

# Cbfa1/osf2 Transduced Bone Marrow Stromal Cells Facilitate Bone Formation *In Vitro* and *In Vivo*

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**Abstract.** It has been well established that core binding factor a-1/osteoblast-specific factor-2 (cbfa1/osf2) is a key regulator of osteoblast differentiation and function, however, it is not known whether it can induce bone formation *in vitro* and *in vivo*. To investigate the effect of cbfa1/osf2 on bone formation, we used a recombinant adenoviral vector carrying the mouse cbfa1/osf2 gene to transduce primary cultured bone marrow stromal cells (MSCs) of BALB/c mice. We found that Ad-cbfa1/osf2-transduced MSCs produced cbfa1/osf2 protein and differentiated into osteoblast-like cells. The transduced MSCs had increased alkaline phosphatase activity, increased expression of osteocalcin, osteopontin and bone sialoprotein, and increased matrix mineralization *in vitro*. To observe the induction of bone formation *in vivo*, MSCs transduced with Ad-cbfa1/osf2 were transplanted into a 5 mm diameter critical-sized skull defect in BALB/c mice, with type I collagen as scaffolding material. Healing of the defect in treatment and control groups was examined grossly and histologically at four weeks. Skull defects transplanted with Ad-cbfa1/osf2-transduced MSCs had an average of 85% osseous closure at four weeks. Control groups in which the defects were not treated (group 1), treated with collagen only (group 2), or treated with collagen and nontransduced MSCs (group 3) showed little or no osseous healing. These studies indicate that cbfa1/osf2 can induce osteoblast differentiation and bone formation both *in vitro* and *in vivo*, suggesting that MSCs transduced with the cbfa1/osf2 gene may be useful in treating bone defects.

**Key words:** Cbfa1/osf2 — Bone marrow stromal cells — Bone formation

Currently, there are no completely effective treatments for bone defect problems. Conventional therapies of autogenous bone grafts, allograft implants, and prosthetic implants have been used to treat these problems, and can promote reasonable clinical outcome. However, these methods are limited by supply and osteogenic potential. Recent advances in cell and molecular biology

have enabled researchers in the bone tissue engineering field to incorporate cell and gene therapies. This new therapy method offers a solution to bone defect problems, and is under active investigation at this time. Several investigators have shown exciting results using *ex vivo* and *in vivo* regional gene therapy in animal models [1]. These studies have introduced the genes for bone morphogenic proteins (BMPs), including BMP-2, BMP-4, and BMP-7 (OP-1) [2–4]. The BMPs are growth factors that have a powerful capacity to elicit new bone formation [5]. However, the regulation of osteoblast differentiation and bone formation requires the cooperation of many factors [6], including transcription factors [7]. Of these, core binding factor (cbfa1/osf2) has been identified as essential for osteoblast differentiation and bone formation.

Cbfa1/osf2 belongs to the runt-domain gene family, and has a DNA binding domain that is homologous to the *Drosophila* pair-rule gene *runt* [3]. This transcription factor is highly restricted to osteoblast cells [9]. Molecular and genetic evidence has demonstrated that it activates osteoblast differentiation during embryonic development in mice and humans [10]. Cbfa1/osf2 has been shown to regulate the expression of genes that characterize the osteoblast phenotype, including osteocalcin, osteopontin, type I collagen, bone sialoprotein, and collagenase-3. This regulation occurs when the cbfa1 DNA binding domain (cbfa1) [11] interacts with the related DNA sequence element, which is called osteoblast-specific element 2 (OSE2) [12]. In addition, cbfa1/osf2 can induce osteoblast differentiation of non-osteoblastic cells [13]. Patients heterozygous for mutations or deletions of cbfa1/osf2 develop cleidocranial dysplasia (CCD) [14], an autosomal-dominant condition characterized by abnormal skeletal genesis and arrest of osteoblast development [15]. Likewise, heterozygous inactivation of cbfa1/osf2 in mice leads to a CCD phenotype [16]. These data indicate that cbfa1/osf2 is an essential, indispensable regulator of osteoblast differentiation. Furthermore, the skeletons of cbfa1-transgenic

mice are normal at birth, but show decreased bone formation 3 weeks after birth, leading to osteopenia [17], suggesting that *cbfa1/osf2* plays a crucial role in osteoblast function [18].

Given that *cbfa1/osf2* is indispensable for osteoblast differentiation and function, we investigated induction of bone formation by *cbfa1/osf2* *in vitro* and *in vivo*. We found that transduction of a *cbfa1/osf2* construct into bone marrow stromal cells (MSCs) could induce osteoblast-like differentiation, expression of osteoblast proteins, and bone formation. In addition, we showed that transplantation of *cbfa1/osf2*-expressing MSCs into a critical-sized skull defect in mice could facilitate the healing of bone defects *in vivo*. These results suggest that *cbfa1/osf2* may be a good candidate for bone formation gene therapy.

## Materials and Methods

### Materials

The pBS KS<sup>-</sup> plasmid containing full-length mouse *cbfa1/osf2* cDNA was generously provided by Professor Gérard Karsenty (Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX). The AdEasy System was provided by Dr. Tong-Chuan He (Howard Hughes Medical Institute, Baltimore, MD). Type I collagen was from Sigma Chemical Co (St. Louis, MO). Restriction endonucleases (*Xho*I, *Xba*I, *Pme*I, *Pac*I and *Bam*HI) were obtained from BioLab Scientific Ltd (New Zealand). TRIZOL Reagent and LF2000 Reagent were obtained from Life Technology (Gaithersburg, MD). Histostain<sup>TM</sup> SP kit was obtained from Zymed Laboratories Inc (South San Francisco, CA). Polyclonal goat anti-mouse *cbfa1/osf2* primary antibody was obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). One Step RT-PCR kit was from Qiagen Inc (Stanford, CA). Dulbecco's modified Eagle medium (DMEM) and fetal serum was from HyClone (Logan, UT).

