European Review for Medical and Pharmacological Sciences 2015; 19: 3056-3062

Expression of recombinant BMP-7 gene increased ossification activity in the rabbit bone mesenchymal stem cells

Z. XUE, L.-Y. NIU, G. AN, Y.-S. GUO, S.-C. LV, X.-P. REN

The Second Hospital of Harbin Medical University, Harbin, China

Abstract. – OBJECTIVE: The mesenchymal stem cells (MSCs), which were distributed in the bone marrow stroma, become ideal progenitor cells in bone tissue engineering because of their convenient isolation, small injury when obtained, and strong osteogenic capacity. The osteogenic differentiation of MSCs, which is indicated by the increased alkaline phosphatase (ALP) activity and the enhanced accumulation of collagen, could be induced by a strong osteogenic capacity biological factor termed bone morphogenetic protein-7 (BMP-7). Although the chemically synthesized BMP-7 was widely applied to study the osteogenic differentiation of MSCs, transferring and expressing BMP-7 gene in target cells is more desirable, especially for gene therapy, given the advantages and convenience on the stable expression of BMP-7. The aim of this study was to determine whether recombinant BMP-7-expressing MSCs would induce bone formation *in vitro*.

MATERIALS AND METHODS: BMP-7 gene was cloned from human placental tissue to construct a recombinant eukaryotic expression plasmid carrying BMP-7 gene by conjugating with eukaryotic expression vector pcDNA3.1. MSCs were isolated from rabbit bone marrow and cultured *in vitro*. Then they were divided into 3 groups: pcDNA3.1-BMP-7-transfected, pcDNA3.1-transfected, and untransfected. Human healthy fresh placental tissue was provided by the Department of Gynaecology and Obstetrics, Second Affiliated Hospital of Harbin Medical University. Written informed consent was obtained from the women. One healthy male New Zealand rabbit was provided by the Laboratory Animal Center, Harbin Medical University.

RESULTS: A significant increase of ALP activity was detected in the supernatant of pcDNA3.1-BMP-7 transfected MSCs, and the enhanced collagen accumulation, which was inferred by the increased hydroxyproline content and RT-PCR.

CONCLUSIONS: These results implied that BMP-7 gene was expressed in MSCs sufficiently and was involved in inducing differentiation of MSCs into osteoblast.

Key Words:

Bone morphogenetic protein-7, Mesenchymal stem cells, Gene transfection, Alkaline phosphatase activity, Collagen.

Introduction

The bone marrow mesenchymal stem cells (MSCs) are widely used as progenitor cells in bone tissue engineering studies[1]. The MSCs were distributed in the bone marrow stroma, and were progenitors of different cell lineages such as cartilage, bone, and adipocytes[2]. The osteogenic potential of MSCs is well known as evidenced by bone formation following transplantation of MSCs *in vivo*. Because of the feasibility in cell isolation and cultivation[3-6], the multipotent MSCs have been widely investigated as a therapeutic agent in tissue engineering and gene therapy[7,8]. *In vitro* experiment has also shown that, under the stimulation of ascorbic acid, osteogenesis supplement, and beta-glycerophosphate, the cultivated MSCs were able to differentiate into osteoblast lineage cells[9], which was indicated by an increased alkaline phosphatase (ALP) activity and accumulated collagen[10].

Bone morphogenetic protein-7 (BMP-7) has been shown to possess strong osteoinductive properties[11-15]. BMP-7 has been demonstrated to trigger the development of osteoblasts from MSCs[16-19]. BMP-7 gene therapy plays an important role in modulating bone regeneration[20-21]. However, more efficient and safe delivery vectors must be obtained before clinical trials can be carried out successfully. Previous work of recombinant pcDNA3.1 vector for gene therapy has shown some outstanding advantages and has made it an attractive candidate for clinical trials in recent years[22-24].

