Osteogenic differentiation of human mesenchymal stem cells by type I BMP receptor, ALK-2

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ABSTRACT

Osteogenic differentiation of human mesenchymal stem cells by type I BMP receptor, ALK-2

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Human mesenchymal stem cells (hMSCs) render a potential interest to investigators in the fields of tissue engineering, gene therapy and cellular transplantation because hMSCs can differentiate to multifunctional mesenchyme-origin cells. Specially, with respect bone regeneration, many scientists have researched the osteogenic functions of bone morphogenetic proteins (BMPs) with hMSCs. The overexpression of bone morphogenetic protein-2 (BMP-2) gene can stimulate osteogenic differentiation of hMSCs and also induce bone formation in animal model have been reported. As well as, osteogenesis can be induced by the overexpression of type I BMP receptor gene in chondrocytes and myoblasts as similar as BMP-2 gene has been reported. In the present study, we investigated the osteogenic differentiation of hMSCs by the overexpression of activin receptor-like kinase-2 (ALK-2) gene as one of type I BMP receptors by in vitro experiments and verified bone formation in immunodeficiency mice by the implantation of genetic modified hMSCs.

hMSCs were isolated from bone marrow of patients with spinal stenosis. Adenoviral vector containing with ALK-2 gene (Ad/ALK-2) was produced from 293A cell. After hMSCs were transduced with Ad/ALK-2, osteogenic staining and RT-PCR to osteogenic marker genes were performed to observe osteogenic differentiation of hMSCs. To verify the overexpression of ALK-2 gene in hMSCs trigger osteogenic cell signaling, westem blot to Smad1/5/8 and MAPK was performed. For 3-dimensional (3D) culture of Ad/ALK-2-transduced hMSCs, cells were seeded to bovine demineralized bone matrix (bDBM) as scaffold and the cell morphology was observed by scanning electron microscopy (SEM). Also, for analyzing newly synthesized surface material of ALK-2-overexpressed hMSCs, SEM-energy-dispersive X-ray spectroscopy (SEM-EDX) was performed. Finally, Ad/ALK-2-transduced hMSCs-adhered bDBMs were implanted to immunodeficiency mice. At 2 and 4 weeks, each group was sacrificed and the implants were harvested. For histological analysis, hematoxylin and eosin (H&E) staining and immunofluorescent (IF) staining were performed.

We observed Ad/ALK-2-transduced hMSCs were stained by osteogenic staining and mRNA expression of osteocalcin in hMSCs was increased with depending on virus titer of Ad/ALK-2. Also, mRNA expression of osteogenic transcription factors such as *runx2*, *osterix*, *dlx5* was activated by ALK-2. Western blot analysis of osteogenic cell signaling protein showed that the overexpression of ALK-2 activated Smad1/5/8 signaling pathway and increased phosphorylation of p38. On 3D culture, a deposit of calcium phosphate at surface on these cells was observed. On the in vivo experiment, the overexpression of ALK-2 could not stimulated enough to bone regeneration compared to the overexpression of BMP-2 as positive control at 2 weeks, but the ALK-2-overexpressed groups stimulated osteogenesis at 4 weeks were observed by histological staining. And ALK-2 protein in Ad/ALK-2-transduced hMSCs-adhered implants was overexpressed for 4 weeks was verified by immunofluorescent (IF) staining.

Overexpression of type I BMP receptor, ALK-2 induced osteogenic phenotype in hMSC was verified by in vitro and in vivo studies. Therefore, ALK-2 is a potential therapeutic candidate for osteogenic gene therapy was confirmed by this study.

Key words : human mesenchymal stem cells, type I BMP receptor, activin receptor-like kinase-2, gene therapy

Bone formation of human mesenchymal stem cell induced by adenovirus-based gene transfer of ALK-2

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Ⅰ. INTRODUCTION

Bone grafting is commonly used in orthopedic reconstruction surgeries such as spinal fusion, revision of total joint arthroplasty, or repair of skeletal defects following trauma or the removal of tumor [1]. In the past, many experimental and clinical studies have shown that fresh autogenous bone grafts are vastly superior to allogenous bone graft in skeletal repair and remodeling. However, due to the size limitation of autogenous bone grafts, problems with chronic pain at the donor site, and also complications of the procedures, processed allograft remains an attractive substitute for bone grafting [1-3]. Extensive research has shown that the critical difference between autograft and allograft bone healing is the role of the grafted cells [2]. These previous studies demonstrated that there are no significant differences between the healing of an allograft and an isolated and processed graft from a genetically identical animal, using a murine model of femoral graft healing [4,5].

The repair and incorporation of bone graft is regulated process that is very similar to fracture-healing. The initial phase is characterized by inflammation and vascular invasion from the host bed, which facilitates recruitment of MSC that will differentiate into the bone-forming cells. In the case of autograft, both graft and host bones contribute these osteogenic cells. In contrast, since allograft does not contain any live cells, healing relies upon invasion of the graft by host cells and tissues. While the later phases of graft healing are characterized by remodeling, allografts remodel very slowly, and in the case of large structural allografts, remodeling along the allograft is very limited [6-8].

There are two conceivable approaches by which osteoinductive and remodeling properties can be conferred on processed allograft. The first is to engraft MSC that will act as an artificial periosteum to promote bone formation from the graft and subsequent vascular ingrowth and remodeling. While several groups have demonstrated the efficacy of this approach, many issues remain regarding its clinical potential, including the source of the cells, reproducible engraftment of cells onto the graft, and added cost and complexity [6,7,9-15]. The other approach is to introduce the critical biological factor onto the allograft directly. This approach has come to fruition in the form of Food and Drug Administration (FDA)-approved BMP [1,2,16-18]. However, requirement of high dose and short protein half-life of BMP limit this strategy for large structural grafts. So then, the most orthopedic researchers believe gene therapy offers a cost-effective solution to these problems.

BMPs play a particularly important role in skeletal formation as a previous descried [19]. Although BMPs have been shown to have a broad spectrum of action on proliferation, differentiation, and apoptosis in numerous cellular systems, they are the only members of the TGF-β superfamily that have the ability to stimulate ectopic bone formation by recapitulating all the events occurring during endochondral ossification, and to potentiate chondrocyte and

osteoblast differentiation in vitro [19,20]. Because of these reasons, many studies of BMPs and MSC were reported on the orthopeadic parts, especially BMP-2 and also *bmp-2* gene have been used as a candidate gene of gene therapy for bone regeneration [21-28].

BMPs signal cells through two types of serine/threonine kinase receptors, which phosphate and activate the type II and type I receptors upon ligand binding [19,29,30]. This activated signaling pathway finally induces the osteogenesis of MSCs. ALK-2 as the major target of the present study, is one of these BMP type I receptors, originally named activin receptor-like kinase (ALK) have been cloned in mammals [19]. ALK-2 has been shown to bind activin, BMP-2, and BMP-7, and also to mediate Műllerian inhibiting substance signaling. The other receptors of BMP-2 and BMP-7 were reported ALK-3 (termed also BMPR-IA) and its close structural and functional homologue ALK-6 (BMPR-IB). The type I receptors act downstream from type II receptors and are the effectors of the signal transduction. When activated, the type I receptors phosphorylate intracellular mediators, the Smad proteins. Eight Smad proteins have been identified in mammals, and these Smads are classified into three groups according to their function: the receptor-regulated Smad (R-Smad), the common mediator Smad (Co-Smad) and the inhibitory Smads (I-Smad). The R-Smads are phosphorylated by the type I receptors upon ligand binding. Specially, Smads 1, 5, 8 are involved in the osteogenic cell signaling pathway of BMP. The phosphorylated R-Smads interact with the single Co-Smad identified in mammals, Smad4. The heteromeric complex formed then translocates into the nucleus, and is responsible for the transcriptional regulation of target genes such as osteogenic transcriptional factors [20,31-34].

Some researchers have reported that overexpression of active ALK-1, ALK-2, ALK-3, and ALK-6 induces alkaline phosphatase activity in myoblastic C2C12 cells and then ALK-2 induces highly osteogenic differentiation compared to other ALKs [29,35]. In addition, other

research group showed that ALK-2 allows chondrocytes to undergo further differentiation into osteoblastic cells, as attested by *osteocalcin* mRNA expression and by the highest alkaline phosphatase activity observed in mouse chondrocyte cell line, MC615 cells and embryonic chondrocyte producing active ALK-2 [32,36]. They proved that ALK-2 is the only receptor to induce *osteocalcin* gene expression and Smads are not sufficient to promote *osteocalcin* gene expression. It was hypothesized that overexpression of ALK-2 in hMSCs could be similar to osteogenic property in murine cell line at the previous reports and also genetic modification of BMP type I receptor in hMSCs could represent to bone formation in vivo system.

The purposes of the current study are to verify the overexpression of BMP type I receptor, ALK-2 affect to osteogenic differentiation of hMSCs and to prove ALK-2 protein mediates a same or a different MAPK signaling pathway compared to BMP-2 at in vitro experiment. Final goals are to estimate the osteogenic properties of *alk-2* gene-transduced hMSCs-seeded scaffolds induce new bone formation on in vivo murine model by comparing to *bmp-2* gene-transduced hMSC groups.

Ⅱ. MATERIALS AND METHODS

All of the experimental protocols were approved by the human subjects Institutional Review Board of the institution.

1. Cell culture of human mesenchymal stem cells (hMSCs)

A. Primary culture of hMSCs

Bone marrow samples were obtained from iliac crest aspirates from ten patients (average age: 65 years) during surgical procedures of spinal stenosis.

Briefly, 10ml of bone marrow was taken from different donors over heparin (BD lifescience, Franklin Lakes, NJ, USA). Low-density mononuclear cells were separated on Ficoll-Hypaque (Pharmacia Diagnostic, Freiburg, Germany) density gradient. Mononuclear cells were plated at a concentration of 10^6cells/ml of DMEM-LG medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomysin solution (all from Invitrogen, Rockville, MD, USA) in T-25 cm^2 tissue culture flasks (Nalgene Nunc International, Rocklide, Denmark) and incubated at 37℃ in humidified, 5% CO_2 atmosphere. After 1 week, non-adherent cells were removed. hMSCs were expanded to fourth passage and experimented at fifth passage through sixth passage.

