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Osterix Overexpression in Mesenchymal Stem cells Stimulates Healing of Critical-Sized Defects in Murine Calvarial Bone

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Abstract

Osterix (Osx) is a zinc-finger-containing transcription factor that is expressed in osteoblasts of all endochondral and membranous bones. In Osx null mice, osteoblast differentiation is impaired, and bone formation is absent. We hypothesized that overexpression of Osx in bone marrow–derived mesenchymal stem cells (BMSCs) would enhance osteogenic differentiation during bone regeneration *in vivo*. Overexpression of Osx in mouse BMSCs was achieved using retroviral infection together with a green fluorescent protein (GFP) vector to monitor transduction efficiency and determine the source of regenerative cells in implantation studies. Bone regeneration *in vivo* was evaluated by implanting BMSCs overexpressing Osx into 4-mm calvarial bone defects in adult mice using type I collagen sponge as a carrier. New bone formation in the defects was quantified using radiological and histological procedures 5 weeks after implantation. The results showed that implantation of Osx-transduced BMSCs resulted in 85% healing of calvarial bone defects as detected using radiological analyses. Histological examination of the implants demonstrated that the Osx-transduced group exhibited amounts of newly formed bone that was five times as high as in a group transduced with the empty vector. Immunohistochemistry for GFP showed positive immunoreaction localized to areas of newly engineered bone in the Osx-transduced group. Immunohistochemistry with antibodies against the extracellular matrix protein bone sialoprotein resulted in strong staining in areas of new bone formation. In addition, the clonal BMSCs showed an osteogenic potential similar to that of primary cultures of BMSCs, suggesting the usefulness of this model in bone tissue engineering. These results indicate that *ex vivo* gene therapy of Osx is a useful therapeutic approach in regenerating adult bone tissue.

INTRODUCTION

Autologous bone grafts are the preferred surgical approach to repair massive bone defects because of their osteogenic and osteoinductive properties.^{1,2} However, the supply of suitable bone is limited, and its collection is not only painful but also frequently associated with a risk of infection, hemorrhage, cosmetic disability, and nerve damage.^{1,2} Alternatively, tissue engineering approaches are attempting to heal bone lesions above a critical size by using resorbable scaffolds supplemented with regeneration-competent cells, such as embryonic stem cells derived from the inner cell mass of blastocyst-stage embryos or adult bone marrow–derived mesenchymal stem cells (BMSCs).^{3–5} Because of ethical controversy about the use

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of embryonic stem cells, research focus has shifted toward *ex vivo* expansion of adult BMSCs for their use in bone regeneration studies.

