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# Evidence of In Vivo Osteoinduction in Adult Rat Bone by Adeno-Runx2 Intra-Femoral Delivery

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**Abstract** The bone marrow microenvironment provides a unique opportunity in vivo to assess the role of genes in bone remodeling. The objective of this study was to determine whether Runx2 expression is regulated by rhBMP-2 in vivo and to examine the effect of Runx2 overexpression on bone in vivo. In the in vivo calvaria model we used, rhBMP-2 induced Runx2 protein expression in periosteal cells while in vitro, adenovirus-mediated Runx2 overexpression induced mineralization in mesenchymal stem cells. A single injection of adeno-Runx2 directly into the bone marrow of the right femur in mature rats, and subsequent analysis after 3 weeks, showed a significant bone mineral density (BMD) increase (~15%) as compared to the controls. The whole-femur mean BMD of the active virus-injected group was 0.193 ($g/cm^2$) while that of the control virus-injected group was 0.175 ($g/cm^2$) ($P < 0.05$). In addition, a significant increase (36%) in trabecular BMD at the distal end of the femur was observed. These data demonstrate that directly delivering adeno-Runx2 into bone marrow of adult rats induces osteogenesis and illustrates potential advantages of such approaches over ex vivo gene therapy protocols involving marrow cell isolation, gene transduction, and subsequent in vivo transfer. J. Cell. Biochem. 103: 1912–1924, 2008. 

**Key words:** BMP-2; adeno; Runx2; intra-femoral; OSE2; osteogenesis; gene therapy

Musculoskeletal regeneration through gene therapy and gene transduction systems has gained significant interest in recent years [Evans et al., 2005]. Several approaches using viral vectors or stem cells transduced ex vivo with osteogenic genes have been reported [Franceschi et al., 2000; Lieberman et al., 2002; Zhang et al., 2005; Kimelman et al., 2007]. Such studies have revealed that efficient in vivo delivery and expression of the osteoinductive gene is critical for successful therapies for bone and joint disorders including fracture, arthritis, and osteoporosis [Lieberman et al., 2002]. Bone and cartilage formation as well as maintenance is a highly regulated process that is controlled by multiple growth factors, cytokines, and hormones [Lieberman et al., 2002; Barnes et al., 2004; Chadderdon et al., 2004; Gafni et al., 2004; Shimer et al., 2004]. Since the initial observation that bone morphogenetic proteins (BMPs) could induce ectopic bone formation [Urist et al., 1984], BMP-2 has been the most widely studied protein to enhance bone formation in vitro and in vivo [Wozney and Seeherman, 2004; Seeherman and Wozney, 2005]. In addition to the application of BMP2 in fracture repair locally, BMP-2 has also been investigated through gene therapy approaches [Musgrave et al., 1999; Schreiber et al., 2005; Zhao et al., 2005].

It has been established that BMP-2 modulates a host of transcriptional events which stimulate osteoblastogenesis and promote osteoblast function [Wozney, 2002]. One of the key genes regulated by BMP-2 in vitro is the transcription factor, Runx2 [Yamaguchi et al., 2000; Yang et al., 2003; Phimphilai et al., 2006; Bae et al., 2007]. In addition, parathyroid hormone (PTH) has also been shown to induce Runx2 mRNA expression in various cell systems [Krishnan et al., 2003]. The critical role of Runx2 in chondrocyte maturation, as well as cartilage and bone formation is shown in Runx2

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knockout mice which completely lack intramembranous and endochondral ossification due to the maturational arrest of the osteoblasts [Komori et al., 1997]. Moreover, human mutations in Runx2 result in heterozygous loss of function and cause the human disease, Cleidocranial Dysplasia [Mundlos et al., 1997; Ducy et al., 1999]. Runx2 belongs to the runt domain gene family [Banerjee et al., 1997]. One of the two Runx2 isoforms containing the N-terminal amino acid sequence MASNS is expressed primarily in osteoblasts and in hypertrophic chondrocytes [Stewart et al., 1997; Gao et al., 1998].

Runx2 overexpression enhances the osteoblastic genes or reporter genes containing the osteoblast-specific DNA binding element 2 (OSE2) [Ducy and Karsenty, 1995; Geoffroy et al., 1995; Rossert et al., 1996; Ji et al., 1998]. However, Runx2-mediated induction of matrix proteins and mineralization in vitro is cell type dependent. For example, NIH3T3 and IMR-90 fibroblasts overexpressing Runx2 did not produce a mineralized matrix. In contrast, Runx2 overexpression in osteoblast-like cell line UMR-106, led to the induction of osteoblast extracellular matrix protein (ECM) expression and mineralized nodule formation [Geoffroy et al., 2002]. Nonetheless, recent gene transfer studies with Runx2 overexpressing cells have supported its capacity to induce osteogenesis in vitro and in vivo [Byers and Garcia, 2004; Byers et al., 2005; Zhao et al., 2005]. To further evaluate vitro and in vivo effects of Runx2 and its modulation by BMP-2, we first investigated the regulation of Runx2 expression in vivo by rhBMP2 in a mouse calvariae model. We demonstrated that local administration of rhBMP-2 enhances Runx2 protein and increases periosteal thickness as a result of greater proliferation of osteoblasts. We isolated Runx2 cDNAs including splice variants [Bhat et al., 2000] and developed recombinant adeno-Runx2 viruses. The functionality of the splice variants was determined by transactivation of the OSE2 reporter, as well as the induction of mineralization in human mesenchymal stem cells. Finally, we show the in vivo osteogenic potential of Runx2 by a single injection of recombinant adeno-Runx2 virus into the bone marrow, through an intra-osseous model injection developed in adult rats by using adeno-BMP-9 [Bhat et al., 2001]. This work further supports the utility of the model and demonstrates the in vivo effect of intra-femoral injection of adeno-Runx2 in enhancing bone mineral density (BMD). In addition, it supports the feasibility of utilizing viral vectors and the intra-femoral injection approach to study effects of other potential osteogenic genes.