Corresponding Author: Songcen LV, MD; e-mail: lvsongcen@yahoo.com

In order to test the effect of BMP-7 gene transfection and the feasibility in induction of the MSCs, the DNA fragment encoding BMP-7 was recombined into the eukaryotic expression vector (pcDNA3.1 plasmid), which was subsequently transfected into the MSCs of New Zealand rabbit. The effect of the BMP-7 transfection on the MSCs was evaluated by analyzing the ALP activity and collagen accumulation. This study provides fundamental understanding and a platform for future applications of pcDNA3.1-BMP-7 local gene therapy.

Materials and Methods

Isolation and Culture of Rabbit MSCs

Following anesthesia by injection of 2% sodium pentobarbital (35 mg/kg) into ear vein and skin preparation and povidone iodine sterilization, 2 mL bone marrow was taken from medullary cavity and anti-coagulated using 200 units of heparin. Subsequently, bone marrow was mixed with lymphocyte separating medium, centrifuged at 2500 r/min for 5 minutes first, and then at 800 r/min for 5 minutes. All cells were cultured with Dulbecco's modified Eagle's medium containing 12% fetal bovine serum at 37°C in 5% CO^2-enriched environment. The isolated cells were plated at a density of $2\times10^5/2.5$ cm^2 flask and the medium was renewed every 3 days.

Construction of pcDNA3.1- BMP-7 Plasmid

Cloning of human BMP-7 gene: the BMP-7 cDNA was cloned by reverse transcription-polymerase chain reaction from fresh placental tissue cells of healthy human using the following set of primers:

Forward: 5'-gtggtaccgatgcacgtgcgctcactg-3';
Reverse: 5'-agaagatctctcggaggagctagtggcag-3'.

(Introduced Kpn I and BgI II restriction sites were underlined).

PCR was performed by 33 cycles of predenaturation (94, 4 minutes), denaturation (94°C, 45 seconds), annealing (62°C, 1 minute), and extension (72°C, 1.5 minutes), followed by a final extension of 10 minutes at 72°C. The PCR products were separated by 1% agarose gel electrophoresis. Construction of recombinant pcDNA3.1-BMP-7 eukaryotic expression vector and identification: the recombinant plasmid pcDNA3.1-BMP-7 was constructed by inserting full-length human BMP-7 cDNA (1.3 kb) into the restriction sites between Kpn I and Bam I of the multiple cloning site. The recombinant eukaryotic expression plasmid pcDNA3.1-BMP-7 was constructed using the fragments of pcDNA3.1 and BMP-7 treated by the enzymes (Kpn, BgI, Bam, and T4 DNA, Shang Hai Sangon Biotech, Shanghai, China).

Liposome Mediated pcDNA3.1-BMP-7 Transfection into Rabbit MSCs

When the third passage of MSCs reached 80% confluence, pcDNA3.1-BMP-7 plasmid, blank vector was respectively transfected into the MSCs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). G418 (300 g/mL) was added in the culture medium for the cells for screening at 24 hours after the transfection. The untransfected cells were cleaned out by the G418, and MSCs successfully transfected by plasmid survived in the G418 screening. Two weeks later, G418-resistant cell clones were collected and then cultivated with normal medium. Three groups were set: cells transfected with pcDNA3.1-BMP-7, cells transfected with pcDNA3.1, and untransfected cells.

Detecting BMP-7 Expression Using RT-PCR

To identify BMP-7 mRNA expression of cells, total RNA was extracted 72 hours after transfection using TRIzol Reagent (Promega, Madison, WI, USA). RT-PCR was performed as above 5 L of reverse transcription products and a forementioned specific primer were taken for PCR amplification of BMP-7 gene.

Detection of Alkaline Phosphatase (ALP) activity

Cells were inoculated into a 24-well plate at a density of 2×10^4/well. Four parallel wells were set for each group. Supernatant was taken at 0, 2, 4, 6, 8, and 10 days to detect ALP activity using ALP assay kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China).