B. Three-dimensional (3D) culture of hMSCs

hMSCs and genetic modified hMSCs were trypsinized and resuspended in 2 x 10^5 cells/ 20μl culture medium supplemented with 50μg/ml of ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) and 5mM of β-glycerol phosphate (Sigma-Aldrich, St. Louis, MO, USA) as osteogenic media. The concentrated cell suspensions were seeded onto the prepared scaffolds. The used scaffold was bDBM sponge. Briefly, bDBM sponge was prepared to 0.5 x 0.5 x 0.1 cm^3 size and demineralized used by standard HCl/Triton X-100 treatment. The demineralized bDBM sponges were sterilized by the γ-irradiation at 25kGy. Cells were allowed to adhere to bDBM for 1hour and transferred to 125ml Erlenmeyer flask (Corning, Steuben County, NY, USA). Each flask was contained 5 each cells-seeded scaffolds. 50ml of osteogenic media was added to flask (Figure 1). For animal study, a part of scaffold was collected from flask at 24 hour and implanted to subcutaneous on nude mouse. The rest of scaffolds were cultured for 14 days and analyzed cell morphology by a scanning electron microscopy (SEM) and SEM-energy-dispersive X-ray spectroscopy (SEM-EDX). Culture media were changed every 3 days.

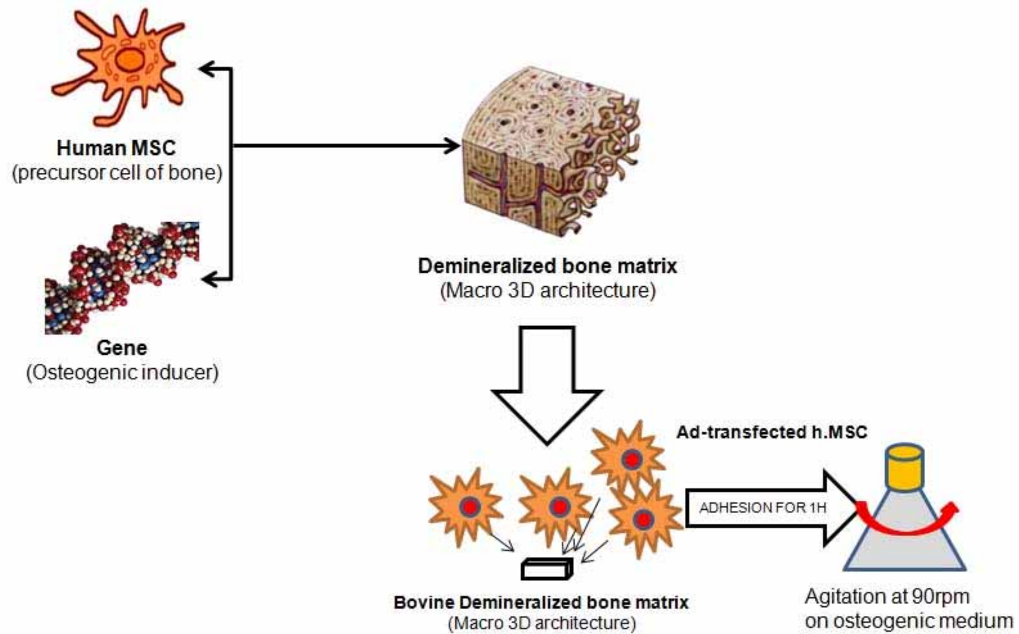

Figure 1. Scheme of 3D culture with adenovirus-transduced hMSCs on bDBM scaffolds bDBM sponges were prepared to 0.5 x 0.5 x 0.1 cm^3 size and adhered adenovirus-transduced hMSCs for 1 hour. Cells-adhered sponges were transferred to 125ml Erlenmeyer flask. Every group was cultured with agitating at 90 rpm.

2. Construction and preparation of adenoviral vectors

The human *alk-2*(*halk-2*) cDNA was generated by the standard PCR protocol using set consisting of *BamHI* restriction site contained upstream primer and *HindIII* restriction site contained downstream primer. The former had a Kosak sequence at position -3 in the original sequence. The latter had a stop codon (encodes 509 amino acids). The PCR product of human *alk-2* cDNA contains 1 GS domain and 1 protein kinase domain. The human *bmp-2* (*hbmp-2*) cDNA as the positive control to osteogenesis was also generated by a similar method, and it encoded 396 amino acids. These receptor and ligand cDNAs were primarily subcloned into TOPO-TA cloning vector (Invitrogen, San Diego, CA, USA). The nucleotide sequence of the amplified cDNA was verified using an ABI PRISM377 automatic DNA sequencer. The sequence confirmed cDNA were digested by *BamHI* (NEB, Beverly, MA, USA) and *HindIII* (NEB, Beverly, MA, USA) at 37℃ for 1hour and eluted by Gel extraction kit (QIAGEN Inc., Hilden, Germany). The pCA14 shuttle vector was also excised by *BamHI* and *HindIII* at same condition and prepared by cleanup method for ligation with cDNAs. Restriction enzyme digested pCA14 vector and *halk-2* cDNA or *hbmp-2* cDNA were ligated at room temperature for 2 hours and then transformed to *Escherichia coli* DH5α competent cell. Positive transformants (named each vector, pCA14/*halk-2* and pCA14/*hbmp-2*) were collected and verified used by the standard PCR method. To finally clone each cDNA to adenoviral vector, type 5 adenoviral vector, vmRL-H5dl324Bst was linearized by *BstBI* (BMS, IN, USA) digestion and pCA14/halk2 was linearized by *NdeI/ScaI* (BMS, IN, USA) digestion. Also, pCA14/*hbmp2* was linearized by *XmnI* (NEB, Beverly, MA, USA) digestion. Type 5 adenoviral vector was kindly provided by Dr. Yoon at Cancer Research Center, Yonsei university. The linearized pCA14/*halk2* or pCA14/*hbmp2* was co-tranformed into *E. coli*

BJ5183 competent cell together with *BstBI*-digested vmRL-H5dl324Bst for homologous recombination. To verify the respective homologous recombinants, the plasmid DNA purified from overnight *E.coli* culture was digested with *HindIII*, and the digestion pattern was analysis. Also, the plasmid DNA (vmRL-H5dl324Bst/*halk-2* and vmRL-H5dl324Bst/*hbmp-2*) was checked PCR to each cDNA to verify finally. The proper homologous recombinant adenoviral plasmid DNA was digested with *PacI* (NEB, Beverly, MA, USA) and transfected into 293A cells (Invitrogen, Rockville, MD, USA) to generate each cDNA-contained adenovirus, Ad/ALK-2 and Ad/BMP-2.

To generate LacZ-expressing adenovirus, a shuttle vector was created in which the *lacZ* gene was excised from plasmid pcDNA/hygro/LacZ (Invitrogen, Rockville, MD, USA) and cloned into pCA14 shuttle vector. Ad/lacZ preparation method was same as the previous Ad/ALK-2.

The titer (multiplicity of infection, MOI) used in this study was determined by absorbency of the dissociated virus at *A260*nm, where 1 absorbency unit is equivalent to 10^{12} viral particles per milliliter. The particle-to-infection unit (PFU) ratio was 100:1.

3. Transduction efficiency of adenovirus vector to hMSCs

A. 5-bromo-4-chloro-3-indolyl- β-D-galactopyranoside (X-gal) staining

hMSCs were plated onto 24-well plates at 2 x 10^4 cells per well. At 24 hour, cells were transduced with Ad/lacZ adenovirus at various MOIs. β-Galactosidase (β-gal) activity was visualized 48hours later by 5-bromo-4-chloro-3-indolyl- β-D-galactopyranoside (X-gal;

Sigma-Aldrich, Steinheim, Germany) staining according to standard technique. Individual experiments were carried out with three sets of cells and all experiments were repeated at least three times.

B. The expression of ALK-2 and BMP-2 in hMSCs

To verify the expression of ALK-2 on Ad/ALK-2-transduced hMSCs, hMSCs were plated onto 6-well plates at 2 x 10^5 cells per well and maintained for 24 hours in DMEM-LG medium supplemented with 10% FBS. The medium was replaced with fresh Hank's balanced salt solution (HBSS; Invitrogen, Rockville, MD, USA) containing with 1% FBS at 1 hour before the transduction of adenovirus. The cells were transduced with Ad/ALK-2 adenovirus at 150 MOI and were added at 1 hour with DMEM-LG containing with 10% FBS. Negative control and mock (Ad/lacZ) group were treated at the same condition. After overnight incubation, the medium was replaced with fresh complete culture medium and transduced cells were cultured for another 2 days at 37℃, 5% CO_2 atmosphere.

Cells transduced with Ad/ALK-2 and control were washed twice with PBS and solubilized in a cell lysis buffer containing with 20mM Tris-HCl, pH7.4, 150mM NaCl, 1% (w/v) Triton X-100, and protease inhibitor cocktail (Roche Applied Science, Branford, CT, USA). Lysates were briefly sonicated and cleared by centrifugation. Protein concentration was determined by the Bradford method (Bio-Rad Laboratories Inc., Hercules, CA, USA). 10μg of each sample was separated by SDS-PAGE. The proteins were transferred to nitrocellulose membranes. The membranes were then blocked for 1hour at room temperature in 50mM Tris-HCl, pH 7.6, 150mM NaCl, 0.1% Tween 20 containing with 5% nonfat dry milk (BD Biosciences, Franklin Lakes, NJ, USA). After blocking, the membranes were incubated with

anti-human ACVR antibody (R&D Systems, Minneapolis, MN, USA). The secondary antibody was used by a goat anti-mouse IgG conjugated to horseradish peroxidase (R&D systems, Minneapolis, MN, USA). Finally, the blots were visualized using enhanced chemiluminescence plus (ECL-PLUS; GE, Buckinghamshire, UK).

4. Assay to osteogenic differentiation of hMSCs

A. Histochemical staining of osteogenic phenotype

Histochemical analysis of alkaline phosphatase activity was carried out as describes. hMSCs were plated onto 24-well plates at 2 x 10^4 cells per well and transduced with Ad/ALK-2 adenovirus at a various MOI. The transduced cells were maintained for 7 days and the medium was changed twice during the course of the experiment. The cells were washed with PBS and fixed with 60% citrate buffered acetone at room temperature for 30 seconds. After washing with distilled water, cells were incubated for 30 minutes with a mixture of 0.1mg/ml naphthol AS-MX phosphate (Sigma-Aldrich, St. Louis, MO, USA), 0.5% N,N-dimethylformamide, 2mM $MgCl_2$, and 0.6mg/ml fast blue BB salt (Sigma-Aldrich, St. Louis, MO, USA) in 0.1M Tris-HCl, pH8.5, at room temperature, and rinsed with distilled water. Counter staining was used by Mayer's hematoxylin solution (Sigma-Aldrich, St. Louis, MO, USA). Finally, the stained cells were air-dried and photographed.