## MATERIALS AND METHODS

### In Vivo Calvaria Injection of rhBMP-2 and Immunohistochemistry

To measure the effect of BMP-2 on Runx2 gene expression in vivo, we modified the procedures developed by Mundy and co-workers [Mundy et al., 1999]. Recombinant human BMP-2 (rhBMP-2) or vehicle (2% DMS, 0.1% BSA in PBS) were injected into 6-week-old male Swiss mice (Taconic Farm, Germantown, NY) at 30 ug/kg/day subcutaneously three times per day for 1 and 5 days, over the right side of the calvaria along with vehicle control injection group. The animal protocol was approved by the Internal Animal Care and Use Committee and conformed to National Institute of Health guidelines. After sacrificing by $CO_2$ narcosis, mouse calvaria was removed intact, soft tissues were gently dissected, and the bones were fixed in 10% phosphate-buffered formalin for 24 h for further processing and analysis. Fixed calvaria was decalcified in TBD-2® decalcifying agent (Shandon-Thermo Inc., Waltham, MA) for 7–8 h and then dehydrated in graded alcohol. The calvaria was then bisected perpendicular to the sagittal suture through the central portion of the parietal bones parallel to the lambdoidal and coronal sutures and embedded in paraffin. Four to six 5 μm-thick representative, nonconsecutive step sections were cut and stained with hematoxylin and eosin (H&E). To further identify if BMP-2 could up-regulate the expression of Runx2 in vivo, immunohistochemistry of calvaria sections was performed by utilizing the Runx2 polyclonal antibody. Runx2 polyclonal antibody was generated in rabbits by using the N-terminal synthetic peptide PSTSRRFSPP-SSSLQPGKMS as the immunogen. The specificity of the Runx2 antibody to recognize Runx2 protein was first confirmed by the ability of the antibody to recognize Runx2 in the nuclear extract of COS7 cells (ATCC) only when they were transfected with Runx2 cDNA as compared to the untransfected control cells. In addition, the polyclonal antibody could recognize endogenous Runx2 in the nuclear cell fraction of mouse, rat, and human osteoblast

(HOB)-derived cell lines by Western blot analysis. In calvaria sections, the specificity of the Runx2 antibody was confirmed by the ability of the N-terminal immunogen peptide to block the binding during immunohistochemistry. The binding of antibody to Runx2 protein was assayed using an avidin-linked peroxidase system (Vector Laboratories, Burlingame, CA). Controls were comprised of samples with the avidin–peroxidase but without primary antibody.

### Cell Culture

Human embryonic kidney 293A cells (HEK293A) (ATCC, Gaithersburg, MD) were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Gibco/BRL-Invitrogen, Carlsbad, CA). One day before processing for adeno-cloning vector transfection or adeno recombinant infection, 60 mm culture dishes or T-150 flasks were plated with $2.5 \times 10^5$ cells/dish or $1 \times 10^6$ cells/flask, respectively, and incubated at 37°C $CO_2$ incubator. HEK-293A cells were involved in the recombinant adeno-Runx2 construction, purification, and amplification, as well as in Runx2 protein expression analysis. Immortalized HOB cell lines used (HOBO2O2 and HOBO3C5) [Bodine and Komm, 1999] were maintained at 34°C in DMEM/F-12 medium containing 10% (v/v) heat-inactivated fetal bovine serum, 1% (v/v) penicillin-streptomycin, and 2 mM glutaMAX-I (all from Invitrogen). Cells were plated at $9.8 \times 10^4$ cells/cm$^2$, allowed to attach overnight at 34°C, and transferred to 39°C in DMEM/F-12 medium containing 0.25% (wt/v) bovine serum albumin (Serologicals Proteins, Inc., Kankakee, IL), 1% (v/v) penicillin-streptomycin, 2 mM glutaMAX-I, 50 μg/ml ascorbate-2-phosphate (Wako Pure Chemical Industries Ltd., Osaka, Japan), and 10 nM menadione sodium bisulfite, vitamin $K_3$ (Sigma, St. Louis, MO). Forty-eight hours later, the cells were either infected with recombinant adenovirus for protein extraction or total RNA analysis.

### Cloning of Runx2 cDNAs and Development of Adeno-Runx2 Virus and 6x-OSE2-Reporter

Total RNA was isolated from human osteoblastic cells (HOBO3C5) [Bodine and Komm, 1999] using Trizol reagent (Invitrogen) as described by the manufacturer. To clone Runx2 with the N-terminal MASN (til-1) sequence [Stewart et al., 1997; Gao et al., 1998], the RNA was reverse transcribed using SuperScript RNase (−) Reverse transcriptase (Invitrogen) as described by the manufacturer. The PCR reactions were performed using the Advantage GC cDNA polymerase mix (Clontech, Mountain View, CA) using primers: (Forward) 5′-GATCT-AGAAGATCTCCATC**ATG**GCATCAAACAGC-CTCTTCAGCACAGTGACA-3′ and (Reverse) 5′-GGATCTAGAGTCGAC**TCA**ATATGGTCG-CCAAACAGATTCATCCATTCTGCCACT-3′.

In order to generate adeno-Runx2 virus, both the Runx2 full length and Runx2Δ4 splice variant were amplified by PCR from the pCR3.1 constructs so that the coding region will contain optimal Kozak consensus sequence just upstream to ATG initiation codon as well as the PmeI and BglII unique cloning sites. The PCR reaction was performed using the Advantage GC cDNA reaction buffer with the addition of PWO DNA polymerase (Boehringer Mannheim, Ridgefield, CT) and the primers: (forward) 5′-GAGAGATCTCCACCATGGCATCAAACAGC-CTCTTCAGCACAGTGACA-3′ and reverse 5′-GAGGTTTAAACTCAATATGGTCGCCAAAC-AGATTCATCCATTC-3′. The amplified DNA was cloned into the PmeI and BglII cloning site of the pQBI-AdCMV5GFP (Q-Biogene Inc., Carlsbad CA) adeno-shuttle vector. Recombinant adenoviruses were generated, purified, confirmed, and amplified, as previously described [Bhat et al., 1986; Paabo et al., 1987]. To demonstrate the transactivating functional activity of Runx2 protein produced by recombinant adeno-Runx2 virus, a reporter construct containing six tandem Runx2 binding OSE2 was fused to the luciferase gene. Specifically, six copies of the OSE2 [Ducy and Karsenty, 1995] sequences (5′-AGCCGCAGCTCCCAACCACA-TAT-3′) were synthesized and cloned upstream of the human basal osteocalcin promoter sequence (5′-AGGGTATAAACAGTGCTGGAGGC-TGGCGGGGCAGGCCAGCTGAGTCC-3′) that was pre-cloned into the pGL3-Basic luciferase reporter vector (Promega Corp. Madison, WI).