### Construction of Adenovirus Encoding Mouse *Cbfa1/osf2* Gene

For construction of adenovirus, the AdEasy system was used. This system is composed of a replication-defective E1 and E3-gene-deleted adenoviral vector (pAdEasy-1), and a shuttle vector (pShuttle-CMV), and has been previously described [19]. Briefly, the pBS KS<sup>-</sup> plasmid containing full-length mouse *cbfa1/osf2* cDNA was digested with *Xho*I and *Xba*I, resulting in a 3000 bp fragment containing the mouse *cbfa1/osf2* cDNA. The target fragment was inserted into the pShuttle-CMV vector. The resultant plasmid was linearized by *Pme*I digestion, and subsequently cotransformed into *E. coli* BJ5183 cells with the adenoviral backbone vector pAdEasy-1. Recombinants were selected with kanamycin, and recombinants were confirmed by restriction digestion with *Pac*I and *Bam*HI. Finally, the recombinant plasmids were linearized by digestion with *Pac*I and transfected into 293T cells with LF2000 Reagent for adenovirus packaging. Recombinant adenoviruses carrying the *cbfa1/osf2* gene (called Ad-*cbfa1/osf2*) were purified by CsCl gradient centrifugation. Viruses were titered by standard limiting dilution methods.

### Primary Cultures of Bone Marrow Stromal Cells

Bone marrow cells were obtained from the femurs and tibias of 6 to 8-week-old female BALB/c mice sacrificed by pentobar-

bitol sodium overdose. Femurs and tibias were removed and soft tissues were detached aseptically. Metaphyses were resected from both ends, the diaphyses were flushed with Hank's balanced salt solution, and bone marrow cells were collected. A suspension of bone marrow cells was obtained by repeated aspiration of the cell preparation through a 20-gauge needle. The cell suspension was centrifuged at 1000g for 5 minutes, resuspended in growth medium (DMEM supplemented with 10% fetal bovine serum, 2% penicillin/streptomycin), aliquoted into 250 ml tissue culture flasks (for transplantation *in vivo*), 6-well and 24-well culture plates (for examination *in vitro*), and cultured at 37°C and 5% CO<sub>2</sub>. Three days later, nonadherent cells were removed by replacing the medium. To confirm that the differentiation of MSCs into osteoblasts was due to the overexpression of *cbfa1/osf2*, in our experiments, the growth medium was not supplemented with dexamethasone, ascorbic acid, and β-glycerol phosphate because these reagents themselves may induce the differentiation of MSCs into osteoblasts.

### Infection of Bone Marrow Stromal Cells with Ad-*cbfa1/osf2*

Infection of bone marrow stromal cells was performed at 80% confluency. Cells were washed with PBS solution, and then incubated with Ad-*cbfa1/osf2* (multiplicity of infection = 60) in serum-free DMEM at 37°C. After 4 h, an equal volume of growth medium was added. For examination *in vitro*, 24 h later, cells were reseeded with complete medium and the medium was changed every 3 days. For transplantation *in vivo*, the cells were allowed to recover for 24 h prior to harvesting.

### Alkaline Phosphatase (ALP) Assay

An alkaline phosphatase assay was performed as previously described [20]. Confluent mouse MSCs in 24-well plates were transfected with Ad-*cbfa1/osf2*, Ad-LacZ or left alone. Cells were further incubated for another 7 days, then the cell layers were washed three times with TBS (50 mM Tris, pH 7.4 and 0.15 M NaCl) and stored at -20°C. For the assay, the cell layer from each well was scraped into 0.5 ml of 50 mM Tris, pH 7.4, and sonicated. ALP activity in the sonicate was measured using *p*-nitrophenyl phosphate (3 mM final concentration) as the substrate in 0.7 M 2-amino-methyl-1-propanol, pH 10.3, and 6.7 mM MgCl<sub>2</sub>. Protein in the cell layers was measured by Coomassie blue staining. The enzyme activity was expressed as *p*-nitrophenol produced in nmol/min/mg protein.

### Reverse Transcription-Polymerase Chain Reaction Analysis

Expression of *cbfa1*, osteopontin, osteocalcin, and bone sialoprotein mRNA in MSCs was analyzed by reverse transcription-polymerase chain reaction (RT-PCR). MSCs in 6-well culture plates were transfected with Ad-*cbfa1/osf2*, Ad-LacZ or left alone. Seven days after transduction, the cells were washed with PBS solution, and total RNA was isolated with the TRIZOL Reagent. RT-PCR was performed with the One Step RT-PCR kit; reverse transcription and PCR were carried out sequentially in the same tube. According to the manufacturer's instructions, 2 μg total RNA was used per RT-PCR reaction in a total volume of 50 μl. A typical thermal cycler program, including steps for both reverse transcription and PCR, was designed according to the One Step RT-PCR kit protocol. Conditions for PCR were optimized, and a condition suitable for all the genes was used. Each cycle consisted of 94°C for 45 sec, 55°C for 30 sec, and 72°C for 45 sec, and 32 cycles were performed. RT-PCR primers are listed in Table 1, and β-actin was reverse transcribed as an internal control. RT-PCR products were electrophoresed through 1.5% agarose gel, stained with ethidium bromide, and photographed.