For the determination of mineralized nodules, hMSCs were plated and transduced as same as the previous describes. Cells were maintained for 7 days and the medium was changed twice during the course of the experiment. The cultured medium was supplemented with 50μg/ml

ascorbic acid and 5mM β-glycerol phosphate. At the end of the treatment, the cells were washed twice with PBS, and the formation of in vitro mineralized nodules was determined by alizarin red-S staining and von Kossa staining. Alizarin red-S is a dye that binds selectively to calcium salts and binds about 2 mol of calcium/mol of dye.

For alizarin red-S staining, cells were fixed at 4℃ for 1 hour with 70% ethanol and rinsed with distilled water. The fixed cells were stained for 10 minutes at room temperature with 40mM alizarin red-S (Sigma-Aldrich, St. Louis, MO, USA) in 0.1M borate buffer, pH4.0 and washed with PBS for 5 minutes. Finally, the stained cells were air-dried and photographed.

For von Kossa staining, cells were fixed with 10% neutral buffered formalin for 1hour and rinsed with distilled water. The fixed cells were stained with 3% fresh made $AgNO_3$ (Sigma-Aldrich, St. Louis, MO, USA) and developed in front of a 60W lamp until the calcium turn to black. And then stained cells were counterstained with nuclear fast red (Sigma-Aldrich, St. Louis, MO, USA). Finally, the stained cells were air-dried and photographed.

B. Reverse-transcription-polymerase chain reaction (RT-PCR) of osteogenic marker genes

Total cellular RNA was eluted by selective binding to a silica gel-based membrane using an RNeasy mini kit (QIAGEN Inc., Hilden, Germany). For reverse transcription, a 50μl RT premix reaction (Bioneer, Deajun, Korea) contained 1μg total RNA and 12.5ng/μl oligo $(dT)_{12\text{-}18}$ primers (Invitrogen, Rockville, MD, USA). Reactions were carried out at 42℃ for 1 hour, followed by inactivation of the enzyme at 95℃ for 5 minutes. For PCR amplification, each 20μl PCR premix reaction (Bioneer, Deajun, Korea) contained 1μl of RT aliquot and 10nmol each primer set (Table 1; Bioneer, Deajun, Korea). Following an initial denaturation step of 2

minutes at 94℃, amplification consisted of 25~35cycles of 5 seconds at 94℃, 5~30 seconds at optimal temperature (Table 2), and 30 seconds at 72℃, followed by final extension step for 5 minutes at 72℃. Amplification was performed in a ThermoHybrid Px2 system (ThemoHybaid, Franklin, MA, USA). Specific primers were designed from sequence available in the data banks (Table 2). The RT-PCR products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide. The intensity of the products was quantified using the BioImage Visage 110 system (Bio-Rad Laboratories Inc., Hercules, CA, USA).

No PCR product was observed when the RT reaction volume was replaced with water in PCR reaction (data not shown). *β-actin* gene was used to ascertain that an equivalent amount of cDNA was synthesized from the different samples.

C. SEM and SEM-EDX of Ad/ALK-2-transduced hMSCs-seeded bDBM sponge

For the biological sample preparation of SEM, hMSCs-seeded bDBM sponges were fixed with 2.5% glutaraldehyde (Sigma-Aldrich, St. Louis, MO, USA), and subjected to a serial graded ethanol for dehydration (50, 70, 95, and 100%). After dehydration, the specimens were immersed in hexamethyldisilazane (HMDS; EM sciences, Hatfield, AR, USA) and air-dried. Upon drying, they were mounted on the SEM stubs. The specimens were coated with gold to improve the conductivity. The secondary electron mode was applied during SEM observation. EDX was also obtained during SEM observation, which was coupled with EDX detector.

5. Assay of MAPK signaling in ALK-2-overexpressed hMSCs

To determine the correlation of ALK-2 or BMP-2 to MAPK inhibition, MAPK inhibitors and ALK inhibitor were treated to hMSCs at 72 hours after adenovirus transduction. Cells were incubated for 16 hours with 50μM PD98059, 5 μM SB203580, 10 μM SP600125 (all reagent from Sigma-Aldrich, St. Louis, MO, USA), or 5 μM dorsomorphin dihydrochloride (Tocris Bioscience, Ellisville, MO, USA).

MAPK or ALK inhibitor treated hMSCs were washed with PBS and solubilized in a cell lysis buffer containing containing 20mM Tris-HCl, pH7.4, 150mM NaCl, 1%(w/v) Triton X-100, and protease inhibitor cocktail. Lysates were briefly sonicated and cleared by centrifugation. Protein concentration was determined by the Bradford method. 10μg of each sample was separated by SDS-PAGE. The proteins were transferred to nitrocellulose membranes and immunoblotted with anti-Smad1 antibody, anti-phospho-Smad1/5/8 antibody, anti-p44/42 antibody, anti-phsopho-p44/42 antibody, anti-p38 antibody, anti-phospho-p38 antibody, anti-SAPK/JNK antibody, or anti-phospho-SAPK/JNK antibody (all from Cell Signaling, Beverly, MA, USA). The blots were visualized using enhanced chemiluminascence detection system (GE, Buckinghamshire, UK).

TABLE 1. Sequence of the oligonucleotide primers for PCR analysis

Gene	Primer	Strand	Length
Human *β-actin*	GGCGGACTATGACTTAGTTG	+	20
	AAACAACAATGTGCAATCAA	-	20
Human *osteocalcin*	CACTCCTCGCCCTATTGGCC	+	20
	GCCAACTCGTCACAGTCCGG	-	20
Human *runx2*	AGATGGGACTGTGGTTACTG	+	20
	GTCGCTACTTGGGGAGGATT	-	20
Human *dlx5*	TGACAGGAGTGTTTGACAGA	+	20
	TGATACTGGTAGGGGTTGAG	-	20
Human *osterix*	CCTTTACAAGCACTAATGGG	+	20
	CACCATGGAGTAGGAGTGTT	-	20

TABLE 2. Amplification condition of osteogenic marker genes

Gene	Temperature (℃)	Cycle	Product size(bp)
Human *β-actin*	53	26	238
Human *osteocalcin*	62	28	299
Human *runx2*	58	30	189
Human *dlx5*	58	30	225
Human *osterix*	55	30	299

6. Histological assay to the in vivo implantation of Ad/ALK-2-transduced hMSCs-seeded bDBMs

A. Implantation of genetic modified hMSCs-seeded bDBM scaffold

All animal studies were conducted in accordance with principles and procedures approval by the University of Yonsei Committee for Animal Resources. Implantation of Ad/ALK-2 or Ad/BMP-2-transduced hMSCs-seeded bDBM scaffolds was performed at specific pathogen free (SPF) zone. Briefly, 8-week-old *Balb/c-nu/nu* mice were anesthetized, and 5mm-long incision was made on the back skin. hMSCs-adhered scaffolds were implanted to subcutaneous part and the incision was closed with absorbable sutures. The mice were maintained for 2 weeks or 4 weeks (Figure 2). At the times, each group was sacrificed by CO_2 inhalation and the implanted scaffolds were harvested. The harvested scaffolds were performed to histological analysis.

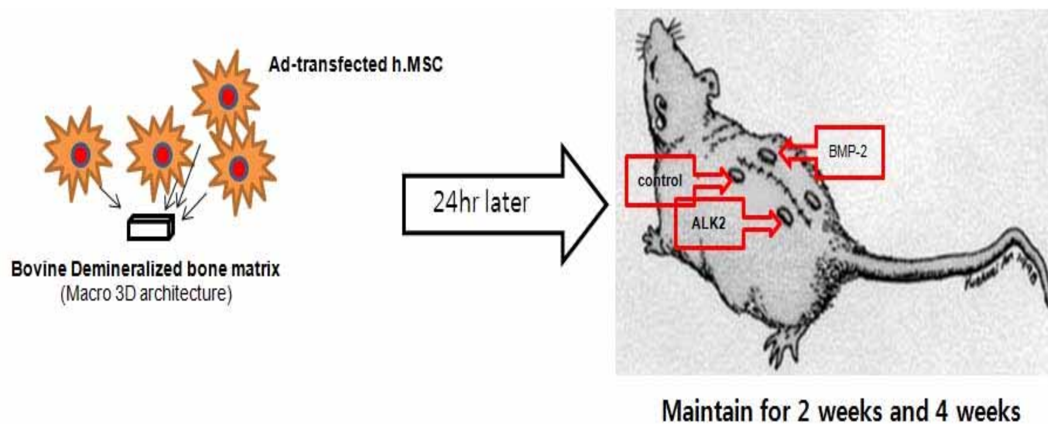

Figure 2. Implantation of genetic modified hMSCs-adhered bDBM sponges to nude mice Sponges were implanted to subcutaneous part of mice and maintained for 2 or 4 weeks.

B. Hematoxylin & eosin (H&E) staining of the implants

The harvested scaffolds were fixed in 10% neutral buffered formalin (NBF) and decalcified with 5% formic acid in 10% NBF for 3 day with gently shaking. During the decalcification, the decalcified solution was changed every day. And then 3μm paraffin-embedded sections were prepared and stained by standard H&E staining method. The H&E stained slides were observed by bright-field microscopy (Olympus, Tokyo, Japan).

C. Immunofluorescent (IF) staining of ALK-2 protein

To verify the expression of ALK-2 on the harvested implants, IF staining was performed with anti-human ACVR antibody (R&D Systems, Minneapolis, MN, USA). For antigen retrieval of formalin-fixed paraffin embedded specimens, paraffin sections were immersed in 10mM Tris, 1mM EDTA, and 0.05% Tween-20, pH9.0 for 30 minutes and allowed to cool for 20 minutes at room temperature. Antigen retrieval sections were rinsed with 0.05% Tween-20 and blocked for 30 minutes. The sections were followed by incubation with primary antibody to human ALK-2 and then incubated with Alexa488-conjugated antibody against mouse immunoglobulin (Molecular Probes Inc., Eugene, OR, USA). After a final wash, the sections were covered with the mounting media contained DAPI (Vector Laboratories Inc., Burlingame, Canada) and analyzed by confocal laser-scanning microscopy (Carl Zeiss Microimaging GmbH, Göttingen, Germany).

Ⅲ. RESULTS

1. Transduction efficiency of the prepared adenovirus vectors to hMSCs

For the decision of optimal virus titer, Ad/LacZ was transduced to hMSCs and stained by X-gal staining (Figure 3). The difference of transduction efficiency of hMSCs from different patients was observed. However, the average transduction efficiency was over 80% at 150MOI of Ad/LacZ. These experiments were repeated at three times.