Detection of Hydroxyproline Content by Chloramine-T Method

Two weeks later, cells were inoculated into a 24-well plate at a density of 2×10^4/well. Four parallel cells were set in each group. Hydroxyproline content was detected using hydroxyproline assay kit (Wuhan Boshide Bioengineering Institute, Hubei, China). The formula to calculatehydrox-

yproline content was: Hydroxyproline (mg/L) = $Absorbance_{detected\ tubes}$/ $Absorbance_{standard\ tubes}$ × concentration of standard tubes × diluted times.

RT-PCR of the Type I Collagen and β-actin mRNA

Two weeks after the adding of the mineralization solution in the medium, Total RNA of the different groups of MSCs was extracted as previously described. After the first strand synthesis, the expression of type collagen and -actin was detected using RT-PCR. The photodensity ratio of type collagen/ -actin mRNA was used in the analysis, and 3 replicates were conducted.

Detection of Osteocalcin Production

Two weeks later, cells were inoculated into a 24-well plate at a density of 2×10^4/well. Four parallel cells were set in each group. Supernatant was taken for detection of osteocalcin by radioimmunoassay.

Statistical Analysis

The statistical analyses were processed using SPSS 10.0 software (SPSS Inc, Chicago, IL, USA). ALP activity, the hydroxyproline content, and photodensity ratio of different groups were compared using *t* test. The level of significance was set at $p < 0.05$.

Results

Successful Construction of pcDNA3.1-BMP-7 Plasmid

Gel electrophoresis showed the fragment of BMP-7 was 1.3 kb, plasmid pcDNA3.1-BMP-7 was 6.6 kb, the fragments treated by restriction endonuclease Kpn I and Bam I were 5.3 kb and1.3 kb, respectively, which met physical map, demonstrating that BMP-7 cDNA sequence had been correctly inserted into pcDNA3.1 (Figure 1).

Cell Changed to Polygonal Shape After pcDNA3.1-BMP-7 Transfection

Initially the rabbit MSCs were round-shaped. After inoculation of 48 hours, MSCs became long spindle shape gradually (Figure 2). The G418 screening was performed after 24 h of transfection, and nearly 80% of the cultivated MSCs were killed in the next week. Two weeks after the G418 screening of the transfected MSCs, the pcDNA3.1-BMP-7 plasmid transfected MSCs formed colonies, and these transfected G418-resistant MSCs had polygonal shape, which was similar to the osteoblasts (Figure 3). In contrast, the MSCs transfected by blank vectors and the untransfected MSCs were still in long spindle shape.

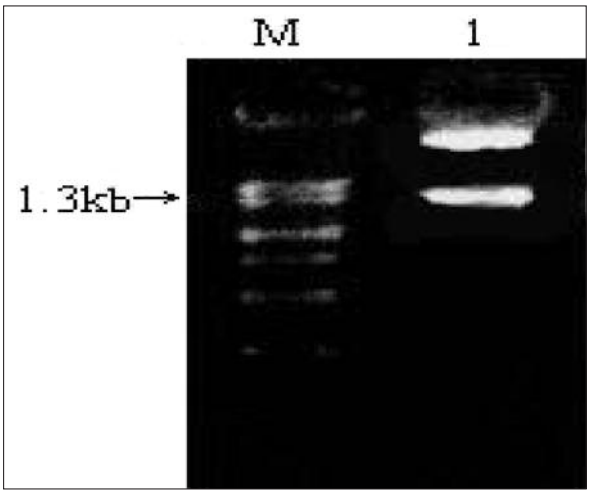

Figure 1. Electrophoresis of the reconstructed pcDNA3.1-BMP-7 plasmid. Lane 1: pcDNA3.1-hBMP-7; Lane M: DNA marker. The length of the marker was also shown.

BMP-7 Expressed in the pcDNA3.1-BMP-7 Transfected MSCs

After 72-hours transfection, RT-PCR clearly demonstrated the mRNA expression of BMP-7 in pcDNA3.1-BMP-7-transfected cells. A 1.3 kb fragment was seen in pcDNA3.1-BMP-7-transfected cells, but not seen in pcDNA3.1-transfected cells and untransfected cells (Figure 4).