### Adeno-Runx2 Viral Infection and Reporter Assay

Adeno-Runx2 infection of HOB0202 cells was performed by plating cells in growth media at 250,000 cells per well in 6-well plates and incubated at 37°C for 48 h in 5% $CO_2$. The media was then removed and the cells were infected

with Runx2Δ4 adenovirus or control adenovirus in media containing 1% FBS (0.5 ml) (Hyclone, Logan, UT) and subsequently incubated at 37°C for 1 h. Following a 3 h incubation at 37°C, an equal volume of DMEM containing 20% FBS was added to each of the wells and then the cells were cultured for 24 h prior to harvest. The cells were washed twice with PBS and then lysed in reporter lysis buffer (Promega Corp). Luciferase assays using the Luciferase Assay System (Promega Corp) and the β-galactosidase assays using Galacto Reaction Buffer/Substrate Diluent and Light Emission Accelerator (Tropix, Bedford, MA) were performed with 25:l and 4:l of sample lysate, respectively.

### Nuclear Extract Isolation and Western Blot Analysis

Nuclear extracts were obtained as previously described [Sadowski and Gilman, 1993; Sadowski et al., 1993; Shuai et al., 1993]. Briefly, cold hypotonic buffer (20 mM HEPES pH 7.9, 20 mM NaF, 1 mM $NaH_2PO_4$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride, and mammalian protease inhibitor cocktail) (Sigma) with 0.2% NP40 were added to the dishes to lyse the cells. The samples were centrifuged at 16,000*g* for 20 s to pellet the nuclei. A volume of 5 M NaCl was added to the supernatant to yield a final concentration of 120 mM NaCl. The sample was then centrifuged for 20 min at 16,000*g*. The nuclear pellet was resuspended in hypertonic buffer (hypotonic buffer containing 420 mM NaCl and 20% glycerol) in 0.2% NP40. The pellets were incubated in the hypertonic buffer for 30 min at 4°C, centrifuged at 16,000*g* for 20 min and the supernatant was collected. The nuclear protein concentration was determined by the BCA protein assay (Pierce Biotech, Rockford, IL). The Western blot was performed by running the samples on a 4–12% Bis Tris SDS–PAGE (Invitrogen) and then transferring the proteins on to nitrocellulose filters (Invitrogen). Immunoblotting was performed using a rabbit polyclonal antibody recognizing the Runx2 N-terminal peptide. A Western Breeze kit (Invitrogen) was used as described by the manufacturer to detect the Runx2, primary antibody.

### Immunocytostaining

HEK293A cells were plated in mini 8-well Lab-Tec II chamber slides (Nalge Nunc Intl., Sewell, NJ) at low confluence ($\sim 5 \times 10^4$/well) to get a single cell population the next day. The cells were infected with adeno-Runx2 virus at low (1–5×) multiplicity of infection (MOI). Following overnight incubation, the cells were rinsed with cold PBS (+4°C), fixed with ice-cold methanol (−20°C), treated with Runx2 polyclonal primary antibody that was generated using the N-terminal synthetic peptide. After incubation at room temperature for 1 h, cells were rinsed with cold PBS and finally stained with the fluorescein isothiocyanate (FITC)-conjugated anti-rabbit goat IgG (Jackson Immono Research, West Grove, PA) as secondary antibody. During this indirect immunostaining process, controls without primary antibody treatment were also included in the test. Single cell staining was examined florescent microscopically at low (4×) and high (40×) magnifications to detect the nuclear expression of Runx2 protein.

### Human Mesenchymal Stem Cell Infection by Adeno-Runx2 and Mineralization Assay

Recombinant Runx2 adenoviral infection of human mesenchymal stem cells (BioWhittaker, Walkersville, MD) was performed by plating cells at 50,000 cells/well in a 6-well dish and then growing them to confluence (approximately 6 days) in growth media provided by BioWhittaker. The cells were then infected in 0.75 ml media containing 1% serum with 50 plaque-forming units (pfu) per cell of Runx2 adenovirus. Following a 1 h infection, the cells were washed with serum free media and then cultured with growth media or growth media with an osteogenic cocktail containing 100 nM dexamethasone, 50 μg/ml ascorbic acid, 10 mM β-glycerol phosphate, and 100 nM vitamin K for the indicated times. The formation of mineralized nodules was determined by alizarin red-S histochemical staining as previously described [Stanford et al., 1995]. Briefly, the cells and matrix were washed with calcium free PBS three times and then fixed with cold 70% ethanol for 1 h. The samples were then washed three times with distilled water and stained for 10 min with rotation using 40 mM alizarin red-S pH 4.2. The samples were then rinsed five times with distilled water and then once with PBS. Stained samples were photographed with a Nikon Eclipse TE300 Inverted microscope equipped with a DXM1200 digital camera.