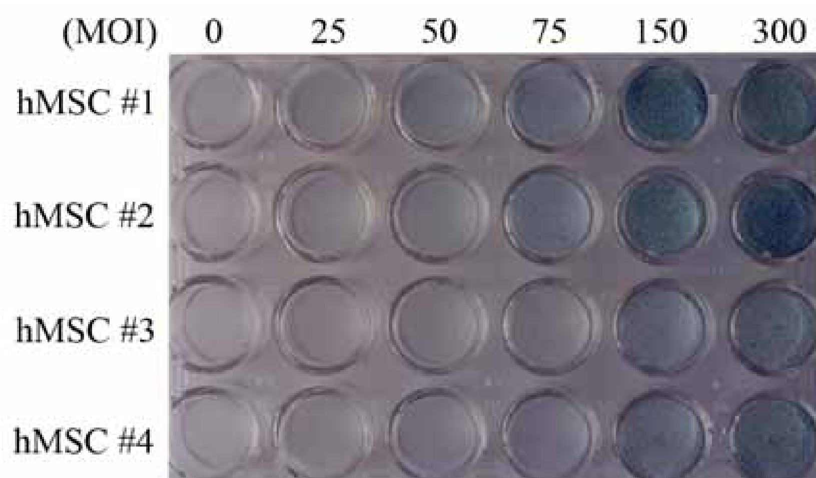

Figure 3. X-gal staining of Ad/LacZ-transduced hMSCs Cells were plated onto 24-well plate at 2 x 10^4 cells per well. Ad/LacZ was diluted with HBSS and transduced for 1 hour. X-gal staining was performed at 48 hours after adenovirus transduction. Negative control (0 MOI) was applied with the same volume of HBSS solution.

2. Overexpression of ALK-2 in hMSCs induces expression of *osteocalcin* mRNA

ALK-2 proteins were over-expressed in Ad/ALK-2-transduced hMSCs compared to control and Ad/LacZ group (Figure 4-a). These increased expression of ALK-2 stimulated expression of *osteocalcin* mRNA, osteogenic marker gene in hMSCs (Figure 4-b). Expression level of *osteocalcin* mRNA at 300 MOI of Ad/ALK-2 increased 1.5-fold compared to control group (Figure 4-c). These results suggested ALK-2 protein activates osteogenic cell signaling and then stimulates the expression of terminal differentiation marker gene.

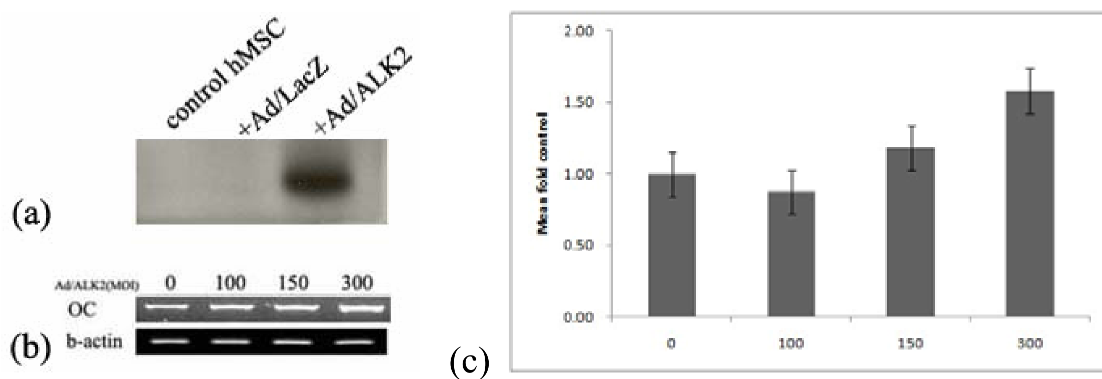

Figure 4. Expression of ALK-2 protein and *osteocalcin* mRNA in Ad/ALK-2-transduced hMSCs Cell extract and total RNA were extracted at 72 hours after virus transduction. (a) ALK-2 protein expression in adenovirus-transduced hMSCs was detected by western blot analysis. Cells were transduced with 150MOI of adenoviral vector and control group was treated with HBSS as same as virus volume. (b) Result of RT-PCR to human o*steocalcin* mRNA. *β-actin* mRNA was used as internal control. (c) Densitometric analysis of *osteocalcin* mRNA expression according to increase of Ad/ALK-2-transduction titer to hMSCs ($p < 0.001$).

3. Overexpression of ALK-2 in hMSCs stimulates mRNA expression of osteogenic transcription factor

To verify the overexpression of ALK-2 stimulates another osteogenic marker gene expression at transcriptional level, RT-PCR of a typical osteogenic transcription factors such as *runx2, dlx5,* and *osterix* was performed (Figure 5). Overexpression of ALK-2 stimulated expression of these mRNA in hMSCs. Expression of *dlx5* mRNAs was upregulated at 3 hours after transduction, and *osterix* and *runx2* mRNA was upregulated at 24 hours and 48 hours after transduction. These results demonstrated the overexpression of ALK-2 in hMSCs activates expression of *runx2, dlx5,* and *osterix* mRNA.

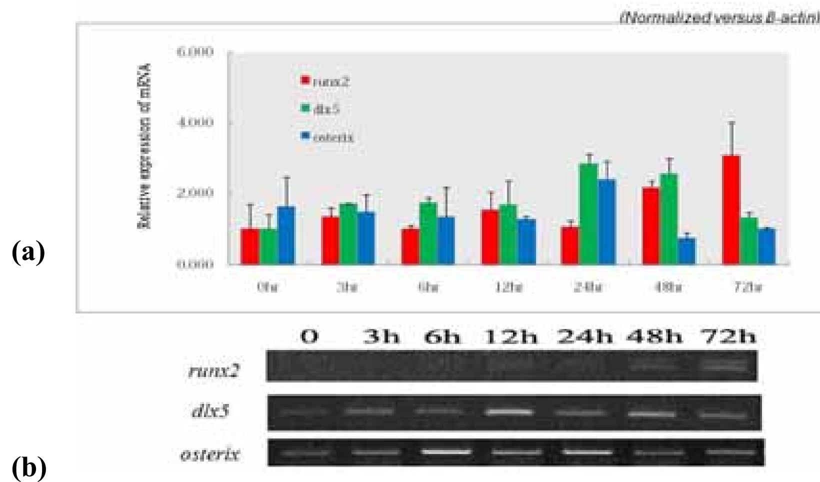

Figure 5. RT-PCR of *runx2*, *dlx5*, and *osterix* mRNA as the osteogenic transcription factor

Total RNA was extracted from Ad/ALK-2-transduced hMSCs and virus titer was 150MOI. Every total RNA was extracted according to time period after virus transduction. (a) Densitometric analysis to RT-PCR of *runx2*, *dlx5*, and *osterix* mRNA. (b) Electrophoresis of RT-PCR products. Every end product was loaded 5μl and visualized by EtBr staining.

4. Overexpression of ALK-2 differentiates hMSCs to osteoblastic cells

To verify the osteogenic phenotype of Ad/ALK-2-transduced hMSCs, alkaline phosphatase staining, Von kossa staining, and alizarin-red staining were performed (Figure 6). Alkaline phosphatase staining is a very common staining to osteoblastic differentiation. Von Kossa staining and alizarin-red staining show a mineral deposit of the surface on osteoblasts. At 7 day after transfection of Ad/ALK-2 to hMSCs, all osteogenic stainings showed the titer-dependent increased intensity of pigments. Specially, a mineral deposit of hMSCs induced by the transduction of Ad/ALK-2 was observed at very early time by von Kossa and alizarin-red staining. Most studies related to osteogenesis have demonstrated the mineral deposit was the terminal osteogenic differentiation marker and this mineral deposit was observed at over 2 weeks with stimulating continuously. However, the mineral deposit of Ad/ALK-2-transduced hMSCs was observed at 7 days. These results showed the accumulation of osteogenic stimulation by the overexpression of ALK-2 accelerated the mineral deposit surface on hMSCs.

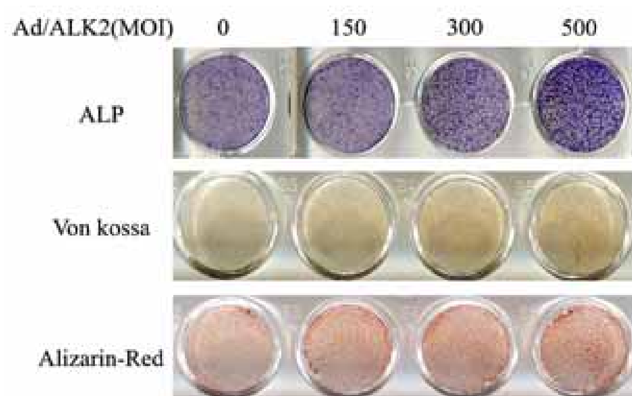

Figure 6. Ostegenic phenotype staining of Ad/ALK-2-transduced hMSCs All stainings were performed at 7 days after adenovirus transduction. Culture medium was supplemented with 50μg/ml ascorbic acid and 5mM β-glycerol phosphate.

5. 3D culture of Ad/ALK-2-transduced hMSCs used by bDBM sponges

To prove the feasibility of Ad/ALK-2-transduced hMSCs for developing the optional tools of ex vivo bone regeneration therapy, Ad/ALK-2-transduced hMSCs was adhered on bDBM sponge and cultured with agitating. And to observe cell morphology with hMSCs-adhered scaffolds, SEM and SEM-EDX were performed.

The prepared bDBMs were observed to a collagenous matrix, which maintained a bony structure and had not any other cells (Figure 7-a~c). Average diameter of inner pore was 300μm over. To check cell adhesion surface on bDBM sponge, only hMSCs were adhered for 1hour on bDBM sponge and immediately transferred to Erlenmeyer culture flask for the agitation culture. hMSCs were well adhered to bDBM sponge and spread out in spite of harsh agitation at 90rpm (Figure 7-d~f). However, any other morphological change without well adhesion was not observed.

To use mock control, Ad/LacZ was transduced to hMSCs and adhered to bDBM as same method as only MSC group (Figure 8). Unexpectedly, proliferation of Ad/LacZ-transduced hMSCs increased and spread out widely surface on bDBM. In this group, the inner pore of bDBM sponge was covered in Ad/LacZ-transduced hMSCs. β-Galactosidase has been well known as a control marker gene in vector system. But in animal, β-galactosidase also has been known which plays functional roles in the formation of extracellular elastic fibers (elastogenesis) and in the development of connective tissue. It seems to be identical to the elastin-binding protein (EBP), a major component of the non-integrin cell surface receptor expressed on fibroblasts, smooth muscle cells, chondroblasts, leukocytes, and certain cancer cell types [37]**.** Accordingly, we thought this function of β-galactosidase could demonstrate the increase proliferation of Ad/LacZ-transduced hMSCs on DBM sponge with agitating culture.