Figure 2. The Long, spindle-shaped MSCs BMP-7 -transfection (× 100).

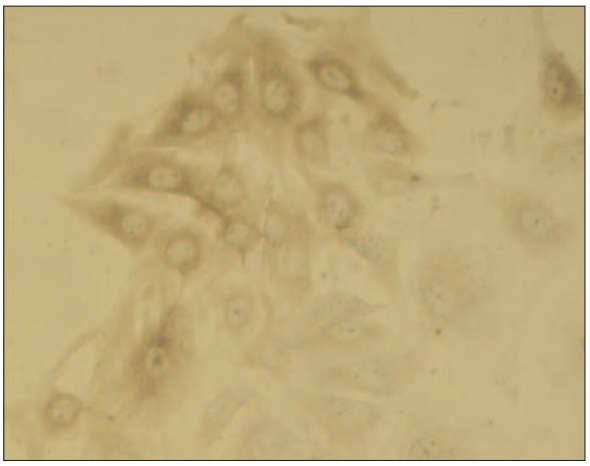

Figure 3. The polygonal-shaped MSCs before after BMP-7 -transfection (× 200).

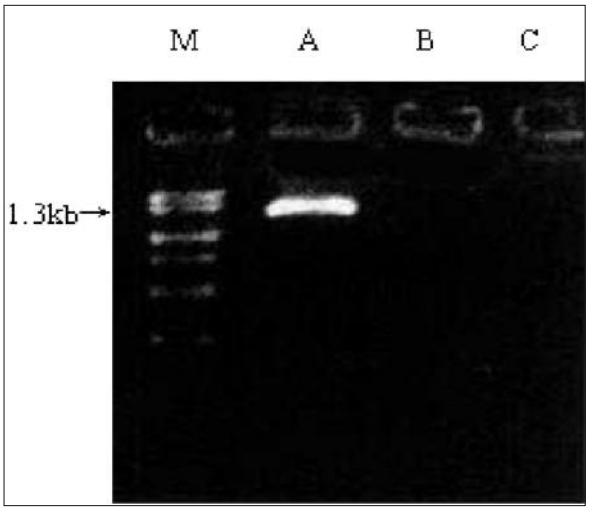

Figure 4. RT-PCR analysis of BMP-7 gene expression Lane M: DNA marker; Lane A: pcDNA3.1-BMP-7-transfected cells; Lane B: pcDNA3.1-transfected cells; Lane C: untransfected cells.

Increased ALP Activity in Rabbit MSCs Transfected by pcDNA3.1-BMP-7

ALP activity in the pcDNA3.1-BMP-7-transfected group significantly increased at 2 days after transfection, peaked at 8 days, and still increased at 10 days. ALP activity in the pcDNA3.1-transfected, and untransfected groups was basically unchanged (Figure 5). At each time point, ALP activity was significantly higher in the pcDNA3.1-BMP-7-treated group than in the pcDNA3.1-transfected, and untransfected groups ($p < 0.05$).

Increased Collagen Accumulation for pcDNA3.1-BMP-7 Transfected MSCs

In order to study the type I collagen accumulation of the cultured MSCs, mineralization solution was added to the culture medium and cultivated the MSCs for two weeks. Then the accumulation of the type I collagen was examined by estimating the hydroxyproline content in the culture medium. The hydroxyproline content was significantly higher in the pcDNA3.1-BMP-7