### Intra-Osseous Adenoviral Administration

Recombinant virus was purified for in vivo tests by large-scale amplifications in HEK-293A cells and subsequent banding in cesium chloride (optical grade) in 50 mM Tris, pH 7.5 (Sigma). The concentrated virus band was collected and dialyzed against PBS pH 7.5 (Gibco/Invitrogen). The virus titer was determined by plaque assay in HEK293A cell monolayers. The intra-osseous injection study included two groups of seven mature (3-month-old), Sprague–Dawley rats weighing approximately 250 g (Taconic Farm). The animal protocol was approved by Internal Animal Care and Use Committee and conformed to National Institute of Health guidelines. Recombinant adeno-Runx2 virus and the control virus that has been inactivated by UV-irradiation were prepared to get a high titer ($10^{10}$ pfu/ml). The rats were anesthetized and a longitudinal anterio-medial incision of 2 cm length made on the right limb to expose the muscles covering the femur. Without cutting the muscle, an orifice of about 0.5 mm diameter was made through the marrow space in the exposed femur at a site approximately 15 mm away from the proximal end. A sterilized, 50 gauge, polyethylene tube was introduced into the marrow space through the orifice and fed ~2 mm in toward the distal end of the femur. To the test group of animals, the active adeno-Runx2 virus (25:l) was then injected into the catheter and followed by a 25:l flush of the citrate solution. The catheter was then quickly removed and bone wax used to seal the orifice in the femur. The muscles around the femur were then sutured back together and the skin closed with wound clips. Similarly, the control groups of rats were injected with the UV-inactivated adeno-Runx2 virus into the right femur. Three weeks post-surgery, the animals were euthanized via $CO_2$ asphyxiation and both femurs harvested and placed in 40% ethanol for bone density measurements.

### Bone Mineral Density Measurements

The BMD of the excised femurs was evaluated by Peripheral Dual Energy X-ray Absorptiometry (pDEXA) using a pDEXA scanner (Sabre$^{TM}$, Norland Corp, Fort Atkinson, WI). Both injected right femur and non-injected left femur of all the animals were harvested 3 weeks after the injection and pDEXA analysis for BMD measurement was carried out on (a) whole femur, (b) section 17 mm from distal end, (c) section 10 mm from proximal end, and (d) section 10 mm at the injected region (mid-shaft). The measurement scan ($4 \times 4$ cm) of the entire femur was performed at a scan speed of 5 mm/s with resolution of $0.2 \times 0.2$ mm. The BMD values were computed by the software as a function of attenuation of the dual beam (28 and 48 KeV) X-ray generated by the source underneath the specimen and the detector traveling along the defined area above the specimen. Analysis of variance with Dennett's *t*-test was used for statistical evaluation of the data. BMD was also assessed by peripheral Quantitative Computed Tomography (pQCT; XCT Research M, Stratec Medizintechnik, Florzheim, Germany). Each femur analysis included the distal end and the area comprised a 2.5 mm segment in the metaphysis located between 6.5 and 9 mm proximal from the distal end of the femur. Volumetric BMD (vBMD) determinations of the segment was achieved by analyzing five continuous 0.5 mm "slices" that completely covered the 2.5 mm segments. The CT scans were 0.5 mm thick, had a voxel (three-dimensional pixel) size of 0.07 mm, and consisted of 360 projections through the slice. The outer 55% of the bone was peeled away in a concentric spiral. The density of the remaining bone was reported as trabecular vBMD. Trabecular vBMD of each segment was calculated as the average vBMD of the five slices that comprised each segment. Analysis of variance with Dennett's *t*-test was used for statistical evaluation of the data.

## RESULTS

### rhBMP2 Protein Induces Runx2 Protein Expression in an In Vivo Calvaria Model

Although there have been multiple reports demonstrating that BMP-2 induces Runx2 mRNA expression in vitro, the responses have been cell type dependent. Furthermore, the experimental evidence on the regulation of Runx2 protein by BMP-2 in vivo has been limited. Therefore, to investigate the effect of BMP-2 on Runx2 protein expression in vivo, male Swiss mice were injected locally on calvaria with rhBMP-2 and immunohistochemical analysis was performed on the calvaria sections. Five days following rhBMP-2 injections, there was a marked increase in the thickness of the periosteum when compared with the

vehicle treatment (Fig. 1A,B, panels 1–4). The increased periosteal thickness was more pronounced with daily rhBMP-2 injections for 5 days than a 1-day injection (Fig. 1A,B, panels 2 and 3). Under these experimental conditions, using a Runx2 polyclonal antibody, we showed that rhBMP-2 injection increased the Runx2 expression within the periosteum. In addition, Runx2 expression markedly increased in parallel with the periosteal thickness following 5 days of daily injection of BMP-2 and harvesting the samples either 24 or 72 h after the last injection (Fig. 1B3,B4).

### Efficient Expression of Runx2 Protein by Adeno-Runx2 Virus

Having established that Runx2 is induced by BMP-2 in vivo and is associated with increased bone formation, we hypothesized that by increasing Runx2 expression locally in vivo we might activate osteogenesis. As the first step to overexpress Runx2, we sought to isolate the cDNAs from HOB cells. During this process, we isolated two Runx2 splice variant cDNAs (Fig. 2) from the immortalized HOB cell lines [Bodine and Komm, 1999]. One clone designated as Runx2 contained the full-length sequence and the second clone designated as Runx2Δ4 contained a deletion of exon 4 based on previously described exon structure [Mundlos et al., 1997]. We hypothesized that the exon 4 could play a role in the osteoinduction. To investigate on these aspects, by using the two splice variants we generated recombinant adeno-Runx2 and adeno-Runx2Δ4 viruses. Infection of HOB cell lines with adeno-Runx2 and adeno-Runx2Δ4 resulted in overexpression of both Runx2 and Runx2Δ4 proteins in the nuclear extract of the cells (Fig. 3A). As expected, in Western blot analysis the Runx2Δ4 protein is slightly smaller than the full-length Runx2 (Fig. 3A). In addition, the expression of both spliced forms of Runx2 was greater as compared to the control lane, which shows no significant level of endogenous Runx2. Further, the nuclear localization of recombinant Runx2 in HEK293A cells demonstrates the intense punctuate staining in the nucleus of a cell (Fig. 3B) infected with the adeno-Runx2, while staining was not observed the absence of the primary antibody for Runx2 (Fig. 3C).