Newly formation of porous channel on Ad/ALK-2-transduced hMSCs at 150 MOI was observed (Figure 9), which was not observed at only hMSC group or Ad/LacZ-transduced group. Also, mineral deposits on cell surface were observed when hMSCs were treated with 1,000 MOI of Ad/ALK-2 (Figure 10), which was very similar to hydroxyapatite crystal.

To analyze these materials, SEM-EDX was performed (Figure 12). Because the specimen of SEM-EDX was used with the same specimen of SEM (Figure 12-a), the gold (Au) peak in the result of SEM-EDX was withdrawn from final analysis. Hydroxyapatite is a major component of bone mineral, which consist of calcium (Ca) and phosphate (PO_4). Figure 12-b showed three peaks of calcium and this portion was over 10% versus total composite. Finally, we demonstrated that 3D culture of high titer of Ad/ALK-2-transduced hMSCs with agitating induces deposit of calcium phosphate surface on hMSCs within 14 days.

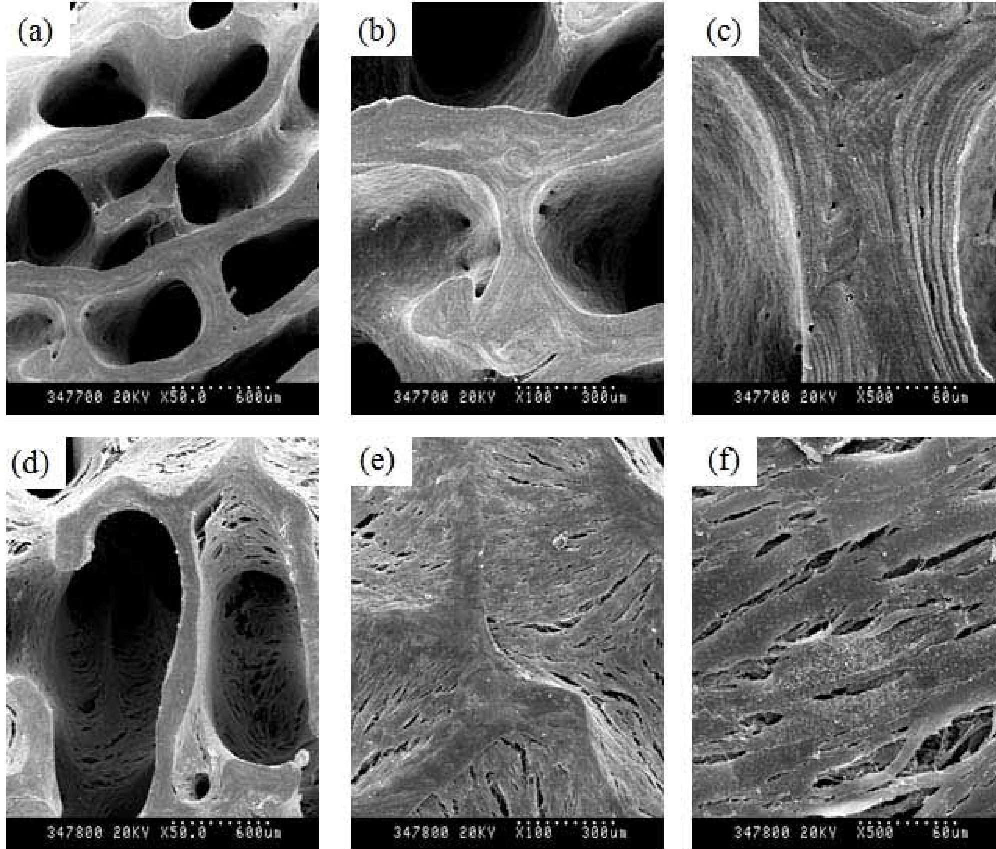

Figure 7. SEM photograph of only bDBM sponge (a~c) and hMSCs-adhered bDBM sponge (d~f) All groups were cultured with the osteogenic media for 14 days at 90rpm. About 2 x 10^5 cells of hMSCs were adhered on bDBM (d~f). (a, d) magnification x 50, (b, e) magnification x 100, (c, f) magnification x 500.

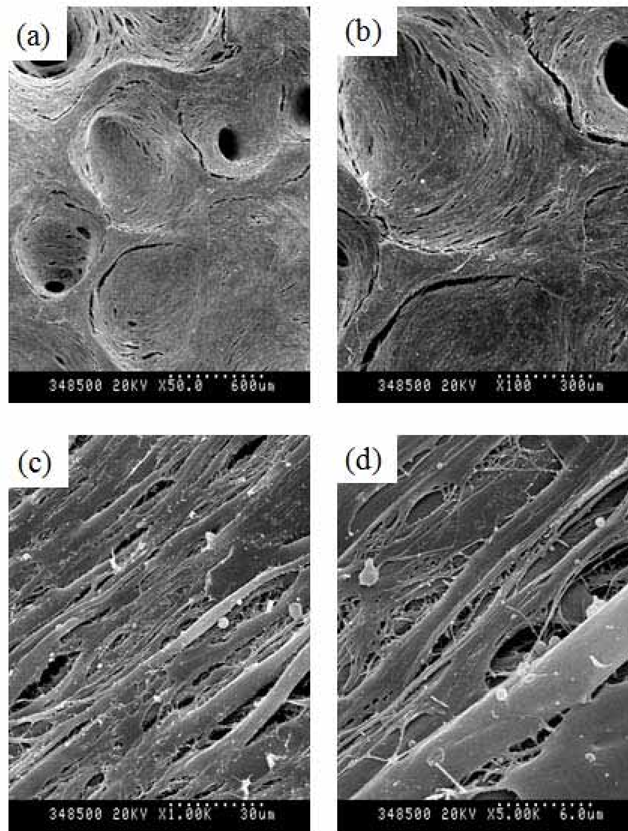

Figure 8. SEM photograph of Ad/LacZ-transduced hMSCs-adhered bDBM sponge Cells-adhered bDBM sponges were cultured with the osteogenic media for 14 days at 90rpm. Virus titer of Ad/LacZ was 150MOI. (a) magnification x 50, (b) magnification x 100, (c) magnification x 1,000, (d) magnification x 5,000.

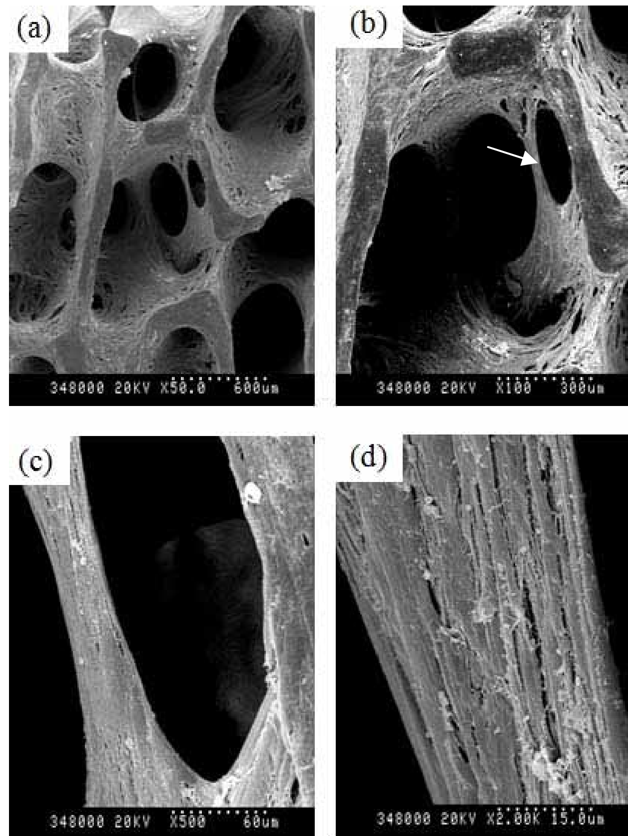

Figure 9. SEM photograph of Ad/ALK-2-transduced hMSCs-adhered bDBM sponge Cell-adhered bDBM sponges were cultured with the osteogenic media for 14 days at 90rpm. Virus titer of Ad/ALK-2 was 150MOI. (a) magnification x 50, (b) magnification x 100, (c) magnification x 500, (d) magnification x 2,000. Arrow mark; newly cell made porous channel.

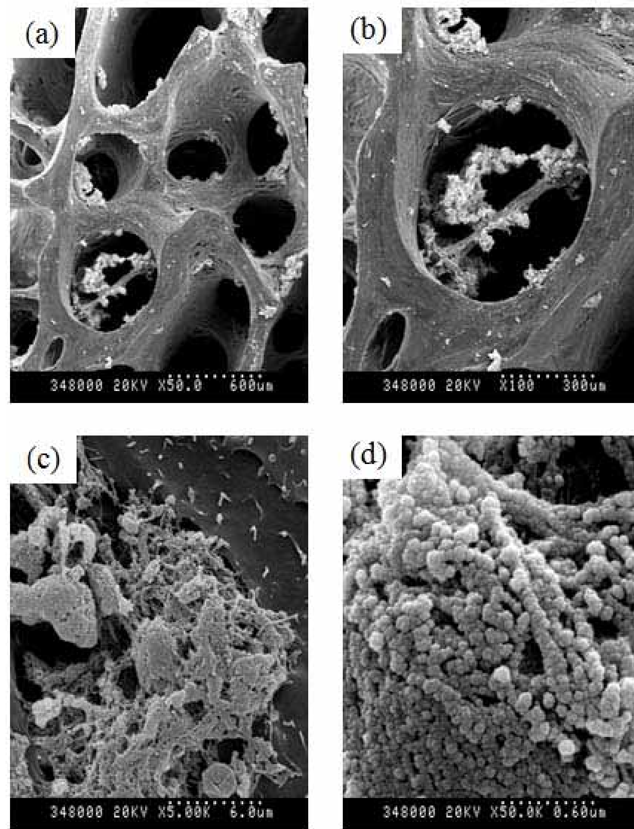

Figure 10. SEM photograph of Ad/ALK-2-transduced hMSCs-adhered bDBM sponge

Cells-adhered bDBM sponges were cultured with the osteogenic media for 14 days at 90rpm. Virus titer of Ad/ALK-2 was 1,000 MOI. (a) magnification x 50, (b) magnification x 100, (c) magnification x 5,000, (d) magnification x 50,000.