**Table 1.** Primers used in reverse transcription-polymerase chain reaction (RT-PCR)

Gene (mouse)	Primer sequences	Product size (bp)
Cbfa1/osl2	5'-AGTCCCAACTTCCTGTGCT-3' 5'-GTGTCATCATCTGAAATACGC-3'	680
Osteocalcin	5'-AGCAGGAGGGCAATAAGG-3' 5'-CGTAGATGCGTTTGTAGGC-3'	165
Osteopontin	5'-ATGAGATTGGCAGTGATTTG-3' 5'-GTAGGGACGATTGGAGTGAA-3'	410
Bone sialoprotein	5'-AAATGGAGACGGCGATAGTTC-3' 5'-CTTCTTGGCAGTTGGAGTG-3'	217
$\beta$ -actin	5'-CTACGTGGGCCGCTCTAGGCA-3' 5'-CCGCCAGCCAAGTCCAGACGC-3'	461

### Immunohistochemical Examination and Matrix Mineralization Analysis In Vitro

The expression of cbfa1/osl2 protein was confirmed by immunohistochemical staining. MSCs were transduced with Ad-cbfa1/osl2, Ad-LacZ, or left alone. Seven days after transduction, cells in 24-well culture plates were washed with PBS and fixed with ice-cold acetone for 5 minutes. Immunohistochemical staining was performed with the Histostain<sup>TM</sup> SP kit. Briefly, the MSCs were incubated in 1% hydrogen peroxide to quench endogenous peroxidase. Following incubation in blocking solution, cells were incubated in polyclonal goat anti-mouse cbfa1/osl2 primary antibody at a 1:200 dilution, followed by incubation with a biotinylated rabbit anti-goat secondary antibody and a streptavidin-peroxidase amplification reagent. Immunoreactivity was detected with the 3,3'-diaminobenzidine (DAB) peroxidase substrate. The positive cells were stained brown. The percentage of cbfa1/osl2-positive cells was measured under a microscope at 200 X magnification by counting the number of cbfa1/osl2-positive cells and total number of cells in six random fields from each well (total of six wells).

In order to analyze the mineralization potential, MSCs in 6-well culture plates were transduced with either Ad-cbfa1/osl2 or Ad-LacZ, or left alone. Cells were further incubated for another 14 days in growth medium. The mineralized matrix was stained for calcium by Von Kossa staining. Cells were washed three times with PBS and fixed in ice-cold acetone for 5 minutes. After washing with water three times, cells were soaked in 5% AgNO<sub>3</sub> solution for 15 minutes, and then exposed to light for at least 20 minutes. The cells were reduced with 5% sodium hyposulfite for 5 minutes and washed with PBS.

### Skull Defect Assay

For the skull defect assay, we used the method described by Lee et al. [21]. Female BALB/c mice (8 mice for each group) were used, as this inbred line is unable to mount an apparent immune response against each other. The mice were anesthetized with pentobarbital sodium (0.07 g/kg) and placed in a ventral decubitus position on the operating table. After sterilization, the scalp was dissected to the skull and the periosteum was stripped. A 5-mm-diameter full-thickness circular skull defect, which is a nonhealing critical-sized defect, was created at the apex of the skull with a minityp drill, with minimal penetration of the dura. Collagen type I was used as a matrix. The mice were divided into four groups. Group 1 did not receive any treatment with the skull defect. Group 2 received collagen only. Group 3 received collagen mixed with nontransduced MSCs. Group 4 received collagen mixed with Ad-cbfa1/osl2 transduced MSCs. The collagen or collagen-wrapped MSCs were placed into the skull defect (groups 2, 3, 4), and the scalp was dosed with a 4-0 nylon suture. All mice were allowed food and activity *ad libitum* after the operation. Four

weeks after surgery, the mice were sacrificed and the skull specimens were dissected free from soft tissue for digital imaging. The area of the original defect was calculated with a 5-mm circular standard on the digital image, and the area of the closed defect was drawn and calculated with the ImageJ program (NIH). The area of the defect filled with new bone divided by the area of the original defect yielded the fraction of skull defect closure. Skull specimens were then fixed in 4% polymerisatum buffered with PBS solution for 24 h.

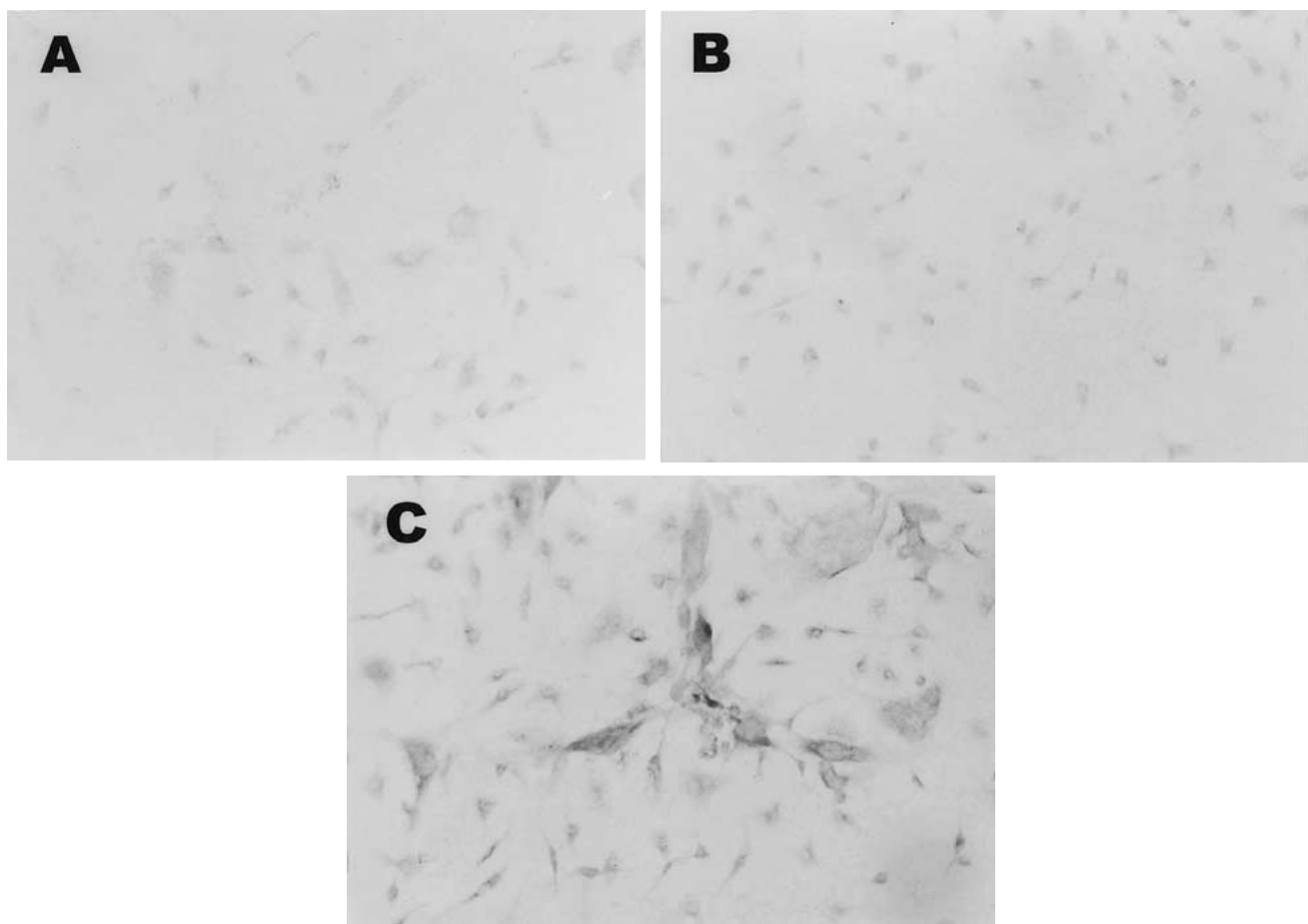
### HE Staining, Alcian Blue Staining, and Von Kossa Staining