BMSCs are adherent cells of non-hematopoietic origin that proliferate and exhibit many of the characteristics attributed to bone marrow stroma *in vivo*.^{6,7} Within the diverse population of BMSCs, there exists a subset of stem cells that have been called mesenchymal stem cells or skeletal stem cells that maintain the multipotential, differentiative features that define a stem cell. These cells are capable of self-renewal and can differentiate into several phenotypes, including bone, cartilage, and adipocytes.^{8–10} BMSCs are capable of forming the stroma that supports myelopoiesis. *In vitro* expanded BMSCs are a rich source of osteogenic progenitor cells that are capable of promoting the repair or regeneration of skeletal defects. Under optimal culture condition, billions of BMSCs can be generated from a limited amount of skeletal material.¹¹ After their isolation and expansion in tissue culture, BMSCs are capable of repairing bone defects upon their transplantation into various animal models.^{12–14} However, long-term *ex vivo* expansion of BMSCs progressively leads to a decrease in their proliferation and loss of their osteogenic potential.^{6–8} One approach that can be used to enhance and maintain a robust osteoblastic differentiation capacity in BMSCs is through the use of *ex vivo* gene delivery,^{5,12} in which viral vectors are used to genetically modify osteoprogenitor cells to overexpress soluble osteoinductive factors such as bone morphogenetic proteins (BMPs).^{15, 16} Overexpression of BMPs in osteoprogenitor cells has allowed for the successful repair of critical-sized defects in model systems.^{5,12,17} However, diffusional limitations, unintended targeting of neighboring non-osseous tissues, and the requirement for cell surface receptors and cofactors may limit the effectiveness of this approach. To achieve an alternative to the *ex vivo* gene therapy of BMPs, other bone regeneration studies have focused on overexpressing core binding factor $\alpha 1$ (Cbfa1)/runt-related gene (Runx)2¹⁸ in BMSCs for transplantation.^{19, 20} As an essential transcription factor in osteoblast differentiation and bone formation,^{21,22} Cbfa1/Runx2 is expressed in an early multi-potential mesenchymal cell population that can give rise to chondrogenic, osteogenic, and dentinogenic tissues, as well as other lineages.²³ Cbfa1/Runx2 enhances osteoblast differentiation at an early stage and inhibits the late stage of osteoblast maturation.^{18,21,24} Overexpression of Cbfa1/Runx2 in BMSCs has been reported to enhance osteoblastic differentiation and mineralization *in vitro*^{19,25} and promote bone formation in heterotopic (subcutaneous and intramuscular) and orthotopic (bone defect) sites.^{19,20} Recently, it has also been demonstrated that a zinc-finger-containing transcription factor named osterix (Osx) is critical in osteoblast differentiation and bone formation.²⁶ In Osx-null mice, no bone formation occurs. Similarly, cells in the periosteum and in the condensed mesenchyme of membranous skeletal elements fail to differentiate into osteoblasts. These cells do, however, express Cbfa1/Runx2. In contrast, Osx is not expressed in Cbfa1/Runx2-null mice. Thus, Osx acts downstream of Runx2/Cbfa1 to induce differentiation of preosteoblasts into fully functional osteoblasts,²⁶ and overexpression of Osx has been shown to be sufficient to guide differentiation of murine embryonic stem cells toward the osteoblastic lineage *in vitro*.²⁷ Our recent study also showed that Osx could enhance *in vitro* proliferation and osteogenic lineage commitment of BMSCs.¹⁰ To extend these observations, the current studies were proposed to ascertain that overexpression of Osx in BMSCs would facilitate osteogenic differentiation in bone regeneration *in vivo*. Our results showed that overexpression of Osx promoted osteogenic commitment of BMSCs in murine calvarial critical-sized defects.

MATERIALS AND METHODS

Animals

Six- to 8-week-old B6D2F1 mice were maintained and used in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals, prepared by the Institute on Laboratory Animal Resources, National Research Council (DHHS Publ. NIH

86-23, 1985). The Institutional Animal Use and Care Committee at the Tufts–New England Medical Center, in Boston, MA, approved the animal protocol.

Subcloning of viral constructs

To transduce BMSCs, we used the avian retroviral receptor (TVA)-based retroviral system.^{28,29} In this system, ectopic expression of the TVA receptor makes cells susceptible to infection with a replication-competent, subgroup-A avian leukosis viral vector (RCAS). The advantage of using this model is that cells expressing TVA can be repeatedly infected using RCAS vectors expressing different genes simultaneously or sequentially. To create the construct-expressing TVA receptor, the *tva* fragment was cleaved out with *EcoR* I from pCR-tva-800 vector (a gift from Dr. Varmus's laboratory, Memorial Sloan-Kettering Cancer Center, New York, NY) and subsequently cloned into the *EcoR* I site of pcDNA3.1 (Invitrogen, San Diego, CA). This new construct was named pcDNA3.1tva (Fig. 1). The RCAS-green fluorescent protein (GFP) and empty viral vector RCAS (previously named by others RCASBP(A))^{28,29} were generously provided by Dr. Stephen Hughes (National Cancer Institute/National Institutes of Health). The 1.2-kb fragment of mouse *Osx* complementary deoxyribonucleic acid (cDNA) was kindly provided by Dr. Benoît de Crombrughe (MD Anderson, Houston, TX). *Osx* cDNA was cloned into *Cla* I sites of RCAS construct through the *Cla* 12 *Nco* shuttle vector. This viral vector carrying the *Osx* cDNA was named RCAS-*Osx*. The orientation of the inserts was confirmed by restriction mapping and sequencing.