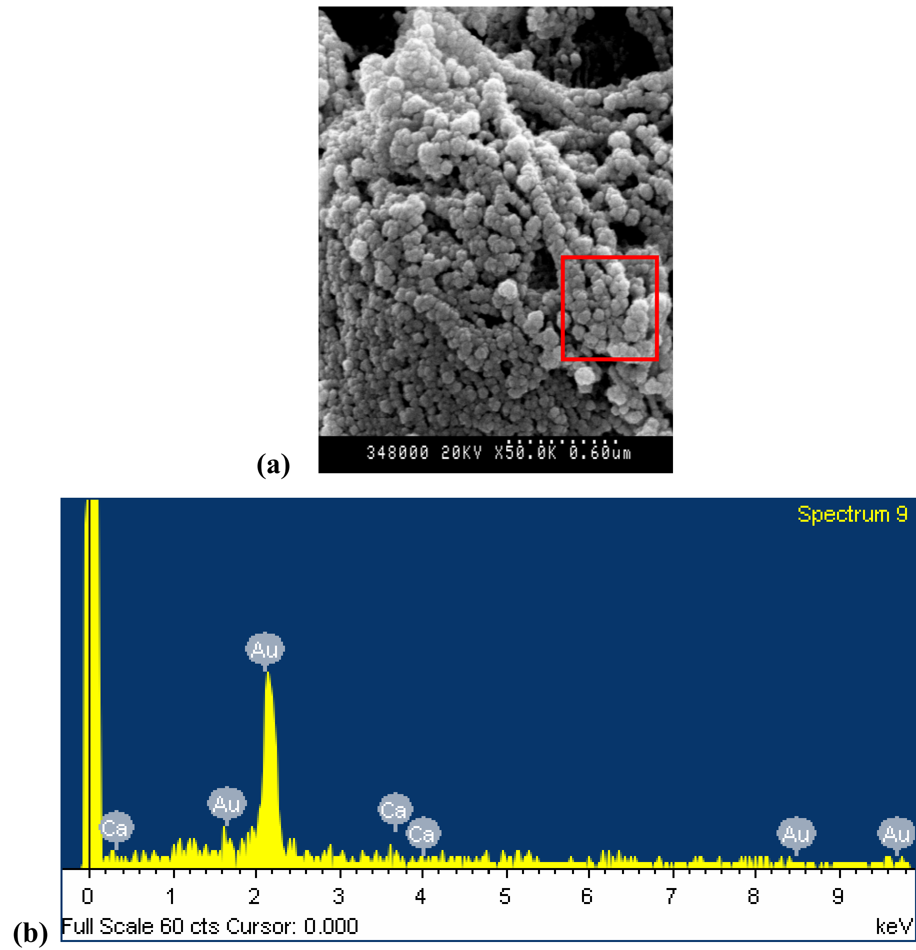

Figure 11. SEM-EDX analysis of the minerals surface on Ad/ALK-2-transduced hMSCs

(a) Pre-scanning photograph of Ad/ALK-2 (1,000MOI)-transduced hMSCs-adhered bDBM sponge before EDX analysis. Red square is a target of EDX analysis, Spectrum 9. (b) Analysis graph of spectrum 9. Au is gold and Ca is calcium.

6. ALK-2 activates Smad1/5/8 signaling and the phosphorylation of p38 protein

ALK-2 protein is a membrane receptor to TGF-β family. To verify the overexpression of ALK-2 in hMSCs activates type I TGF-β receptor-mediated cell signaling, western blot to Smad proteins was performed. Smad1/5/8 proteins are known to BMP-mediated osteogenic cell signaling molecule. These proteins are phosphorylated by type I TGF-β receptor such as ALK-2. Phosphorylation of smad1/5/8 proteins was activated by increasing virus titer of Ad/ALK-2 to hMSCs (Figure12-a). This demonstrated the overexpression of ALK-2 only without a ligand stimulated downstream cell signaling over membrane receptor.

BMP-2 protein was known also as MAPK stimulator because BMP-2 poses a various function for cell differentiation and proliferation. To verify the cell signaling by overexpression of ALK-2 represent to the same pattern of BMP-2 cell signaling, western blot to Smad1/5/8 and MAPK proteins was performed after adenovirus transduction and MAPK or ALK inhibition (Figure12-b,c). Phosphorylation of Smad1/5/8 protein on Ad/ALK-2-transduced hMSCs showed as same as Ad/BMP-2-transduced group, which was verified by western blot after the treatment of ALK inhibitor, dorsomorphin. This result demonstrated that phosphorylation of Smad1/5/8 depend on the activation of type I receptor protein. Expression pattern of Erk2 (p44/42) and SAPK/JNK proteins showed as same as both group. However, phosphorylation of p38 on Ad/ALK-2-transduced group increased over 20-fold compared to Ad/BMP-2-transduced group. This over-phosphorylation of p38 in Ad/ALK-2-transduced group was reported in Fibrodysplasia ossificans progressive (FOP), which disease is a rare autosomal dominant disorder characterized by congenital malformation of the great toes and by progressive heterotopic bone formation in muscle tissue. This disorder was reported by the mutation of BMP type I receptor, ALK-2.

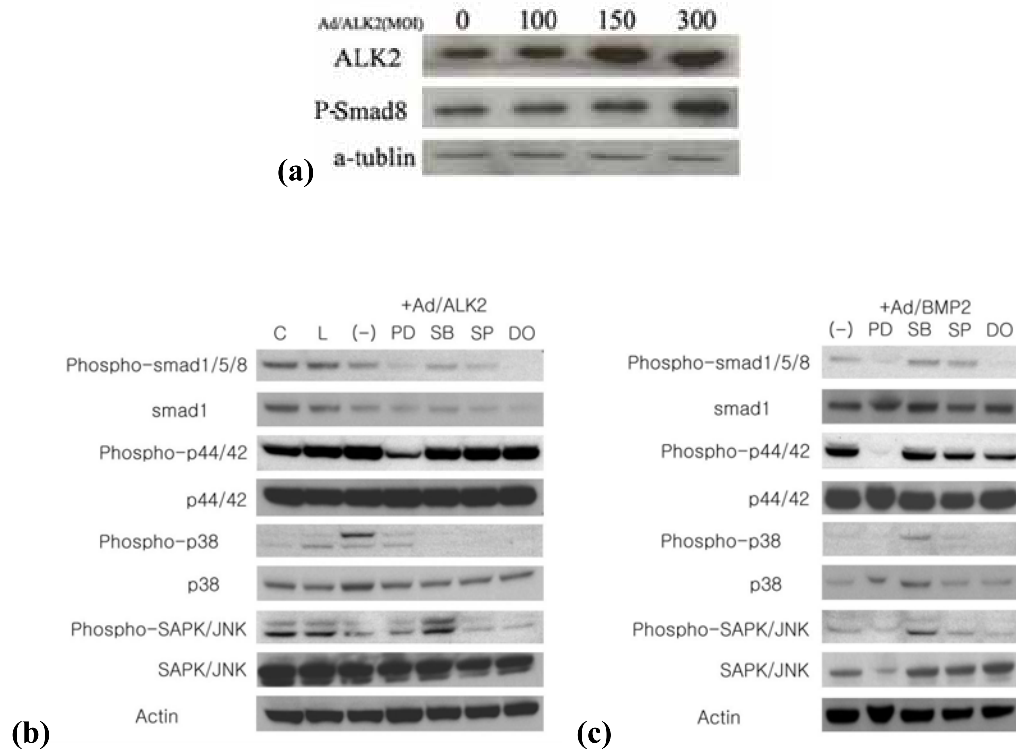

Figure 12. Smad1/5/8 and MAPK expression in Ad/ALK-2-transduced and Ad/BMP-2-transduced hMSCs (a) Western blot to ALK-2 and phospho-Smad1/5/8 in Ad/ALK-2-transduced hMSCs. Virus titer of Ad/ALK-2 was 0, 100, 150 and 300 MOI. (b) Western blot of Smad1/5/8 and MAPK in Ad/ALK-2-transduced hMSCs and (c) Ad/BMP-2-transduced hMSCs. (b) and (c) were treated with 150MOI virus titer. 10μg protein sample was loaded for electrophoresis. ; C, control; L, Ad/LacZ-transduced group; (-), transduction of only Ad/ALK-2 or Ad/BMP-2 ; the following groups was treated with MAPK inhibitor or ALK inhibitor after adenovirus-transduced hMSCs, PD, treatment of 50μM PD98059; SB, treatment of 5 μM SB203580; SP, 10 μM of SP600125, or DO, 5 μM of dorsomorphin dihydrochloride.

7. Implantation of Ad/ALK-2-transduced hMSCs to immunodeficient mice

To prove the osteogenic activity of ALK-2 in vivo model, 3D cultured adenovirus-transduced hMSCs-adhered bDBM sponges were implanted to subcutaneous in nude mice (Figure2). Each group was divided 2 weeks and 4 weeks and maintained. At 2 weeks, only Ad/BMP-2-transduced group as the positive control showed the histological phenotype of osteogenic differentiation, which group was observed osteoblastic lining cells and fat marrow around hMSCs-adhered implants. All groups were observed the implanted cell and sponge well took its place, and the boundary between the implanted cell and mouse cell was observed clearly (Figure 13). At 4 weeks, control groups were not observed osteogenic differentiation, but Ad/BMP-2-transduced groups were observed newly synthesized bone matrix and bone marrow formation. Most of newly bone in Ad/BMP-2 groups was generated from the outside of implanted sponge. This demonstrated a common phenotype of BMP-2 action related to new bone formation because a fresh nutrient and oxygen for new tissue generation was existed abundant around the outside of implantation. Ad/ALK-2 groups were observed the newly synthesized the collagenous matrix, but did not show osteogenic differentiation phenotype at 300 MOI of Ad/ALK-2 (Figure 14). However, 1,000MOI-applied Ad/ALK-2 groups showed osteogenic differentiation phenotype in some part of the implants compared to control. This phenotype was observed at the outside of implants (Figure 15).

IF staining to ALK-2 in the implants showed ALK-2 protein was overexpressed in the implanted hMSCs at 4 weeks (Figure 16). However, these results demonstrated the overexpression of ALK-2 protein could not stimulate enough to induce osteogenesis at in vivo model compared to BMP-2 overexpressed groups, and on the case of Ad/BMP-2 groups, a paracrine effect of BMP-2 induced a robust osteogenic differentiation at in vivo. Ad/ALK-2

groups showed a delayed new bone formation at in vivo, which demonstrated that because ALK-2 protein is a membrane intercalating receptor, which could not induced a powerful bone formation even though they had the stimulating potential of osteogenic cell signaling and *osteocalcin* mRNA expression at in vitro.