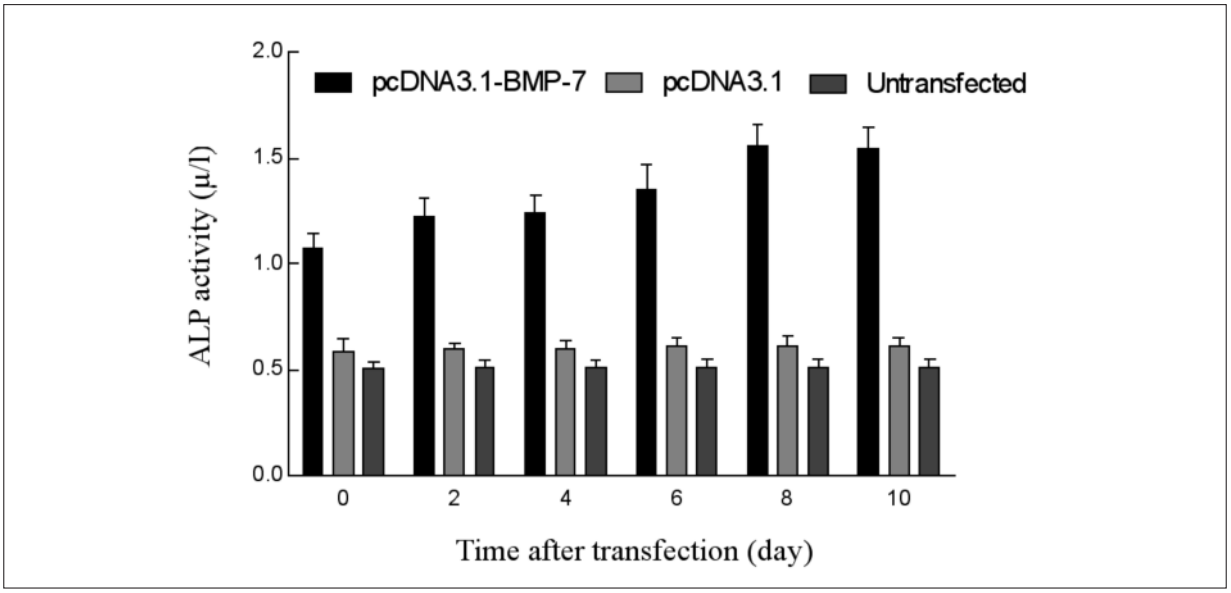

Figure 5. Effect of BMP-7 gene transfection on ALP activity in bone marrow mesenchymal stem cells.

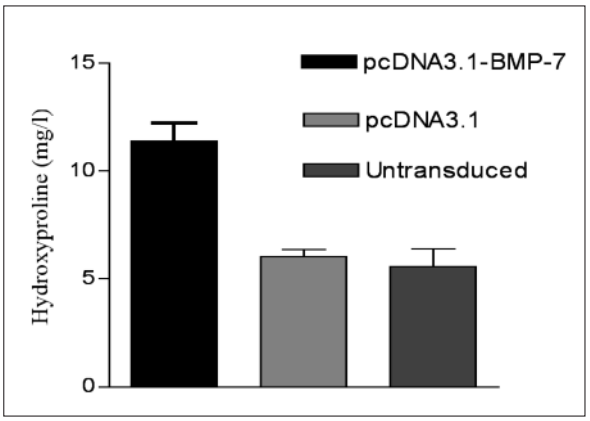

Figure 6. Hydroxyproline production in pcDNA3.1-hBMP-7-transfected, pcDNA3.1-transfected, and untransfected cells.

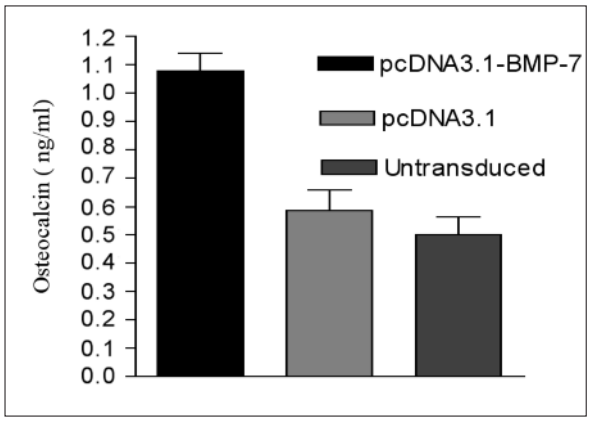

Figure 7. Osteocalcin production in pcDNA3.1- BMP-7-transfected, pcDNA3.1-transfected, and untransfected cells.

transfected group than that in the pcDNA3.1-transfected and the untransfected MSCs ($p < 0.01$, Figure 6). RT-PCR on the type I collagen mRNA also demonstrated that the expression of type I collagen was significantly higher in the pcDNA3.1-BMP-7 transfected MSCs than that in the pcDNA3.1-transfected and the untransfected MSCs ($p < 0.05$, Table I).