The structure of new bone was examined by HE staining. The polymerisatum-fixed skull specimens were decalcified and paraffin-embedded, after which 5  $\mu$ m sections were cut using a microtome, dewaxed with xylene, and rehydrated through graded alcohols. Then HE staining was performed using the standard methods [22]. Alcian Blue staining was performed to examine if there was cartilage formation in the defect. Paraffin sections were deparaffinized in xylene, hydrated in graduated ethanols, and pretreated with 3% acetic acid for 3 min. Sections were then stained with 1% Alcian blue 8GX at pH 2.5 for 30 min, thoroughly rinsed with tap water, and counterstained with HE [23]. To examine the mineralization of bone, the nondecalcified polymerisatum-fixed specimens were embedded in methyl methacrylate and dibutyl phthalate (3:1 v/v). Five micrometer sections were cut using an ultramicrotome (Reichert Jung) and deplasticked with ethylene glycol monoethyl acetate. After rehydration through graded alcohols, the sections were stained with von Kossa staining, as described above, then washed with PBS solution and stained with eosin for viewing.

## Results

### Gene Expression In MSCs Transduced with pAd-cbfa1/osl2

Immunohistochemical staining (Fig. 1) showed the expression of cbfa1/osl2 protein in most Ad-cbfa1/osl2 transduced MSCs, with a positive percentage of  $80 \pm 7.6\%$ . The Ad-LacZ transduced MSCs and nontransduced MSCs did not show staining. RT-PCR was used to examine the gene expression of cbfa1/osl2, osteocalcin, osteopontin, and bone sialoprotein (Fig. 2). The mRNA expression of Cbfa1/osl2, osteocalcin and bone sialoprotein were detected in Ad-cbfa1/osl2 transduced MSCs, but not in Ad-LacZ transduced MSCs and nontransduced MSCs. Expression of osteo-



**Fig. 1.** Representative immunohistochemical staining of primary cultured MSCs. Primary cultured MSCs were transduced with Ad-cbfa1/ostf2, Ad-LacZ or left alone. Seven days after transduction, expression of cbfa1/ostf2 was detected by immunohistochemical staining. No expression of cbfa1/ostf2 was

detected in Ad-LacZ transduced MSCs (A) and parent MSCs (B). In Ad-cbfa1/ostf2 transduced MSCs (C), there was notable expression of cbfa1/ostf2. The experiment was repeated three times, each in triplicate ( $\times 100$ ).

osteo-*pontin* mRNA was detected in Ad-cbfa1/ostf2 transduced MSCs, Ad-LacZ transduced MSCs and nontransduced MSCs with different levels. Expression of osteo-*pontin* in Ad-cbfa1/ostf2 transduced MSCs is much higher than that in Ad-LacZ transduced MSCs and nontransduced MSCs. These results suggested that osteoblast differentiation was triggered by transduction of Ad-cbfa1/ostf2.