### Runx2 and Runx2Δ4 Transactivate OSE2 Reporter Efficiently

It is established that Runx2 transactivates osteogenic genes or reporter genes containing the OSE2 sequences. Accordingly, to confirm that Runx2 and Runx2Δ4 produced by adeno-recombinants are functional, HOB cell lines were co-infected with adeno-OSE2-luciferase reporter virus and the adeno-Runx2 or adeno-Runx2Δ4 viruses. Both Runx2 and Runx2Δ4 viruses at different MOIs resulted in a dose-dependent induction (~12–25 fold) of the reporter activity (Fig. 4).

### Adeno-Runx2 Induces Mineralization in Human Mesenchymal Stem Cells In Vitro

To further evaluate the functional properties of Runx2 expression by adenovirus on

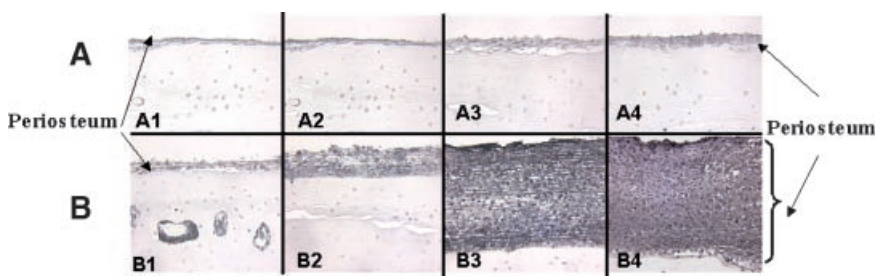


**Fig. 1.** Immunohistochemistry of BMP-2 treated mouse calvaria shows increased thickness of the periosteum and Runx2 expression. Mouse calvaria was injected subcutaneously with vehicle (**A panels**) or with rhBMP-2 (30 ug/kg/day) (**B panels**) for different durations and processed after 1 or 5 days of treatment. The sections were stained for thickness measurement and Runx2 protein expression by using Runx2-specific polyclonal antibody staining. Bound antibody was detected by avidin-linked peroxidase system (Vector labs). Panels A1 and B1 are from mouse calvaria with one injection and processed after 24 h; A2 and B2 are from mice calvaria with one injection and processed after 72 h. Panels A3 and B3 are form mice calvaria with five daily injections and processed after 24 h of last treatment. Panels A4 and B4 are from mice calvariae injected daily for 5 days and processed after 72 h after last injection. The panels indicate that with rhBMP-2 injection there is dramatic increase in the thickness of periosteum, number of osteoblasts, and consequently increased overall expression of Runx2 protein as compared to vehicle treatment.

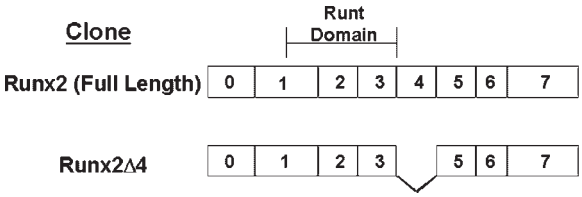


**Fig. 2.** Schemetic of Runx2 cDNAs structure showing 0-7 exons. Two splice variants were isolated from human osteoblast cells. Exon 0 represents one of the two isoforms of Runx2 and contains the MASN amino acids at the N-terminus. Runx2Δ4 is a splice variant lacking the exon 4. Reports indicate that exon 4 could interact partially with a co-repressor mSinA [Westendorf, 2006].

osteogenic differentiation, we infected the human bone marrow-derived mesenchymal stem cells (hMSCs) with adeno-Runx2 or adeno-Runx2Δ4. Twenty-one days following the infection in the presence of growth media, both adeno-Runx2 (Fig. 5A3,B3) and adeno-Runx2Δ4 (Fig. 5A2,B2) induced mineralization compared to the control virus (Fig. 5A1,B1). The effects of Runx2 overexpression on mineralization are enhanced when cells are cultured in osteoblast differentiating media containing ascorbic acid, β-glycerol phosphate, and dexamethasone (Fig. 5A5,B5).

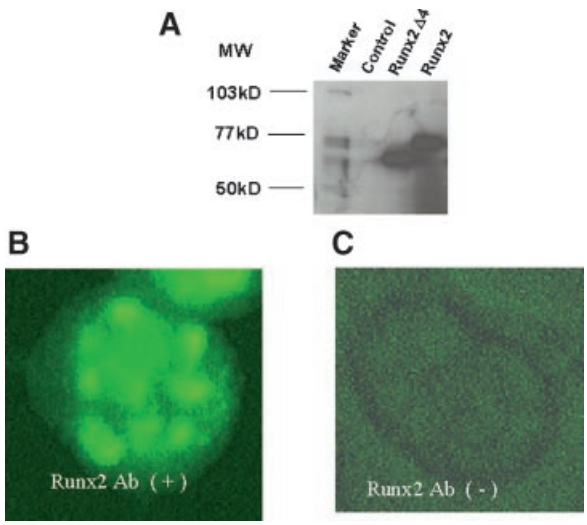


**Fig. 3.** Efficient Runx2 protein expression by adeno-Runx2 virus. **A**: Western blot analysis of adeno-Runx2/adeno-Runx2Δ4-infected human osteoblastic (HOB) cells. After 48 h of infection, nuclear fractions were subjected to SDS–PAGE. The Western blot using Runx2 polyclonal antibodies shows abundant expression of Runx2 and Runx2Δ4. **B**, **C**: HEK-293A cells are infected with adeno-Runx2 virus and after overnight incubation, the cells are processed for immunoflorescence detection by using primary antibody to Runx2 with FITC staining. The intense and punctuate staining was observed only in presence of Runx2 antibody (B) and not in its absence (C) indicates the strong expression Runx2 in the nucleus of a single cell.