Production of high-titer RCAS viral stocks

RCAS constructs were transfected into the established chicken fibroblast cell line DF1 (CL-12203) to produce the viral stocks. DF1 cells were purchased from ATCC (Manassas, VA) and propagated in Dulbecco's modified Eagle medium with 4 mM L-glutamine, 1.5 g/L sodium bicarbonate and 4.5 g/L glucose (ATCC), 10 % fetal bovine serum (GIBCO/BRL, Gaithersburg, MD), and 1% penicillin/streptomycin at 39°C and 5% carbon dioxide. RCAS constructs were transfected into 80% confluent DF-1 cells using Superfect lipofectin solution (Qiagen, Valencia, CA). Viral supernatant was harvested over 3 days and then concentrated approximately 30-fold using ultracentrifugation at 26,000 rpm at 4°C. The viral pellet was re-suspended to a titer of 10⁸ colony forming units/mL and stored at –80 °C.

BMSC culture conditions and viral transduction

Primary bone marrow stromal cells were harvested from 6- to 8-week-old B6D2F1 mice using methods described previously.^{10,30} Briefly, the femora and tibiae were excised immediately after euthanasia, and the soft tissue was removed. The proximal end of the femora and the distal end of the tibiae were excised. Alpha minimum essential medium (α -MEM) was used to flush marrow gently from the shafts with a 25-gauge needle. A single-cell suspension was obtained by gently aspirating the cells sequentially through 20- and 23-gauge needles. The bone marrow cells were then seeded into T-75 flasks (Falcon Labware, Meylan Cedex, France) at a cell density of 4.0×10⁵ nucleated cells/cm² and cultured under non-differentiating conditions (α -MEM supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μ g/mL streptomycin) and incubated until the cells became confluent (approximately 2 weeks). Clonal BMSCs were obtained essentially as previously described,³¹ and spontaneously immortalized clonal cells were chosen for the experiments presented in this article.

To control the specificity of the viral targeting, we stably transfected BMSCs with a pcDNA3.1 vector-encoding TVA and then infected these cells with RCAS vector, as previously described.¹⁰ Briefly, BMSCs were transfected with pcDNA3.1tva using the liposome reagent (Qiagen).³² Positive clones were then selected in G418-containing media for 14 days. Infection of BMSCs was performed at 50% confluence. These BMSC clones stably expressing TVA were incubated with RCAS viral supernatant for 3 h at 37°C, and then an equal volume of culture

medium was added. For transplantation *in vivo*, the cells were allowed to recover for 24 h before harvesting.

Reverse transcriptase polymerase chain reaction analysis

Total ribonucleic acid (RNA) was extracted using an RNeasy MiniKit (Qiagen). Freshly isolated RNA was reverse transcribed with a SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA), following the manufacturer's recommendations. The resulting cDNA was then amplified using polymerase chain reaction (PCR) with the Platinum PCR supermix (Invitrogen, Carlsbad, CA). The sequences of the primers were 5'-ATTCTCCCATTCTCCCTCCCT-3' and 5'-GGAAGGGTGGGTAGTCATTTC-3' (product size: 376 bp) for amplification of mouse *Osx* and 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTG CTGTA-3' (product size: 450 bp) for mouse glyceraldehyde-3-phosphate dehydrogenase. Linearity of the PCR conditions was determined for each primer pair in preliminary experiments (data not shown). Images of the amplified products in 1.2% agarose gels were captured using a UVP bioimaging system (UVP, Upland, CA) and processed by Adobe Photoshop 6.0 (Adobe Systems Inc., San Jose, CA) and Scion Image Beta 4.02 (Scion Corp., Frederick, MD).

Calvarial defect assay

For experiments *in vivo*, BMSCs expressing TVA were infected with RCAS-*Osx* or the empty RCAS viral particles and the marker virus RCAS-GFP. The RCAS-GFP was used to follow the fate of the transplanted cells and to record the infection efficiency. Infected BMSCs (5×10^6) were then incubated with 4-mm bovine type I collagen sponge discs (Collagen Matrix, Inc., Franklin Lakes, NJ) for 60 min at 37°C with agitation and then cultured for an extra 24 h before their use in implantation. Six- to 8-week-old B6D2F1 mice were anesthetized, and then a 4-mm-diameter calvarial critical-sized defect was created on each side of the calvarial bone using a dental bur attached to a slow-speed handpiece with minimal invasion of the *dura mater*. The critical-sized defects in mice were randomly divided into 6 groups (defects unfilled, defects filled with collagen alone, defects filled with collagen seeded with primary BMSCs, defects filled with collagen seeded with clonal BMSCs, defects filled with collagen seeded with empty RCAS vector-transduced clonal BMSCs, and defects filled with collagen seeded with RCAS-*Osx*-transduced clonal BMSCs) with 6 animals each group.

