone-destructive diseases.

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