Increased Osteocalcin Production in Rabbit MSCs Transfected by pcDNA3.1-BMP-7

Two weeks later, osteocalcin production was significantly greater in the pcDNA3.1-BMP-7-transfected group than in the pcDNA3.1-transfected, and untransfected cells ($p < 0.05$) (Figure 7).

Discussion

The multipotent MSCs are appropriate seed cells in tissue engineering because they are easy to isolate and cultivate. However, enhancing the differentiation of MSCs into osteoblasts stably and massively became a key issue in bone tissue engineering and related gene therapy. Gene transfer technology is to make exogenous gene into cells and induces its expression in the cell by the development of molecular biology[25-27].

In this study, rabbit MSCs were transfected with pcDNA3.1-BMP-7 eukaryotic expression vector *in vitro*. Seventy-two hours after the transfection, BMP-7 gene transcription was detected using RT-PCR. The BMP-7 gene was induced into MSCs through gene transfection to maintain the osteogenic capacity and the continued expression of local BMP-7 protein[28-30]. This may be an ideal method in treatment of bone regeneration and bone defects[31-32]. Before the gene transfection procedure, it is necessary to determine whether the BMP-7 gene could be efficiently transfected into MSCs with eukaryotic vector. Subsequent studies demonstrated that pcDNA3.1-BMP-7-transfected MSCs could express BMP-7 protein in the cytoplasm and secrete it into culture medium. ALP and collagen were regarded as indicator of osteoblastic differentiation[33]. To further investigate the biological activities of the generated BMP-7 protein, we measured the ALP activity and osteocalcin production as well as type I collagen accumulation in MSCs. In the present study, the distinct effects of pcDNA3.1-BMP-7 on induction of the early osteogenic marker ALP and late osteogenic marker type I collagen accumulation with osteocalcin were demonstrated in transfected MSCs, suggesting that these MSCs underwent differentiation into functional osteoblasts. Since osteoblas-

Table I. Photodensity ratio of the type I collagen/β-actin mRNA among different groups (n = 3, mean ± SD).

MSCs group	Photodensity ratio
Untransfected MSCs	.45 ± 0.13
pcDNA3.1-transfected MSCs	1.36 ± 0.12
pcDNA3.1-BMP-7 transfected MSCs	2.78 ± 0.18

The type I collagen/β-actin photodensity ratio was significantly higher in the pcDNA3.1-BMP-7 transfected MSCs than that of pcDNA3.1-transfected MSCs and untransfected MSCs ($p < 0.05$).

tic differentiation was seen exclusively in pcDNA3.1-BMP-7- transfected cells and no BMP-7 protein was observed in the untransfected or pcDNA3.1- transfected cells, we regarded the osteogenic stimulus (BMP-7) as the major determinant driving MSCs differentiation into the osteogenic lineage.

Conclusions

In the present study, harvested MSCs were cultured and transfected with the BMP-7 gene via eukaryotic vector *in vitro* and eukaryotic expression vector of recombinant pcDNA3.1-BMP-7 was constructed successfully. These results indicated that BMP-7 was expressed in MSCs sufficiently and was involved in inducing differentiation of MSCs into osteoblast. The method would provide substantial basement for BMP-7 gene therapy. Further studies on the pathways regulatedby BMP-7, transfecting BMP-7 into MSCs *in vivo*, and controlling BMP-7 expression was needed to facilitate the practical and clinical application of BMP-7 as gene therapy treatment.