Analysis of calvarial defect healing

Healing of calvarial defects was examined using radiology and histological examination 5 weeks after BMSC implantation. Radiographs were taken with a Faxitron radiographic inspection unit (Faxitron X-ray Corp., Buffalo, IL). The kVP and exposure time were kept constant for all samples. Areas of new bone formation in the calvarial defects were measured in the radiographs using Scion Image Beta 4.02 software (Scion Corp.). The ratio of the area of the defect filled with new bone divided by the area of the original defect yielded the percentage of healing. For histological examination, skull specimens were fixed in a 4% phosphate-buffered formalin solution and then decalcified with 18% ethylene-diaminetetraacetic acid (pH 8.0). Samples were then dehydrated in an ascending series of ethanol and embedded in paraffin wax. Five- μ m sections were cut using a microtome and mounted on glass slides. Hematoxylin and eosin (H&E) staining was performed using standard methods, as previously described.^{33,34} Expression of bone sialoprotein (BSP) or GFP in the sections was detected using immunohistochemical staining for BSP or GFP followed by counter-staining in hematoxylin using a Histostain SP kit (Zymed Laboratories, Inc., S. San Francisco, CA) and a BSP monoclonal antibody (1:25; Chemicon International, Temecula, CA) or GFP monoclonal antibody (dilution 1:100; BD Biosciences, San Jose, CA) using methods described earlier.^{33,34} H&E staining and immunostaining sections were examined

and photographed with a Nikon Eclipse E600 microscope and Spot Advanced software (Diagnostic Instruments, Sterling Heights, MI). Newly formed bone in H&E-stained sections was quantified in 4 sections of at least 4 different defects for each treatment at 40× magnification using Spot Advanced software. New bone formation in each section was expressed as a percentage of the total area of the defect.

The localization and intensity of BSP staining was studied on transverse sections of the cranium using previously described semi-quantitative methods.³⁵ In brief, the relative intensities of BSP staining were classified as intense, moderate, weak, or negative. For each slide, the intensity of BSP staining was determined by comparing the staining at each site with surrounding host bone in the same section. Adjacent host bone was classified as moderate staining intensity, and the areas of newly formed bone were compared with this internal standard. Stronger staining than internal controls was classified as intense, lower than internal controls as low, and no staining as negative. The localization of protein was studied in the newly formed bone, in the adjacent host bone, and in the fibrous connective tissue that filled in the defect. All slides were coded to prevent the introduction of examiner bias.

Statistical analysis

All results are expressed as means $\pm$ standard errors of the mean of 3 or more independent experiments. One-way analysis of variance was used to test significance using the software package Statgraphic statistical graphics system (STSC, Inc., Rockville, MD). *P*-values <0.05 were considered statistically significant.

RESULTS

Expression pattern of *Osx* in BMSCs transduced with RCAS/TVA retroviral infection system

To overexpress *Osx* in BMSCs, we used an RCAS/TVA retroviral system in which BMSCs expressing TVA were transduced with RCAS-*Osx* or empty viral vector (Fig. 1). We then evaluated the expression pattern of *Osx* upon BMSC transduction with the RCAS vectors. According to semiquantitative reverse-transcriptase PCR analyses, RCAS-*Osx*-transduced BMSC cultures showed a linear increase with time in their normalized levels of *Osx* (Fig. 2). Thus, at 4 weeks, levels of *Osx* were 5 times as high as those exhibited at 1 week. In contrast, empty vector-transduced cells did not express *Osx* at any of the time points examined.