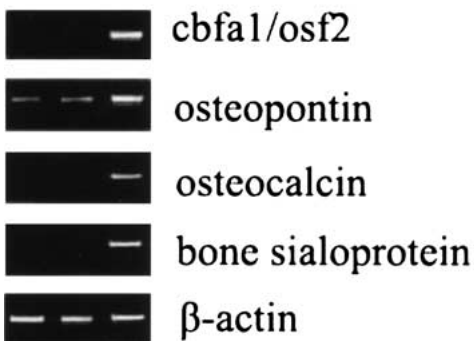
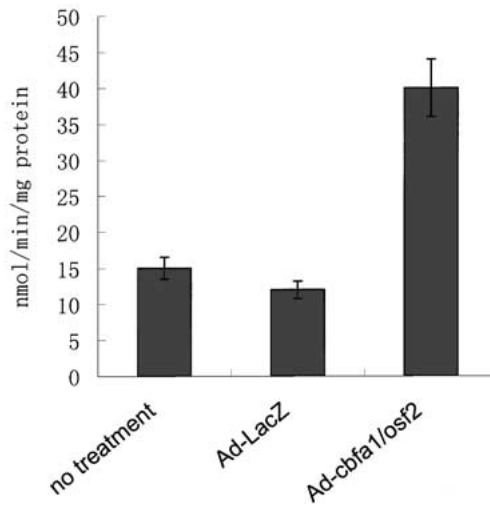
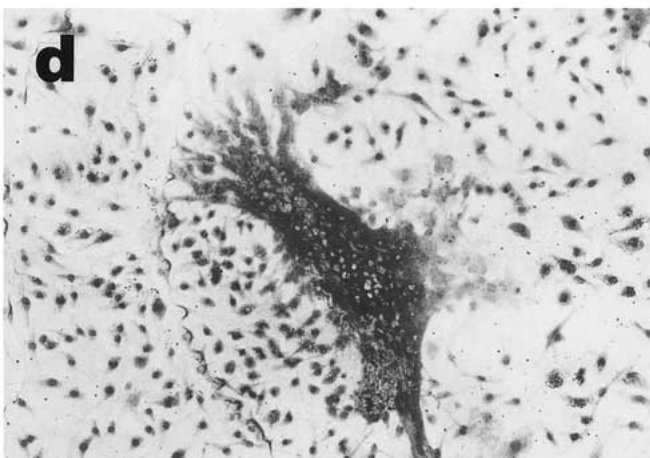
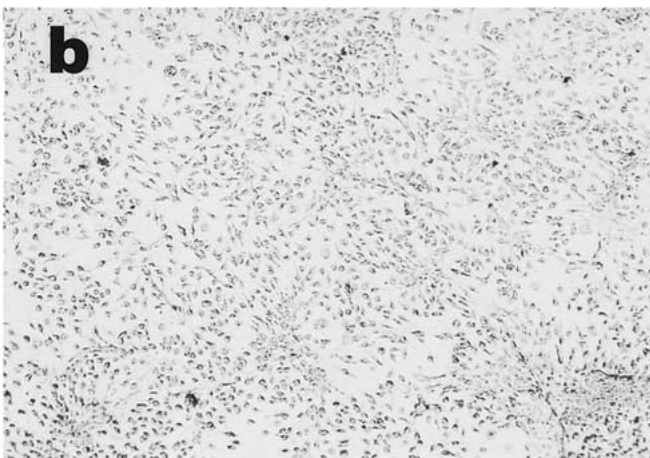
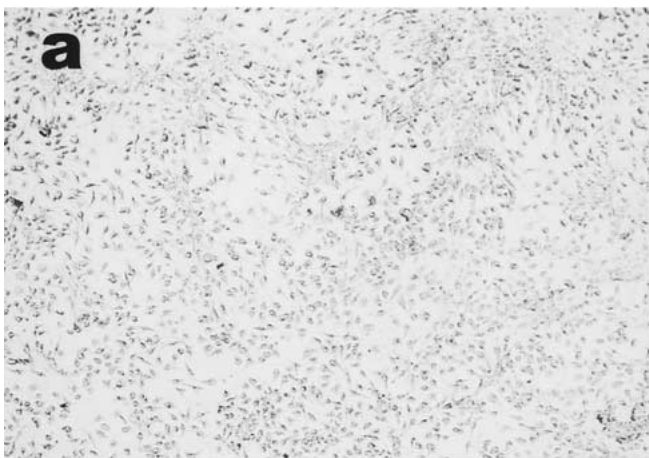
#### *ALP Assay and Examination of Mineralization*

Since ALP has been implicated as a marker of osteoblast differentiation [24], the ALP activity of transduced and nontransduced MSCs was measured (Fig. 2). We found that the ALP activity of Ad-cbfa1/ostf2 transduced MSCs was about 40 nmol/min/mg protein, which was much higher than that of Ad-LacZ transduced MSCs (about 12 nmol/min/mg protein) and nontransduced MSCs (15 nmol/min/mg protein). Next, von Kossa staining was performed to allow visualization of bone mineralization (Fig. 2). We found that the matrix of the MSCs transduced with Ad-cbfa1/ostf2 became quite

mineralized by 14 days after transduction, whereas the matrix of Ad-LacZ transduced MSCs and nontransduced MSCs was little mineralized at that time. These results suggest that pAd-cbfa1/ostf2 can induce the differentiation of MSCs to osteoblasts that are able to mineralize.

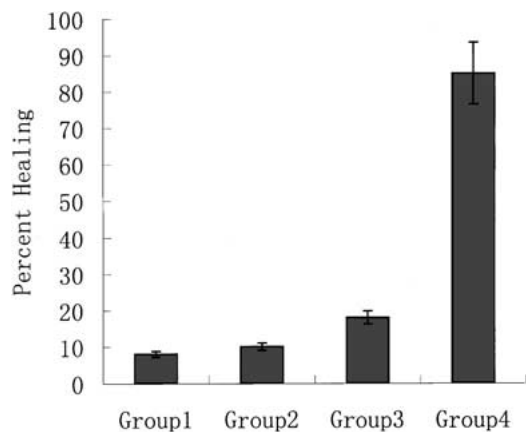
#### *Skull Defect Healing Induced by pAd-cbfa1/ostf2 Transduced MSCs*

We examined the healing of the skull defects by gross image analysis and histological staining. Image analysis (Fig. 3) showed that group 1 (untreated defect) and group 2 (defect treated with collagen alone) had little osseous healing at four weeks. Group 3 (defect treated with collagen and nontransduced MSCs) showed slight healing at four weeks (average 18%). Finally, group 4 (defect treated with collagen and Ad-cbfa1/ostf2 transduced MSCs) showed average 85% osseous healing by 4 weeks. In all groups, where no osseous healing had occurred, the defect was closed by soft tissue membrane.