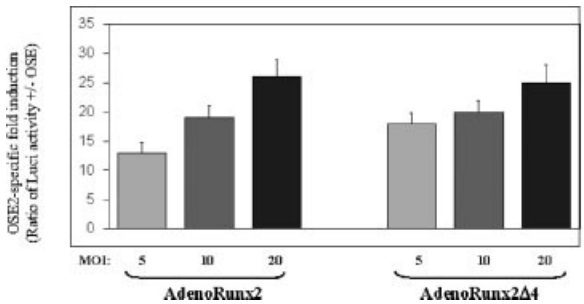


**Fig. 4.** Dose-dependent transactivation of OSE2-luciferase reporter by adeno-Runx2 and adeno-Runx2Δ4 viruses. Osteoblastic cells (HOB) cells were transfected with 6x-OSE2-luciferase reporter or control reporter without OSE2 and subsequently infected with either adeno-Runx2 or adeno-Runx2Δ4 viruses at three multiplicities of infections (MOIs). After 24 h incubation, the luciferase activity was measured. The ratio of luciferase activity with OSE2 and without OSE2 was calculated and represented in the bar graph as the fold induction that is specific to OSE2. Both forms of Runx2 show dose-dependent almost equal pattern of transactivation of the reporter.

### Evidence of Bone Induction In Vivo by Intra-Osseous Injection of Adeno-Runx2 Into Adult Rat Femur

To determine whether adeno-Runx2 infection of bone marrow stem cells is bone anabolic in vivo, a single dose of the virus was injected into the right femur bone marrow space of seven mature Sprague–Dawley rats per group and the femurs were harvested 3 weeks after the injection. All seven right femurs were obtained from the active adeno-Runx2-injected group but only four were successfully harvested without breaking from the UV-inactivated control group. The contra-lateral, non-injected left femur of each animal was also collected. BMD was analyzed by pDEXA on the whole femur: a section 17 mm from the distal end, a section 10 mm from the proximal end, and a section 10 mm at the injection site. Adeno-Runx2 overexpression resulted in a significant 14% increase in total BMD of the right femur compared to the control group that was injected with inactive adeno-Runx2 virus into the right femur ($0.193 \pm 0.005$ vs. $0.168 \pm 0.168$ g/cm$^2$, $P < 0.05$) (Table IA). Furthermore, there was a significant 15% increase ($0.197 \pm 0.005$ vs. $0.171 \pm 0.007$ g/cm$^2$, $P < 0.01$) in BMD at the section 10 mm from the proximal end of the femur compared to inactive control virus

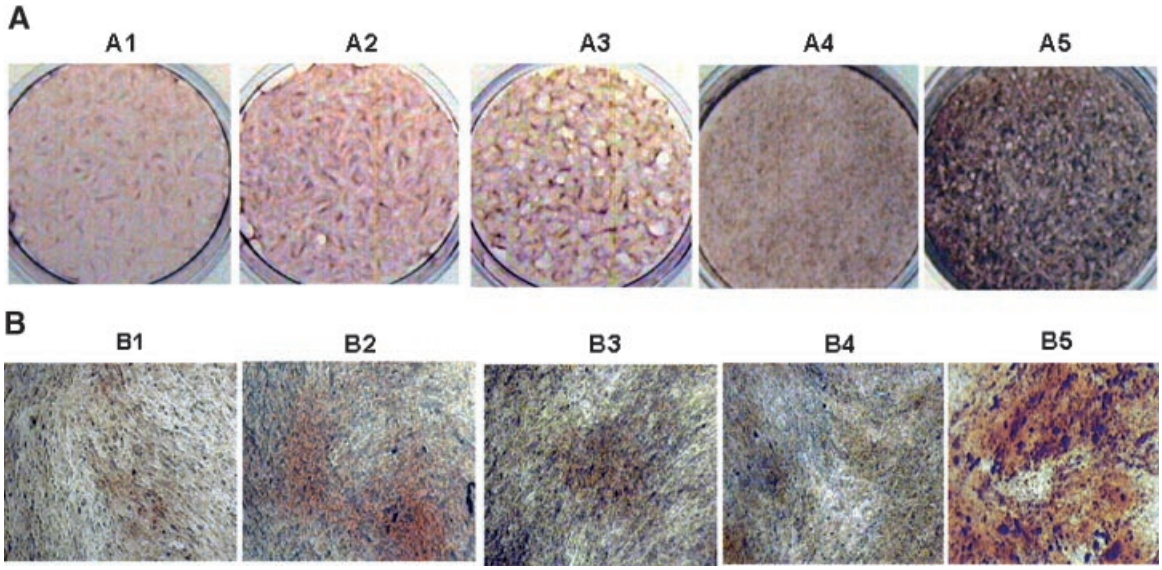


**Fig. 5.** Adeno-Runx2-mediated mineralization of human mesenchymal stem cells (hMSCs). **Part A** shows panels representing 5 full plates of hMSCs and **part B** represents correspondingly magnified region from each of the 5 plates. The hMSCs were infected with 50 plaque-forming units (pfu) per cell of adeno-Runx2 or adeno-Runx2Δ4 viruses and cultured with either growth media (A1–A3 and B1–B3) or growth media containing osteogenic cocktail (A4, A5 and B4, B5). Mineralization was measured after 3 weeks by Alizarin red staining of infected hMSCs. Panels A1 and B1, with control virus and growth medium; panels A2 and B2, adeno-Runx2Δ4 with growth medium; panels A3 and B3, adeno-Runx2 with growth medium; panels A4 and B4, control virus with osteogenic medium; panels A5 and B5, adeno-Runx2 with osteogenic medium which shows greatest staining of mineralized nodules.