Calvarial defect healing by BMSCs overexpressing *Osx*

We examined the ability of RCAS-*Osx*-transduced BMSCs to heal critical-sized defects in murine calvaria 5 weeks after implantation of infected BMSCs. Radiological images of the *Osx*-transduced group of implants (group 6) showed an average 85% healing, with large amounts of newly formed mineralized tissue filling in the defects (Fig. 3A). Group 1 (untreated defect) and group 2 (defect filled with collagen alone) had little osseous healing at 5 weeks (7% and 6%, respectively). Group 3 (defects filled with collagen seeded with primary BMSCs), group 4 (defects filled with collagen seeded with clonal BMSCs), and group 5 (defects filled with collagen seeded with empty RCAS vector-transduced BMSCs) showed a similar healing response and exhibited some mineralization within the defect but to a lower extent than in the *Osx*-transduced group (Fig. 3B).

Further histological examination of the implants indicated that the *Osx*-transduced group exhibited levels of newly formed bone that were 5 and 27 times higher than the BMSC groups (groups 3, 4, and 5) and the control groups (groups 1 and 2), respectively (Fig. 4). Specimens from the *Osx*-transduced group showed nearly complete osseous closure of the defect, where the newly formed bone had a mature bone structure with noticeable marrow spaces and absent cartilage (Fig. 4 and 5). In contrast, bridging of the calvarial defects with new bone was not a

significant feature in mice whose defects were left untreated (group 1) or treated with collagen disk alone (group 2). The healing response in these 2 groups consisted of the formation of membranous connective tissue spanning the defect (Fig. 4A).

To further analyze the extent of *de novo* bone mineralization, immunohistochemistry was performed to determine BSP expression levels. As shown in Fig. 5A (3, 4, 5, and 6), areas of new bone formation within the defect exhibited strong expression for BSP. In contrast, regions within the defect that were filled up with fibrous connective tissue showed a negative immunoreaction with the BSP antibody (Fig. 5A: 1 and 2). The *Osx*-transduced group showed more-intense staining for BSP within the defect region than the empty vector-transduced group (Table 1).

Immunolocalization of GFP

Immunohistochemical analysis for GFP expression within newly formed bone areas was then performed to determine the location of the transplanted BMSCs within the regenerated bone defects. Through this analysis, reddish-brown positive GFP staining (Fig. 5B: 3 and 4) was identified in osteoblasts or osteocytes within the regions of new bone formation. In addition, within the connective tissues surrounding newly formed bone, some cells also showed positive immunoreaction to the GFP antibody. A similar staining pattern was observed in empty RCAS vector and RCAS-*Osx* transduced groups examined (Fig. 5B).

DISCUSSION

The true osteogenic capacity of *Osx in vivo* was tested by implanting RCAS-*Osx*-transduced BMSCs into murine calvarial defects using type I collagen as a carrier. Five weeks after implantation of RCAS-*Osx*-transduced BMSCs, the bone defects demonstrated an average of 85% healing, with newly formed bone 5 times as great as in the empty virus-transduced group of implants and 27 times as great as in defects treated with the carrier alone. In the areas of newly formed bone, active regulation of BSP expression by *Osx* was observed. These results were consistent with those we obtained in our previous *in vitro* experiment.¹⁰ In that study, we found that *Osx* could enhance proliferation and osteogenic potential of BMSCs *in vitro*. Indeed, retroviral transduction of *Osx* in BMSCs cultured in non-differentiating medium did not affect expression of *Runx2/Cbfa1*, another key transcription factor of osteoblast differentiation, but induced an increase in the expressions of other markers associated with the osteoblastic lineage, including alkaline phosphatase, BSP, osteocalcin, and osteopontin. Likewise, *Osx* overexpression in BMSCs also increased their mineralization ability *in vitro*.¹⁰ Thus, we believe that the overexpression of transcription factor *Osx* in BMSCs stimulates the normal cascade of bone anabolic behavior.

The TVA/RCAS retroviral strategy^{28,29} employed in our study provides an alternative delivery system to other viral-based strategies previously used in *ex vivo* gene therapies for bone regeneration.^{19,20} The RCAS virus is replication competent in avian cells but not in mammalian cells and does not spread from cell to cell.³⁶ The viral vectors are efficient in producing stock virus with high titers and achieving high levels of expression after transduction. Pinto *et al.* reported that the persistence of the expression of genes delivered to mice using recombinant RCAS retrovirus was approximately 6 to 12 weeks, because the expression of the genes was confined to skeletal muscle cells.³⁶ This time span of the expression of the foreign genes introduced into the animal may provide an efficient method of tissue engineering.