Acknowledgements

The authors were grateful to colleagues in the Laboratory Animal Center and the Biochemical Department of Harbin Medical University for their generous help. The study was supported by grant (No.12511202) from the Science and Technology Research Foundation of the Education Department of Heilongjiang Province of China.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

1) Bruder SP, Jaiswal N, Ricalton NS, Mosca JD, Kraus KH, Kadiyala S. Mesenchymal stem cells in osteobiology and applied bone regeneration. Clin Orthop Relat Res 1998; 355 Suppl: 247-256.
2) Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284: 143-147.
3) Gronthos S, Zannettino AC, Hay SJ, Shi S, Graves SE, Kortesidis A, Simmons PJ. Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. J Cell Sci 2003; 116: 1827-1835.
4) Tondreau T, Lagneaux L, Dejeneffe M, Massy M, Mortier C, Delforge A, Bron D. Bone marrow-derived mesenchymal stem cells already express specific neural proteins before any differentiation. Differentiation 2004; 72: 319-326.
5) Reyes M, Verfaillie CM. Characterization of multipotent adult progenitor cells, a subpopulation of mesenchymal stem cells. Ann NY Acad Sci 2001; 938: 231-233; discussion 233-235.
6) Sekiya I, Larson BL, Smith JR, Pochampally R, Cui JG, Prockop DJ. Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. Stem Cells 2002; 20: 530-541.
7) Krebsbach PH, Kuznetsov SA, Bianco P, Robey PG. Bone marrow stromal cells: characterization and clinical application. Crit Rev Oral Biol Med 1999; 10: 165-181.
8) Ballas CB, Zielske SP, Gerson SL. Adult bone marrow stem cells for cell and gene therapies: implications for greater use. J Cell Biochem Suppl 2002; 38: 20-28.
9) Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J Cell Biochem 1997; 64: 295-312.
10) Anjos-Afonso F, Bonnet D. Flexible and dynamic organization of bone marrow stromal compartment. Br J Haematol 2007; 139: 373-384.
11) Dorai H, Vukicevic S, Sampath TK. Bone morphogenetic protein-7 (osteogenic protein-1) inhibits smooth muscle cell proliferation and stimulates the expression of markers that are characteristic of SMC phenotype in vitro. J Cell Physiol 2000; 184: 37-45.
12) Chen D, Zhao M, Mundy GR. Bone morphogenetic proteins. Growth Factors 2004; 22: 233-241.
13) Star GP, Giovinazzo M, Langleben D. Effects of bone morphogenic proteins and transforming growth factor-beta on in-vitro production of endothelin-1 by human pulmonary microvascular endothelial cells. Vascul Pharmacol 2009; 50: 45-50.
14) Lee SY, Nakagawa T, Reddi AH. Induction of chondrogenesis and expression of superficial zone protein (SZP)/lubricin by mesenchymal progenitors in the infrapatellar fat pad of the knee joint treated with TGF-beta1 and BMP-7. Biochem Biophys Res Commun 2008; 376: 148-153.
15) Buijs JT, Henriquez NV, van Overveld PG, van der Horst G, ten Dijke P, van der Pluijm G. TGF-beta and BMP7 interactions in tumour progression and bone metastasis. Clin Exp Metastasis 2007; 24: 609-617.
16) Li T, Surendran K, Zawaideh MA, Mathew S, Hruska KA. Bone morphogeneticprotein 7: a novel treatment for chronic renal and bone disease. Curr Opin Nephrol Hypertens 2004; 13: 417-422.
17) Miyamoto C, Matsumoto T, Sakimura K, Shindo H. Osteogenic protein-1 with transforming growth

factor-beta1: potent inducer of chondrogenesis of synovial mesenchymal stem cells *in vitro*. J Orthop Sci 2007; 12: 555-561.

18) Chubinskaya S, Hurtig M, Rueger DC. OP-1/BMP-7 in cartilage repair. Int Orthop 2007; 31: 773-781.

19) Takigami H, Kumagai K, Latson L, Togawa D, Bauer T, Powell K, Butler RS, Muschler GF. Bone formation following OP-1 implantation is improved by addition of autogenous bone marrow cells in a canine femur defect model. J Orthop Res 2007; 25: 1333-1342.