(Table IB). Similar comparisons at a site 17 mm from the distal end of the right femur revealed a significant 12% increase ($0.177 \pm 0.005$ vs. $0.157 \pm 0.006$ g/cm$^2$, $P < 0.001$) in BMD with active adeno-Runx2 virus (Table IIA). Furthermore, there was no significant difference in BMD values of the 10 mm region at the injected site of the right femur with or without active virus (Table IIB). In all the groups, there was no significant effect in the uninjected contralateral left femur. In addition to the pDEXA analysis, pQCT analysis conducted on the trabecular bone with five slices taken at the distal end of the femur showed that administration of active virus had resulted in a significant increase in BMD (Fig. 6). There was 33% increase in trabecular BMD in slice #1 which is closet to the distal end and 36% in slice #5 which is the farthest from the distal end ($P < 0.05$). In addition, the mean BMD for all the

**TABLE I. Comparison of BMD Values of Adeno-Runx2 (Active/Inactive)-Injected Right Femur and Contra-Lateral Left Femur of Adult Rat**

| Injected adeno-Runx2 virus | Left femur[a] (not injected) | Right femur[a] (injected) | Difference between right and left femurs[a] |
|---|---|---|---|
| (A) Whole femur | | | |
| UV-inactivated (n = 4) | $0.178 \pm 0.002$ | $0.168 \pm 0.008$ | $-0.010 \pm 0.006$ |
| Active (n = 7) | $0.183 \pm 0.005$ | $0.193^* \pm 0.005$ | $0.010^* \pm 0.004$ |
| Difference between groups with active and inactivated virus | 0.005 (2.7%) | 0.025 (14%) | |
| (B) ~10mm section from proximal end | | | |
| UV-inactivated (n = 4) | $0.178 \pm 0.004$ | $0.171 \pm 0.007$ | $-0.006 \pm 0.005$ |
| Active (n = 7) | $0.188^* \pm 0.005$ | $0.197^{**} \pm 0.005$ | $0.009^* \pm 0.004$ |
| Difference between groups with active and inactivated virus | 0.010 (5%) | 0.026 (15%) | |

[a]Mean BMD (g/cm$^2$) ± SEM.
*$P < 0.05$ versus corresponding controls.
**$P < 0.01$ versus corresponding controls.

**TABLE II. BMD Values of Adeno-Runx2 (Active/Inactive)-Injected Right Femur and Contra-Lateral Left Femur of Adult Rat**

| Injected adeno-Runx2 virus | Left femur[a] (not injected) | Right femur[a] (injected) | Difference between right and left femurs[a] |
|---|---|---|---|
| (A) 17 mm from the distal end | | | |
| UV-inactivated (n = 4) | 0.165 ± 0.003 | 0.157 ± 0.006 | −0.008 ± 0.003 |
| Active (n = 7) | 0.172 ± 0.005 | 0.177** ± 0.005 | 0.006** ± 0.006 |
| Difference between groups with active and inactivated virus | 0.007 (4.2%) | 0.020 (12.7%) | |
| (B) ~10 mm section of virus-injected region | | | |
| UV-inactivated (n = 4) | 0.210 ± 0.004 | 0.213 ± 0.013 | 0.003 ± 0.011 |
| Active (n = 7) | 0.199 ± 0.006 | 0.217 ± 0.007 | 0.018* ± 0.004 |
| Difference between groups with active and inactivated virus | 0.011 (−5.2%) | 0.004 (1.87%) | |

[a]Mean BMD (g/cm$^2$) ± SEM.
*$P < 0.05$ versus corresponding controls.
**$P < 0.01$ versus corresponding controls.

slices showed a significant increase (36%, $P < 0.05$) with active adeno-Runx2 (221.95 ± 11.3 mg/cm$^3$) compared to the control group injected with the UV-inactivated Runx2 virus (162.7 ± 11.6 mg/cm$^3$) (Fig. 6).

## DISCUSSION

Recently, there has been significant interest in evaluating the osteogenic potential of BMP2 and Runx2 in vivo as a gene therapy approach for treating bone disorders [Byers et al., 2004, 2005; Kadowaki et al., 2004; Zheng et al., 2004; Zhao et al., 2005]. Even though BMP-2 up-regulates Runx2 in vitro, a combination gene transduction approach with Runx2 and BMP-2 has also been reported [Yang et al., 2003]. This strategy resulted in a synergistic osteoinductive effect in vivo and indicated that some aspect of the osteogenic pathway is complementary for BMP-2 and Runx2 expression. In vitro, it has been demonstrated that Runx2 interacts with BMP-2 responsive SMADs and together this enhances osteogenic differentiation. In addition, by using Runx2 null cells, it has been reported that the combination of both BMP2 and Runx2 have a synergistic effect in driving differentiation [Afzal et al., 2005; Young et al.,

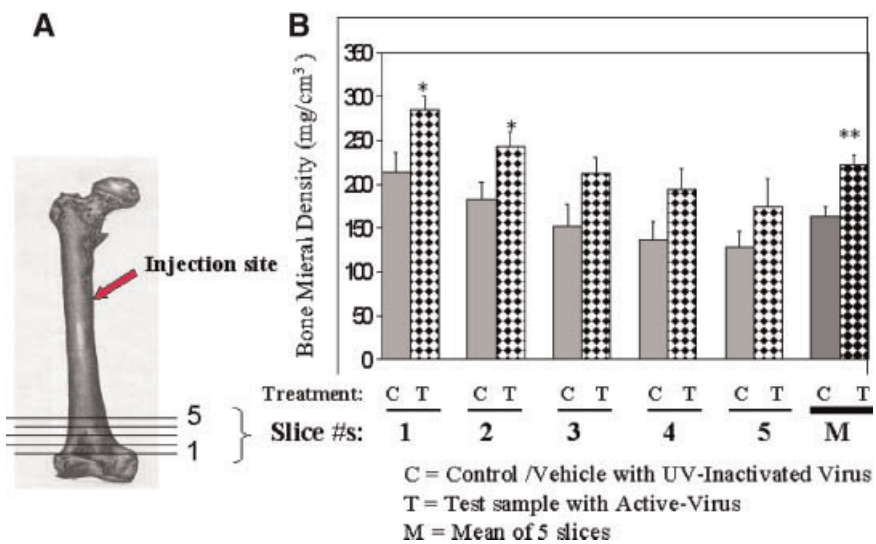


**Fig. 6.** pQCT measurements of adeno-Runx2 (active/inactive) virus-injected right femur (distal region) indicating the trabecular bone mineral density (BMD). **Part A** depicts the virus injection site in the femur and the five slices (#1–5) at the distal end taken during pQCT measurements. **Part B** bar graph shows the BMD values (mg/cm3) of the femurs that were either injected with vehicle control (with inactive adeno-Runx2 virus) or active adeno-Runx2 virus along with the mean of all the measurements under respective control or test group ($P < 0.05$).