Furthermore, multiple genes can be introduced into TVA-expressing cells simultaneously or sequentially. Thus, marker genes can be introduced to visualize cell lineage and bone regeneration *in vivo*. In fact, overexpression of *Cbfa1/Runx2* in osteoprogenitor cells has been found to induce bone regeneration^{19,20} at a rate similar to that described in our study. In those

studies, implanted cells from male donors were detected in recipient females using fluorescence *in situ* hybridization analysis for the Y-chromosome, whereas in our study, we followed the fate of the implanted cells using immunohistochemistry for GFP. In our study, the distribution of GFP-positive cells in regions of newly formed bone was found to be similar in the Osx-transduced group and the empty vector-transduced group. Therefore, location of GFP-positive cells in areas of new bone formation should only be used as a qualitative confirmation of the osteogenic potential of BMSCs *in vivo*. Neither quantification of GFP-positive cells nor of Y-chromosome positive cells in newly formed bone areas can allow us to know whether only the implanted cells and their derived cells are undergoing full differentiation *in vivo* or whether they also induce the surrounding cells to become osteogenic. More-sophisticated post-transplant analyses will be needed to address these important questions.

Even though *ex vivo* gene therapy using Cbfa1 infected into multipotential mesenchymal cells promotes bone formation *in vivo*,^{19,20} transgenic mice overexpressing Cbfa1 in osteoblasts under the control of the collagen I promoter have been shown to exhibit severe osteopenia and fragile bones due to inhibition of the late stage of osteoblast maturation.²⁴ These results suggest that, although Cbfa1 can induce new bone formation in multipotential mesenchymal cells such as BMSCs, this process might occur more efficiently and lead to bone matrix of better quality *in vivo* if Cbfa1 levels are high during the early differentiation stage and low during the osteoblast maturation stage. In agreement with this paradigm, one of the successful bone regeneration studies recently described that the highest levels of expression of Cbfa1/Runx2 occurred at 1 week after transplantation and low levels at 3 weeks after transplantation.²⁰ Unfortunately, levels of Osx *in vivo* or *in vitro* were not reported, and therefore it is difficult to estimate which proportion of the effects of Cbfa1/Runx2 in bone formation could have been due to Osx. Because overexpression of Cbfa1/Runx2 during the late stage of osteoblast differentiation may lead to the formation of bone that is fragile,²⁴ and in our study overexpression of Osx did not increase Cbfa1/Runx2 expression¹⁰ but promoted bone regeneration, a high ratio of Osx to Cbfa1 at the late stages of osteoblast differentiation may be needed for the efficient formation of newly engineered bone. Therefore, the combination of Osx with BMSCs will probably provide a better gene therapy approach than Cbfa1/Runx2 in bone regeneration and wound repairing. In addition, Cbfa1 is known to increase expression of receptor activator of nuclear factor kappa B ligand (RANKL) and vascular endothelial growth factor, which are important in regulating osteoclast bone remodeling and angiogenesis, respectively, during bone formation.^{37–39} Therefore, the function of these cytokines may influence the final outcome of the *ex vivo* gene therapy of Cbfa1/Runx2. Future studies will be needed to determine the contribution of osteoclastic remodeling and scaffold vascularization and angiogenesis to the bone regeneration outcomes resulting from *ex vivo* gene therapies of Osx and Cbfa1/Runx2 in different types of bones. Decreasing the time required for bone regeneration and increasing the quality of the engineered bone are important matters that require careful consideration. In this regard, we are planning to substitute collagen I for synthetic scaffolds^{40,41} that allow implantation of Osx-transduced osteoprogenitor cells in bone defects and delivery of factors with angiogenic properties or that regulate osteoclast function and increase the efficiency of the bone regeneration process. With a RCAS/TVA retroviral system, we will be able to determine those effects at early and late stages of cell differentiation and in the entire process of tissue regeneration using systemic application of viral constructs of gene of interest. We will also be able to control the dosage of the gene expression by modulating the viral titration at different regenerative stages in most *in vivo* models.