**A****a b c****B****c**

**Fig. 2.** **A.** Analysis of expression of mRNA for cbfa1/osf2, osteopontin, osteocalcin, bone sialoprotein by RT-PCR. Four kinds of mRNA were expressed in Ad-cbfa1/osf2 transduced MSCs(c). In Ad-LacZ transduced MSCs(b) and non-transduced MSCs(a), expression of mRNA for osteopontin was detected as well, but the expression is much lower than that in Ad-cbfa1/osf2 transduced MSCs. Expression of mRNA for osteocalcin and bone sialoprotein was detected only in Ad-cbfa1/osf2 transduced MSCs. Experiments were repeated independently three times. **B.** Detection of alkaline phosphatase (ALP) activity. Primary cultured MSCs in 24-well culture plates were transduced with Ad-cbfa1/osf2, Ad-LacZ or left alone. Seven days after transduction, Ad-cbfa1/osf2 trans-

duced MSCs exhibited higher ALP activity than Ad-LacZ transduced cells and parent cells ( $P < 0.01$  vs both of groups). Values are means  $\pm$  SD,  $n = 6$ . Experiments were repeated three times. **C.** Representative von Kossa staining of primary cultured MSCs. Primary cultured MSCs were transduced with Ad-cbfa1/osf2, Ad-LacZ or left alone. After additional growth for 2 weeks in growth medium, the cells were stained for mineralization by von Kossa staining. Individual mineralized nodules with irregular shapes were present in Ad-cbfa1/osf2 transduced MSCs (c,d). No detectable mineralized nodules were present in Ad-LacZ transduced MSCs (b) and non-transduced MSCs (a). Experiments were repeated three times (a,b,c  $\times 40$ ; d  $\times 200$ ).



**Fig. 3.** Percent osseous closure of the skull defect in the four groups. Group 1 = untreated defect, Group 2 = defect treated with collagen alone, Group 3 = defect treated with collagen and nontransduced cells, Group 4 = defect treated with collagen and Ad-cbfa1/osf2 transduced cell. Six animals were used for each group. The percent osseous closure of the skull defect in group 4 was much higher than the control groups ( $P < 0.05$ ). Values are means  $\pm$  SD. Experiment was repeated three times.

Histological specimens were stained with HE and Alcian Blue to further visualize healing (Fig. 4). Typical specimens from groups 1 and 2 showed no evidence of osseous healing. Specimens from group 3 showed slight formation of new bone, while typical specimens from group 4 showed nearly complete osseous closure of the defect. Collagen (groups 2, 3, and 4) was completely absorbed by 4 weeks. No cartilage was observed in the skull defects where there was new bone formed (groups 3 and 4). The results of von Kossa staining (Fig. 5), which was performed to identify mineralized bone, were similar to those of the HE staining. There was little or no mineralized bone in the defect in any of the control groups (1, 2, and 3). However, in the experimental group (group 4), most of the defect was closed by mineralized bone.

## Discussion

Analyses of many cell lineages such as the myoblast, the adipocyte, or the osteoclast lineage have all shown that cell differentiation is triggered by cell-specific transcription factors acting as gene expression switches [25]. *In vitro* experiments and animal studies have shown that cbfa1/osf2 is a specific transcriptional activator and a molecular switch of osteoblast differentiation. Recent data suggest that the genetic mechanisms that regulate fetal skeletogenesis also regulate adult skeletal regeneration, pointing to important regulators of osteoblast differentiation and ossification. The regenerative capacity of adult bone may depend upon the re-induction of the molecular pathways that mediate osteogenesis

during fetal development [26]. Cbfa1/osf2 gene, essential for bone development, is re-induced during adult bone repair [27] suggesting that cbfa1/osf2 may be an important potential target for gene therapy leading to bone regeneration.

For osteoinductive regional gene therapy, *ex vivo* (harvesting, manipulation, and reimplantation) techniques offer the most potential target cells. Several specific types of cell have been suggested for this work, including bone marrow stromal cells (MSCs), myoblasts, or skin fibroblasts [28]. MSCs are an attractive option as they can be easily expanded in cell culture, and they can differentiate into osteoblasts, chondroblasts, and fibroblasts [29]. In our study, primary cultured MSCs from BALB/c mice were used. Since this inbred mouse line shows almost no immune response among individuals, there was little chance that the mice would reject the transplanted MSCs. For transfection of the cultured MSCs with cbfa1/osf2, we used the pAdEasy1 vector, a replication-defective, E1 and E3-deleted adenoviral vector containing a CMV promoter. Unlike retroviruses and adeno-associated viruses, adenoviruses do not integrate into the target cell's genome. Adenoviral vectors are efficient, can be produced in high titers, achieve high levels of expression following transduction, and can transfer genes to both replicating and nonreplicating cells. The duration of expression is generally weeks to months, which is an appropriate timeframe for bone healing. Therefore, we used this system to analyze the effect of cbfa1/osf2 on bone formation *in vitro* and *in vivo*.

Previous studies have reported that cbfa1 could induce the expression of principal osteoblast-specific genes encoding for osteocalcin, osteopontin, and bone sialoprotein in non-osteoblastic cells, and cbfa1/osf2 binding sites were found in their promoter regions of these genes [12, 30]. Overexpression of cbfa1/osf2 in nonosteogenic cells such as C3H10T1/2 and skin fibroblasts induced them to express osteoblast-related genes [31]. Furthermore, it has been shown that disruption of cbfa1/osf2 by antisense oligonucleotides in osteoblast cultures inhibited expression of osteoblastic markers and formation of mineralized nodules [32]. In our *in vitro* studies, transduction with Ad-cbfa1/osf2 induced the MSCs to express osteoblastic proteins (ALP, osteocalcin, osteopontin, and bone sialoprotein), and to increase the formation of mineralized nodules in cultures.