20) Long H, Yuan H, Ma B. Construction of recombinant adenovirus bearing human transforming growth factor beta1 and bone morphogenetic protein 7 genes and its directional inducement effect on marrow stromal cells. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi 2006; 20: 1224-1228.

21) Zhang Y, Song J, Shi B, Wang Y, Chen X, Huang C, Yang X, Xu D, Cheng X, Chen X. Combination of scaffold and adenovirus vectors expressing bone morphogenetic protein-7 for alveolar bone regeneration at dental implant defects. Biomaterials 2007; 28: 4635-4642.

22) Dang HS, Li W, Shen B, Yang J, Zhou ZK, Pei FX, Lu F, Peng WZ. Construction and expression of recombinant plasmid pcDNA3.1(+)-hHGF available in osteoblast. Si chuan Da Xue Xue Bao Yi Xue Ban 2007; 38: 929-933.

23) Ma HB, Wang XJ, Ma J, Xia H, Wang Z, Li Z, Han ZK, Wu CM. Effect of irradiation-induced pcDNA3.1-Egr.1p-p16 on cell apoptosis and cell cycle of JF305 cells. Nan Fang Yi Ke Da Xue Xue Bao 2007; 27: 1183-1186.

24) Ma HB, Wang XJ, Di ZL, Xia H, Li Z, Liu J, Ma J, Kang HF, Wu CM, Bai MH. Construction of targeted plasmid vector pcDNA3.1-Egr.1p-p16 and its expression in pancreatic cancer JF305 cells induced by radiation *in vitro*. World J Gastroenterol 2007; 13: 4214-4218.

25) Franceschi RT, Yang S, Rutherford RB, Krebsbach PH, Zhao M, Wang D. Gene therapy approaches for bone regeneration. Cells Tissues Organs 2004; 176: 95-108.

26) Xiao R, Song Y. Gene therapy on spine fusion. Sheng Wu Yi Xue Gong Cheng Xue Za Zhi 2002; 19: 703-707.

27) Vinge LE, Raake PW, Koch WJ. Gene therapy in heart failure. Circ Res 2008; 102: 1458-1470.

28) Lavery K, Swain P, Falb D, Alaoui-Ismaili MH. BMP-2/4 and BMP-6/7 differentially utilize cell surface receptors to induce osteoblastic differentiation of human bone marrow-derived mesenchymal stem cells. J Biol Chem 2008; 283: 20948-20958.

29) Majewski M, Betz O, Ochsner PE, Liu F, Porter RM, Evans CH. Ex vivo adenoviral transfer of bone morphogenetic protein 12 (BMP-12) cDNA improves Achilles tendon healing in a rat model. Gene Ther 2008; 15: 1139-1146.

30) Seol YJ, Kim KH, Park YJ, Lee YM, Ku Y, Rhyu IC, Lee SJ, Han SB, Chung CP. Osteogenic effects of BMP-2 plasmid gene transfer. Biotechnol Appl Biochem 2008; 49: 85-96.

31) Park J, Lutz R, Felszeghy E, Wiltfang J, Nkenke E, Neukam FW, Schlegel KA. The effect on bone regeneration of a liposomal vector to deliver BMP-2 gene to bone grafts in peri-implant bone defects. Biomaterials 2007; 28: 2772-2782.

32) Hsu WK, Sugiyama O, Park SH, Conduah A, Feeley BT, Liu NQ, Krenek L, Virk MS, An DS, Chen IS, Lieberman JR. Lentiviral-mediated BMP-2 gene transfer enhances healing of segmental femoral defects in rats. Bone 2007; 40: 931-938.

33) Sakano S, Murata Y, Miura T, Iwata H, Sato K, Matsui N, Seo H. Collagen and alkaline phosphatase gene expression during bone morphogenetic protein (BMP)-induced cartilage and bone differentiation. Clin Orthop Relat Res 1993; 292: 337-344.