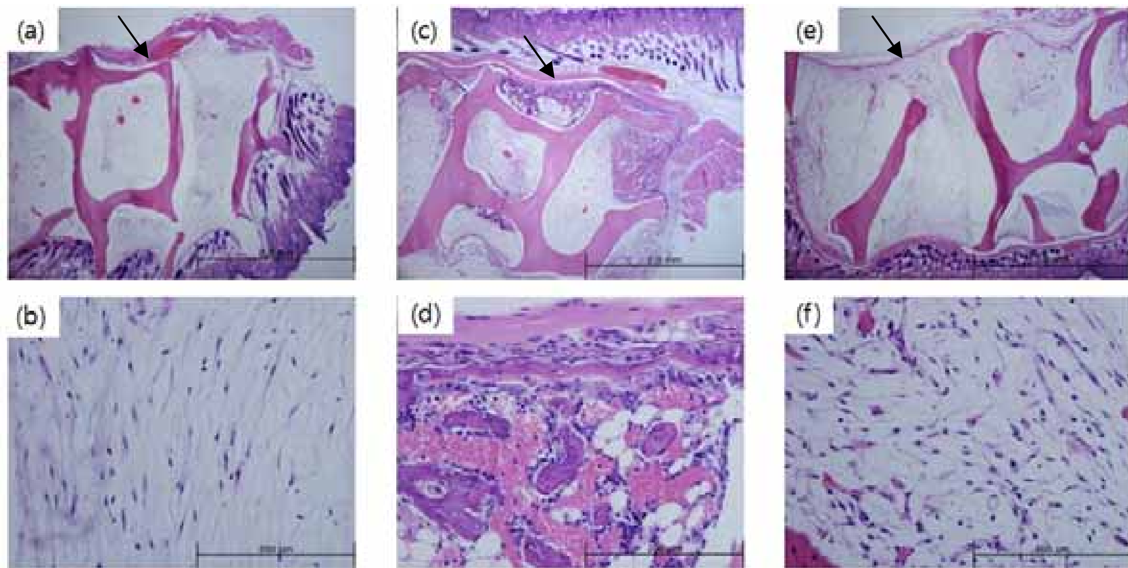

Figure 13. H&E staining of the hMSCs-adhered bDBM sponges from nude mice at 2 weeks after the implantation The used virus titer was 150MOI. (a, b) only hMSC-DBM group, (c, d) Ad/BMP-2-transduced hMSCs-bDBM group, (e, f) Ad/ALK-2-transduced group. (Upper) magnification x 40, (Bottom) magnification x 100.

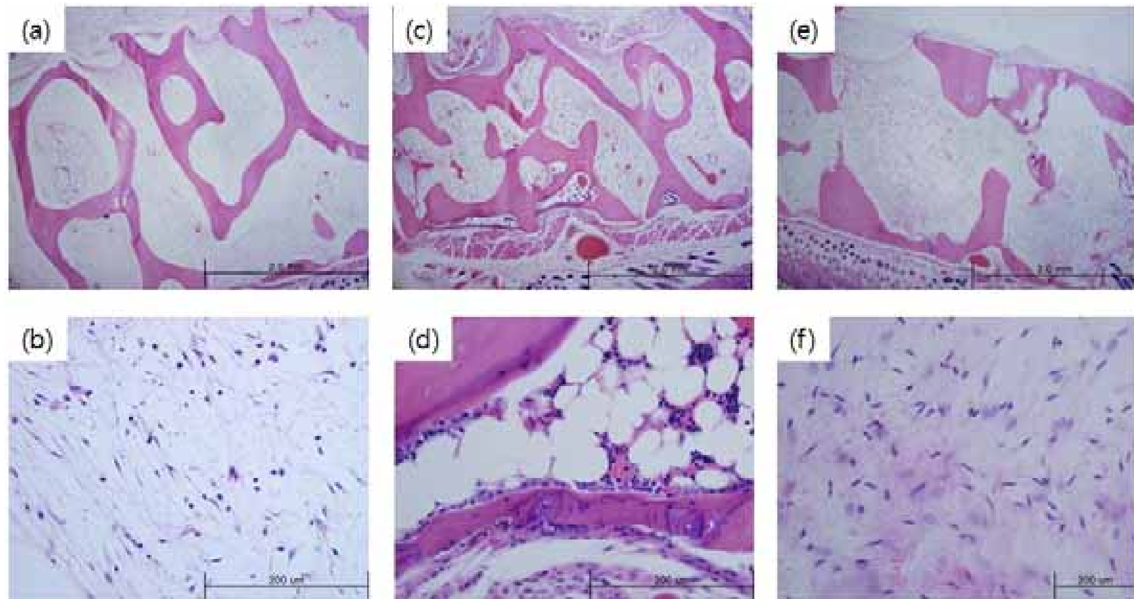

Figure 14. H&E staining of the hMSCs-adhered bDBM sponges from nude mice at 4 weeks after the implantation The used virus titer was 150MOI. (a, b) only hMSCs-bDBM group, (c, d) Ad/BMP-2-transduced hMSCs-bDBM group, (e, f) Ad/ALK-2-transduced group. (Upper) magnification x 40, (Bottom) magnification x 100.

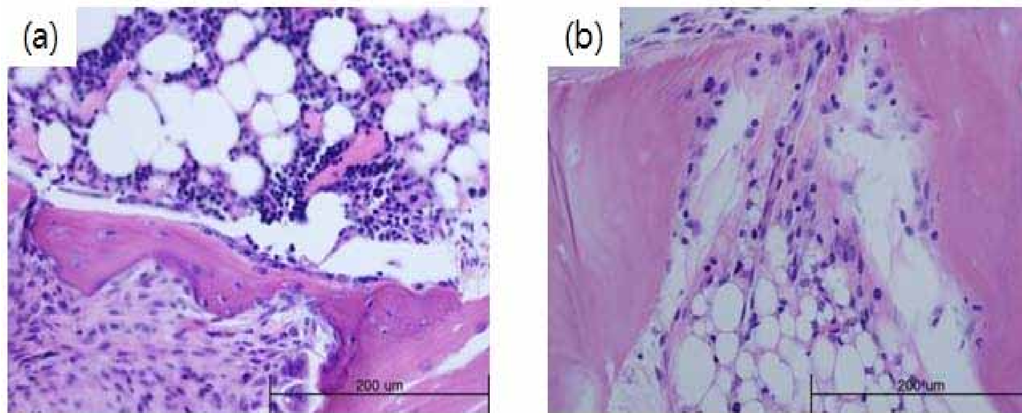

Figure 15. H&E staining of the hMSCs-adhered bDBM sponges from nude mice at 4 weeks after the implantation The used virus titer was 1,000MOI. (a) Ad/BMP-2-transduced hMSCs-bDBM group, (b) Ad/ALK-2-transduced group. Magnification x 100.

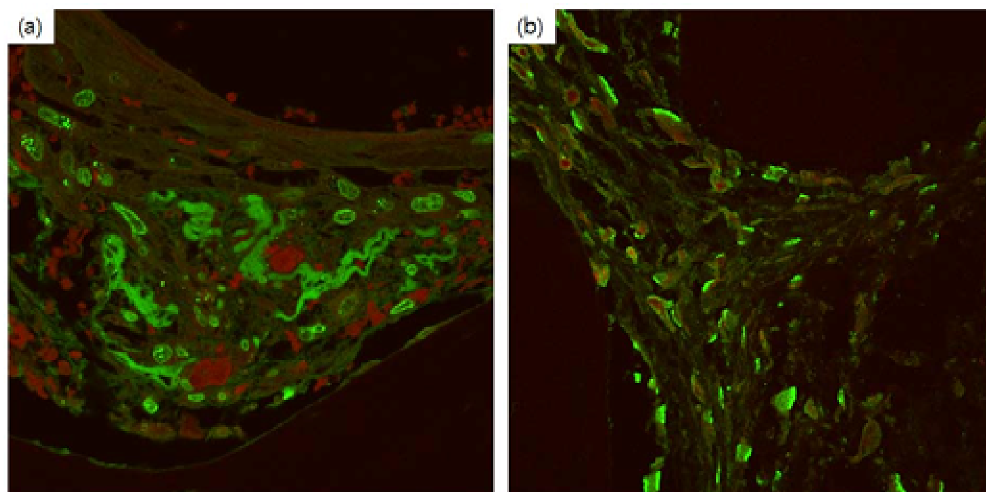

Figure 16. Expression of human ALK-2 protein in the implanted site of nude mouse The implants were Ad/ALK-2-transduced hMSCs-bDBM group at 2 weeks (a) and at 4 weeks (b). Primary antibody to ALK-2 protein was conjugated with Alexa 488 and rodamine F-actin as counterstain was used.

Ⅳ. DISCUSSION

Musculoskeletal conditions are increasingly becoming one of the major health concerns worldwide because of an aging population and increased occurrence of sports-related injuries according to be improve life style [38]. Entering 2005, there is increase approximately to 240 billion dollars worldwide to musculoskeletal disease care, especially fractures healing market, and between 5% and 10% of these result in nonunion or delayed union [39]. Collectively, this represents a substantial cause of morbidity, missed work, and medical cost. Biologics that promote bone-healing are needed in the treatment of established nonunions as well as in the acute treatment of certain fractures associated with many clinical options for stimulating bone formation, but each has substantial limitations [40-43]. To date, autologous bone remains the gold-standard graft material [44,45]. However, its harvest can cause substantial morbidity, including hematoma formation, infection, numbness at the incision site, and persistent pain. In addition, the limited quantity of autologous bone available for harvest may not be sufficient for the treatment of large defects [46].

Recombinant human BMPs (rhBMPs) have recently emerged as a bone-graft substitute. RhBMP-2 has been approved by the U.S. FDA for use in anterior lumbar interbody fusions and the treatment of open tibial fractures [47,48]. RhBMP-7 (osteogenic protein-1) has been approved under a "Humanitarian Device Exemption (HDE)" for the treatment of recalcitrant long-bone nonunions and for use in revision posterolateral spinal arthrodeses [49]. There is evidence that BMPs are more effective than autograft for promoting fracture-healing and spinal fusion; consequently, the introduction of BMPs has been met with a great deal of enthusiasm by orthopaedic community. However, the use of BMP has not been optimized. High doses of growth factor are needed to produce an adequate bone formation response. Presently, rhBMP is

being administrated at doses that a million times greater than its normal concentration in bone, and there are concerns about both safety and the cost of such supraphysiologic doses. Clinical trials and preclinical studies have both shown a potential for ectopic bone formation as well as edema [50-53]. These observations might partly be attributed to the collagen carriers used to deliver BMP, which have been hypothesized to be inefficient protein delivery systems.