For our *in vivo* experiments, MSCs transduced with the cbfa1/osf2 gene were transplanted into murine skull defects. Although parent MSCs have been shown to maintain osteogenic potential after transplantation *in vivo*, with extended culture they display a tendency to lose their multipotentiality, proliferation potential, and *in vivo* bone-forming efficiency [33]. The amount of bone formed in the non-transduced MSC control was not sufficient for bone repair within the 4-week time frame

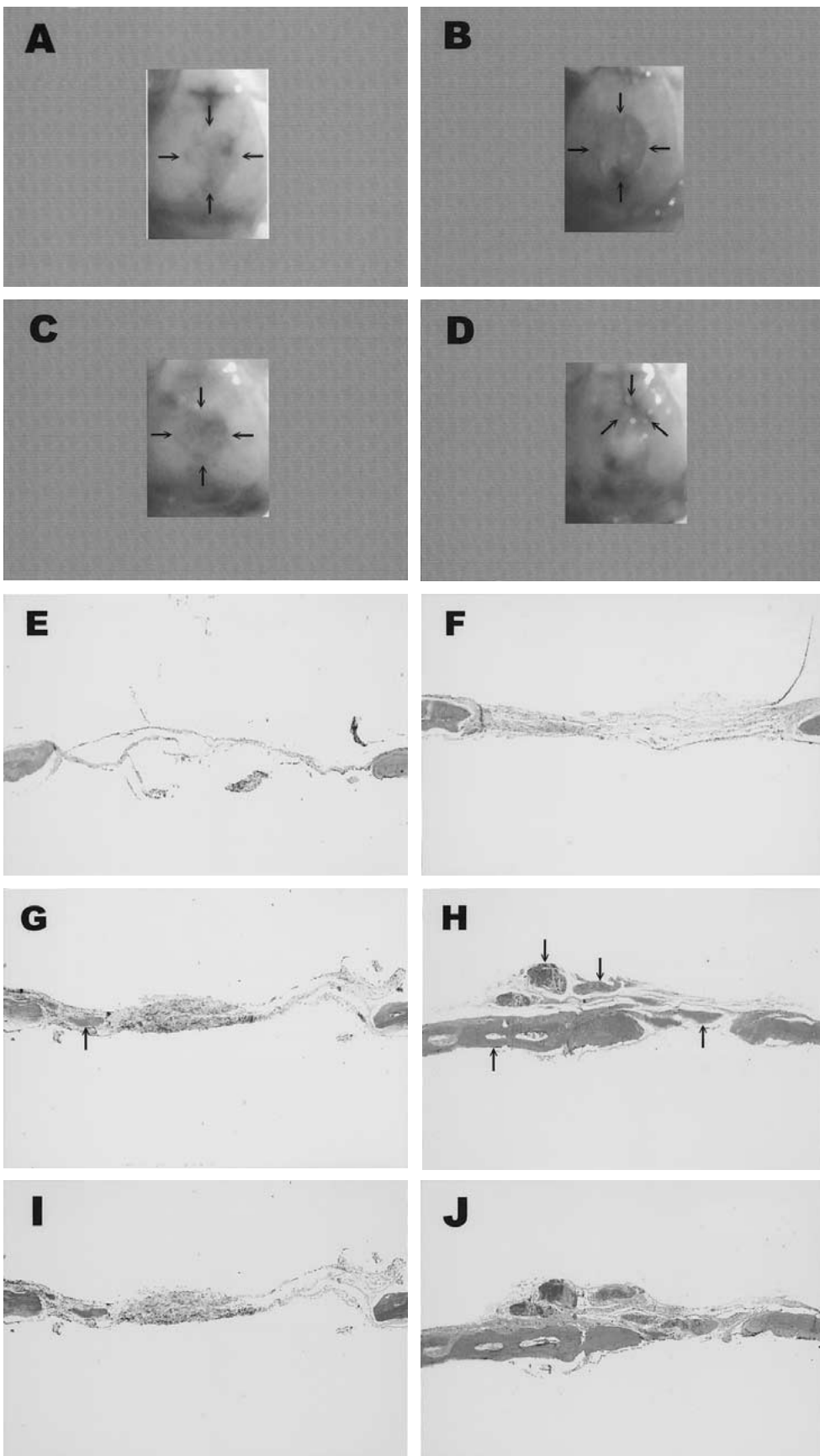
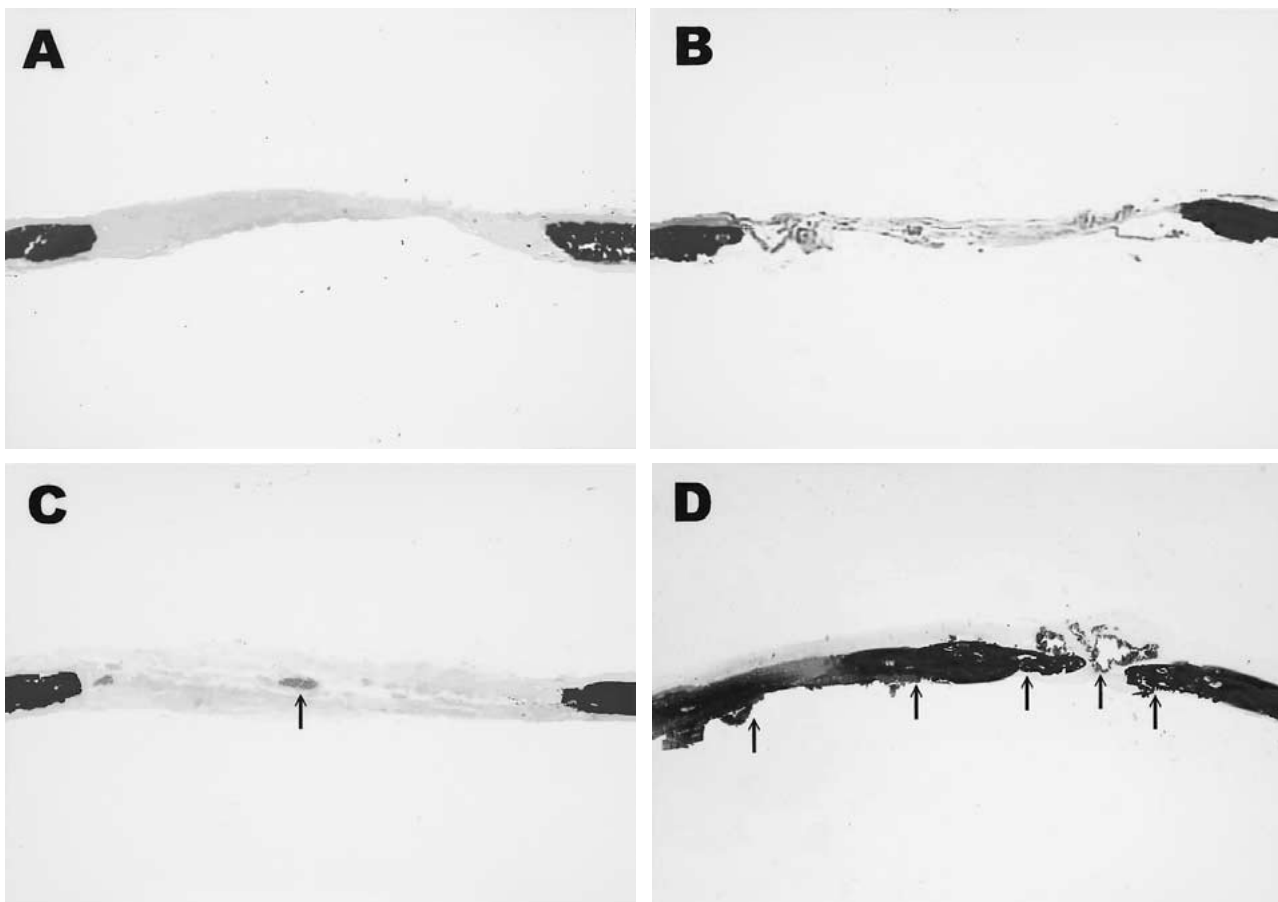