2005; Liu et al., 2007]. Therefore, to evaluate Runx2 regulation by BMP-2 in vivo, we measured Runx2 protein expression in mouse calvaria with rhBMP-2 treatment, in a model known to induce bone formation [Mundy et al., 1999]. The time course experiments showed a clear increase in the overall expression of Runx2 protein as detected by immunohistochemical staining. The enhanced Runx2 staining was in parallel with the increased periosteal thickness and increased numbers of mature osteoblasts due to BMP-2 administration. These in vivo calvaria data suggest that Runx2 expression may be having an important role in stimulating the proliferation of periosteal osteoprogenitor cells as well as their differentiation.

Having established in vivo that rhBMP-2 induces expression of Runx2 concurrent with periosteal osteoblast proliferation, we investigated whether Runx2 could induce osteogenesis without BMP-2 addition in vitro and in vivo. We found that recombinant adeno-Runx2 infection of pluripotent hMSCs induces mineralization confirming reports by others using a murine multipotent C3H10T1/2 cell line, primary murine bone marrow mesenchymal stromal cells or even the calvarial cells isolated from Runx2 −/− mice [Pratap et al., 2003; Yang et al., 2003; Zheng et al., 2004; Zhao et al., 2005]. Furthermore, the current study with the full-length adeno-Runx2 and Runx2Δ4 demonstrates that exon 4 of Runx2 is not essential for its transactivation of Runx2-specific OSE2 reporter and osteoinductive activity in stem cells. Nonetheless, it will be interesting to investigate further on the role of exon 4, since reports indicate that it could interact with a co-repressor mSin3A [Westendorf, 2006]. For example, Runx2 lacking the C-terminal 96 amino acids also showed wild-type protein like osteogenic function despite the region contains a portion of the activation domain [Bae et al., 2007]. Since the isolation of Runx2Δ4 cDNA from another HOB-like cell (SaoS2) was also reported previously [Sevetson et al., 2004], we feel that such an isoform is a natural splice variant that is present in HOB-derived cells. Even though the previous report indicated that this splice variant is active in transcription assay, the current study for the first time demonstrates the osteogecic functional activity of Runx2Δ4 isoform. The data presented here confirm that adeno-Runx2 with or without exon 4 results in a signaling response that leads to the differentiation of stem cells into osteoblasts and induction of mineralization without the addition of BMP-2.

Previous in vivo knockout studies with Runx2 showed that it is important for normal bone formation during the early stages of development [Komori et al., 1997]. In contrast, transgenics overexpressing Runx2 in osteoblasts resulted in the inhibition of osteoblast maturation, high bone resorption, and osteopenia with multiple fractures [Ducy et al., 1999; Liu et al., 2001; Geoffroy et al., 2002]. These conflicting in vivo results indicate the complex role of Runx2 in bone metabolism and suggest the need for additional studies comparing different gene delivery approaches. One such example is the intra-femoral injection method used in adult animals to understand the potential postnatal effect of altering gene expression on bone remodeling and maintenance [Porada et al., 2000; Bhat et al., 2001; McCauslin et al., 2003]. We demonstrated that a single injection of high titer adeno-Runx2 virus after 3 weeks could result in a statistically significant increase in the total bone density of the adult rat femur in 3 weeks. Importantly, the increased BMD was evident at both the proximal and distal ends of the injected right femurs and away from the site of injection. This suggests a generalized infection of stromal or endosteal lining cells by the adeno-Runx2 virus within the injected bone marrow cavity. In addition, the lack of a significant femoral BMD increase in the control group with UV-inactivated virus suggests that the BMD increase we observed with the adeno-Runx2 is associated with the viral infection and gene expression process in the bone marrow or lining cells. This possibility is supported by the pQCT data in which there was a significant (36%) increase in the BMD of the trabecular region of the distal right femur injected with test virus. Taken together, the study shows that adeno-Runx2 can infect the bone marrow cells in vivo and induce bone formation within the bone microenvironment.

The in vivo data from intra-femoral adeno-Runx2 delivery in this study are consistent with the results of previous ex vivo gene therapy approaches wherein, adeno-Runx2 was first transduced into bone marrow stromal cells in vitro. Subsequently, by subcutaneous implantation of such cells into a critical-sized skull defect in mice, the Runx2 expression could induce bone formation without overexpression

of BMP-2 [Zheng et al., 2004]. However, the potential involvement of a low level of constitutively expressed BMP-2 cannot be ruled out. In fact, such a possibility has been implicated in a recent study in C3H10T1/2 cells in which by blocking endogenous BMP-2, it was demonstrated that BMP2 is essential for Runx2-mediated differentiation into osteoblastic cells [Yang et al., 2003; Phimphilai et al., 2006; Bae et al., 2007; Liu et al., 2007].

In this study, demonstrating bone formation in adult rat femur by a single injection of adeno-Runx2 suggests potential applications of such methods in orthopedic regenerative medicine as well as in diseases such as osteoporosis. Direct injection of adeno-BMP-2 or adeno-BMP-7 subcutaneously or into muscle have been reported to induce ectopic bone formation [Musgrave et al., 1999; Franceschi et al., 2000]. Our study demonstrates that recombinant gene delivery directly into the marrow cavity can circumvent the ex vivo cell transduction process used in other studies [Zheng et al., 2004; Zhao et al., 2005; Gersbach et al., 2006]. Similar indications were suggested in the limited reports on intrafemoral approaches using retroviral reporters [Porada et al., 2000; McCauslin et al., 2003]. Moreover, in some diseases such as multiple myeloma with osteolytic lesions it may be more desirable to inject the osteogenic recombinant virus or other therapeutics rather than isolating the bone marrow cells from the patients and utilizing ex vivo approaches [Giuliani et al., 2005]. Importantly, we show that the intrafemoral gene transduction approach in vivo could also be applicable to non-diffusible or non-secreted proteins such as Runx2. In addition, utilizing adenovirus type non-integrating gene delivery vectors could have advantages over retroviral vectors, which integrate and potentially lead to undesired chromosomal insertions or deletions during the regenerative gene therapy process.

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