These concerns have led to investigations of alternative protein delivery mechanisms to promote bone repair [23,26]. Regional gene therapy offers a novel approach to difficult clinical problem. Genetic sequences encoding for growth factors can be transferred to cells at the fracture site, resulting in the production of osteogenic proteins in a localized, sustained, and physiologic manner. Preclinical animal models have demonstrated the tremendous potential of these techniques. In tissue-engineering strategy that will include a spectrum of treatment options such as autologous bone-marrow injection, and stem cell therapies. It is envisioned that gene therapy options will initially be available for the most severe clinical situations such as massive bone loss and recalcitrant nonunions [21]. Despite its tremendous promise, the clinical application of gene therapy must be approached with caution. Thus far, clinical trials for gene therapy for inflammatory arthritis and metabolic disease have led two deaths. Any substantial morbidity will not be accepted in the treatment of nonfatal musculoskeletal conditions [24,25,54].

Gene therapy is a tool that can be used to deliver osteoinductive proteins at a desired location. It may be a more efficient growth factor delivery system than are the current methods of rhBMP delivery, which have substantial limitations, such as a short duration of action. The commercially available products deliver BMPs with a type I collagen carrier. There is an initial burst of BMP release with a half-life of less than ten minutes, followed by a second phase of gradual release with a half-life of between three and five days. A more prolonged expression of BMP might enhance the fracture healing process. Gene therapy may offer a solution. Cells

present within the body can be genetically manipulated to produce osteogenic proteins in the area of interest. This would provide a more physiologic delivery system, with continuous in vivo production of protein at a relative constant level for sustained period [23,49,55].

Stem-cell-based therapies are another emerging option for promoting bone regeneration as the previous describes. Stem cells are defined by their distinct ability to self-renew and to differentiate into multiple cell types. The cell that has been most extensively studied for orthopaedic applications is the mesenchymal stem cell. This is an adult stem cell that is found in tissues of mesoderm origin such as bone marrow, adipose tissue, muscle, and skin [56-58]. When exposed to the appropriate growth factors, these multipotent cells can differentiate into chondrocytes or osteocytes and may contribute to bone formation [27,56,59]. Mesenchymal stem cells seeded onto scaffolds such as hydroxyapatite have induced healing of critical-sized bone defects in severe animal models [60,61]. Moreover, percutaneous injection of bone marrow aspirates has been used to treat tibial nonunions with moderate success [25,62-65].

Stem-cell-based gene therapy may be another way to increase the power of these techniques. Mesenchymal stem cells can be genetically modified to overexpress osteogenic protein such as BMPs. These growth factors stimulate the mesenchymal stem cells to differentiate into bone forming cells through autocrine signaling, and they recruit host osteogenic progenitors through paracrine signaling. Then, almost all scientist possessed of the previous concepts have been questioned how choice the combination of these therapeutic tools.

The purpose of the present study was to find a new candidate gene for bone specific regeneration and a new method for combining stem-cell based tissue engineering. BMP-2 has been chosen and researched by most orthopeadic researchers because which have the robust osteogenic and paracrine effect. However, BMP-2 participates to the many biological processes from embryonic development to adult and from osteogenesis or chondrogenesis to

adipogenesis, which protein is indeed a multiplayer in the human body. There has been thought BMP-2 is a protein very hard to regulate at in vivo environment because of their paracrine effect.

In the past studies, some researchers on orthopeadic therapy have demonstrated the ALK-2 protein as BMP-2 and BMP-7 type I receptor has the osteogenic potential at in vitro. ALK-2 is a protein kinase itself, which possess a GS domain and a protein kinase domain. Using this kinase activity, Ulrich and colleagues also have proved the continuous active ALK-2 (caALK-2) simulates continuously osteogenesis [36,66]. However, the previous study used by caALK-2 was doubted that the continuous stimulation of caALK-2 cannot be safe to bone regeneration therapy in human body because some researchers demonstrated wild type ALK-2 has a potential enough to stimulate osteogenic differentiation of MSCs. Also. the past studies have reported that osteocalcin expression was not upregulated by overexpression of the receptor Smad protein only or Co-Smad. These observations have been raised to the possibility that additional pathway, such as ALK-2-dependent, might be required for osteocalcin expression, a unique trait specific of the osteoblast phenotype.

In this present study, the overexpression of ALK-2 protein used by adenoviral vector system stimulated enough to differentiate hMSCs to osteoblasts in vitro and induced Smad1/5/8 activation and MAPK stimulation in hMSCs was observed. Specially, the up-regulation of p38 MAPK by the overexpression of ALK-2 have recently shown to control BMP-2-induced osteocalcin expression in myoblastic C2C12 cells [29,35,67]. At the present in vivo experiments using by Ad/ALK-2 and 3D scaffolds, bone formation by the overexpression of ALK-2 on the implanted site was failed. These results might be explained that ALK-2 expression did not recruit the precursor cell enough to bone regeneration from nearby environments because ALK-2 protein is not a secreted protein.

However, we thought the present studies demonstrated that on the case of bone union in elderly patients, the restoration of this BMP receptor in hMSCs can increase the sensitivity to growth factors, such as BMP-2, and also if the restoration of this BMP receptor in hMSCs combine with rhBMPs at in vivo therapy, this approach can increase bone regeneration and decrease the using dosage of rhBMPs for bone union. Also, in ex vivo therapy, if the combination of BMP receptor and BMP gene with the appropriate scaffold join to bioreactor culture system, hand-made bone formation could be realized on the extra-corporeal environment.

Based on these results of the present study, we are undergoing studies to verify the potentialities of the combination therapy using by Ad/ALK-2 and Ad/BMP-2 or rhBMP-2 and also to develop the simulated bioreactor system to bone regeneration, which bioreactor can scale-up to 3D culture with Erlenmeyer flask and agitation stress on the previous before.

Ⅴ. CONCLUSION

Overexpression of BMP type I receptor ALK-2 induces osteogenic phenotype in hMSCs. Therefore, ALK-2 gene can be a new therapeutic candidate for osteogenic gene therapy and for restoration of receptor to growth factor in hMSCs.

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초록 (국문 요약)

제 1형 BMP 수용체인 ALK-2에 의한 인간 중간엽 줄기세포의 조골세포 분화

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인간 중간엽 줄기세포 (hMSCs)는 다양한 기능의 중간엽 기원 세포로 분화할 수 있어 조직공학, 유전자 치료 및 세포 이식 분야에서 많은 연구자들에 의해 주목 받고 있는 연구 대상이다. 특히, 골 재생 분야에서는, hMSCs와 골 형태형성 단백질 (BMP)의 조골 세포 분화 기능에 대한 연구가 많은 연구자들에 의해 보고되어 왔다. BMP-2 유전자의 과발현이 hMSCs의 골 분화능을 자극하며, 동물 모델에서도 골 형성을 유도할 수 있음이 보고된 바 있다. 뿐만 아니라, 연골 세포 및 근육세포에서 제 1형 BMP 수용체를 과발현한 경우, BMP-2 유전자와 마찬가지로 조골세포 분화 유도가 가능함도 알려져 왔다. 이에 본 연구에서는, 제 1형 BMP 수용체 중 하나인 activin receptor-like kinase-2 (ALK-2) 유전자를 hMSCs 내에서 과발현 하였을 때, 조골세포 분화능이 유도되는 지를 연구하고, 유전자 변형된 hMSCs를 면역 결핍 쥐에 이식하였을 때, 골 형성이 일어나는 지를 검증하고자 하였다.

hMSCs는 척추관 협착증 환자의 골수로부터 추출하였고, ALK-2 유전자가 포함된 아데노바이러스 벡터 (Ad/ALK-2)는 293A세포로부터 생산하였다. Ad/ALK-2를

hMSCs에 전이시킨 후 조골세포 분화를 관찰하고자, 일반적인 조골세포 분화 염색 및 조골세포 분화 표식 유전자에 대한 RT-PCR를 시행하였다. hMSCs 내에서의 ALK-2 과발현이 조골세포 분화 신호 전달을 유발시키는 지를 검증하고자, Smad1/5/8과 MAPK에 대한 Western blot을 시행하였다. Ad/ALK-2가 전이된 hMSCs의 3차원 배양을 위해, 지지체인 소의 탈회골 (bDBM)에 세포를 분주하고, 주사전자현미경 (SEM)을 이용하여 세포 형태를 관찰하였다. 또한 ALK-2-과발현 hMSCs의 표면에 새로 합성된 표면 물질을 분석하고자, 주사전자현미경-에너지 분산 X-광 스펙트럼 (SEM-EDX)를 수행하였다. 마지막으로, Ad/ALK-2-전이 hMSCs가 흡착된 bDBM을 면역 결핍 쥐에 이식하였다. 2주와 4주째에 각 군을 희생하여 이식체를 수거하였다. 조직 분석을 위해, H&E 염색과 면역 형광 염색 (IF)를 시행하였다.

Ad/ALK-2-전이 hMSCs는 조골세포 염색에서 양성 염색 반응이 관찰되었으며, Ad/ALK-2의 바이러스 농도가 증가함에 따라 *osteocalcin* mRNA의 발현양도 증가되는 것을 관찰할 수 있었다. 조골세포 분화 전사 인자인 *runx2, osterix, dlx5*의 mRNA 발현 또한 ALK-2에 의해 활성화되었다. 조골세포 분화 신호 전달 단백질에 대한 western blot 분석에서도, ALK-2 과발현이 Smad1/5/8 신호 전달과정을 활성화 시키고, p38 단백질의 인산화가 증가되는 것을 관찰할 수 있었다. 3차원 배양에서는, 이러한 유전자 변형된 hMSCs의 세포 표면에 인산 칼슘이 축적되는 것이 관찰되었다. 생체 실험에서는, 이식 2주째에 양성 대조군인 BMP-2 과발현군과 비교하여, ALK-2 단백질만을 과발현한 군에서는 충분한 골 재생을 유도 하지는 못하였으나, 4주 이상 유지한 군에서는 조골세포 분화가 유도됨을 관찰할 수 있었다. 또한, Ad/ALK-2-전이 hMSCs가 흡착된 이식체 내에서 ALK-2 단백질이 4주 동안 과발현되고 있음을 IF 염색을 통해 확인하였다.

제 1형 BMP 수용체인 ALK-2의 과발현이 hMSCs 내에서 조골세포 표현형을

유도한다는 사실을 시험관 및 생체 수준에서 검증하였으며, 본 실험을 통해 ALK-2가 조골세포 분화 유전자 치료를 위한 가능성 있는 치료 후보 물질임을 검증할 수 있었다.

핵심 단어 : 인간 중간엽 줄기 세포, 제 1형 BMP 수용체, activin-receptor like kinase-2, 유전자 치료