Fig. 4.



**Fig. 5.** (A), (B), (C), and (D) shows representative Von Kossa staining of coronal nondecalcified sections from group 1 (defect not treated), group 2 (defect treated with collagen alone), group 3 (defect treated with collagen and nontransduced cells), and group 4 (defect treated with collagen and Ad-cbfa1/osf2

transduced cells), respectively. Six animals were used for each group. Experiments were repeated three times. (A) and (B) shows no new bone formation. (C) shows minimal newly formed bone (arrow). (D) indicates that robust bone was formed (arrows), which almost closes the defect. ( $\times 20$ ).

examined. In contrast, 4 weeks after transplantation of Ad-cbfa1/osf2 transduced MSCs, the test group showed an average defect osseous repair of 85%. This indicates that transduced MSC transplants expressing cbfa1/osf2

are capable of bone formation. However, our healing was not at the 95–100% level described by Lee et al. [21] in their report of Ad-BMP-2 transduced muscle-derived cells healing similar skull defects in the same time frame. This may be due to the fact that cbfa1/osf2 is a transcription factor and therefore works within the host cell. In contrast, BMP-2 can be secreted into the extracellular matrix and affect the surrounding cells. However, our results suggest that cbfa1/osf2 is an effective inducer of bone formation that may be useful in healing adult bone defects.

Although MSCs can differentiate into both osteoblasts and chondrocytes, in this study we found no cartilage formation after transplantation of pAd-cbfa1/osf2 transduced MSCs into the skull defect. This might be due to the following factors. First, cbfa1/osf2 is an osteoblast-specific transcription factor that induces MSCs to differentiate directly into the osteoblast phenotype without an intermediate chondrocyte step. Second, bone formation of calvarium occurs with intramembranous ossification, suggesting that bone for-

**Fig. 4.** Representative images of gross specimens (A, B, C, D). A = group 1 (defect not treated), B = group 2 (defect treated with collagen alone), C = group 3 (defect treated with collagen and nontransduced cells), D = group 4 (defect treated with collagen and Ad-cbfa1/osf2 transduced cells), respectively. In A, B, and C, the defects (arrows) were closed with soft tissue membrane. Only a small defect was left in D, E, F, G, and H show representative HE staining of coronal sections through the skull defect area of group 1, 2, 3, and 4, respectively. Histological evaluation showed no bone formation in group 1 and 2, minimal bone formation (arrow) in group 3 and much new bone formation (arrows) in group 4. I and J show representative Alcian Blue staining of coronal section through the skull defect area of groups 3 and 4, respectively; there was no notable cartilage formation. Six animals were used for each group. Experiments were repeated three times (E, F, G, H, I, J  $\times 20$ ).



mation within the defect might be affected by local microenvironment. Third, skull has an abundant blood supply with high oxygen tension, whereas low oxygen tension is known to effectively induce cartilage formation [34].

Here we have reported the successful transduction of MSCs with vectors containing *cbfa1/osf2*, and the analysis of osteoblast differentiation and skull defect healing by these cells. We have shown that *cbfa1/osf2* can facilitate bone formation *in vitro* and *in vivo*, and suggested that *cbfa1/osf2* may be a valuable candidate for bone regeneration gene therapy. However, further studies will be needed to optimize the carriers and target cells, as well as to increase the expression of *cbfa1/osf2* before this important transcription factor will be of clinical use in treating nonhealing bone defects, bone fractures, spine fusions, and even osteoporosis.

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