Primary BMSCs must be expanded *ex vivo* to obtain large numbers of BMSCs for cell transplantation. A potential obstacle to *ex vivo* expansion, however, is that BMSCs may have a limited replicative life span and gradually lose their osteogenic potential *in vitro*.^{12,42} However, Satomura *et al.*³¹ characterized 5 spontaneously transformed cell lines from a

population of murine BMSCs and found that 3 of 5 clonal cell lines kept osteogenic potential even after 30 to 35 passages. Furthermore, Shi *et al.*⁴³ reported that ectopic expression of human telomerase reverse transcriptase in BMSCs extended their life span and maintained their osteogenic potential. Similarly, we previously showed that murine clonal BMSCs were multipotent and exhibited different levels of expression of *Osx* and *Cbfa1* depending on their differentiation commitment and cell-culture conditions.¹⁰ To facilitate the use of the TVA/RCAS retroviral strategy in the transplantation studies described herein, we chose clonal BMSCs for viral infection and subsequent bone tissue engineering. To address the concern of whether clonal BMSCs may compromise their osteogenic potential, we compared the healing patterns of defects filled with collagen sponge seeded with primary cultured BMSCs and defects filled with a collagen seeded with clonal BMSCs. These two groups of cells with different treatments demonstrated a similar capacity in bone wound healing and tissue regeneration, suggesting that the cloning of the BMSCs does not lead to a loss of osteogenic potential. These data demonstrate that, although the entire (non-clonal) population of marrow stromal cells represents a pool of progenitors differentiating to different cell types,⁶ these stromal cells could be cloned and still possess multiple differentiation potential. These cells can be used in facilitating tissue engineering in a more efficient way.

Given that immortalized clonal cells might exhibit potentially abnormal proliferative characteristics as transformed cells and that we have previously shown that *Osx* can enhance proliferation of BMSCs,¹⁰ the possibility exist that overexpression of *Osx in vivo* could lead to a malignant phenotype in addition to a bone healing response. However, we found that clonal BMSCs alone or BMSCs overexpressing *Osx* were unable to develop a tumorigenic phenotype after being implanted subcutaneously into nude mice (data not shown). In agreement with our results, Cao *et al.*⁴⁴ found that overexpressing *Osx* in mouse osteosarcoma cells inhibited tumor cell growth *in vitro* and *in vivo* and significantly reduced tumor incidence, tumor volume, and lung metastasis after intratibial injection. They determined that *Osx* expression was also associated with decreased osteolysis, which was not related to inhibition of RANKL expression. Whether a reduction in osteolysis incurred by *Osx* may also play a role in *Osx*-mediated bone regeneration needs to be further addressed.

Our studies provide the first demonstration that *ex vivo* gene therapy of *Osx* in adult BMSCs can effectively promote bone regeneration in critical-sized calvarial bone defects. Although additional studies are needed to elucidate the mechanisms by which *Osx* promotes bone formation, our results provide a strong rationale for the development of *ex vivo* therapies using *Osx* to repair bone defects.

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FIG. 1.

Schematic drawing of replication-competent, subgroup-A avian leukosis viral vector (RCAS)/avian retroviral receptor (TVA) system. A viral vector-carrying gene of interest (e.g., osterix (Osx)) will specifically target bone marrow–derived mesenchymal stem cells engineered to express TVA receptor. This infection does not cause viral spreading or immunity, because gag (for a structural protein), pol (for a viral enzyme), or env (for an envelope glycoprotein) are poorly expressed in mammals. Long terminal repeats (LTRs) serve as a constitutive promoter, resulting in expression of the *Osx* gene. CMV, cytomegalvirus; RNA, ribonucleic acid.

FIG. 2.

The replication-competent, subgroup-A avian leukosis viral vector (RCAS)/avian retroviral receptor (TVA) retroviral system induces high levels of expression of osterix (Osx) in bone marrow-derived mesenchymal stem cells (BMSCs) cultured in maintenance media. RCAS-Osx-transduced BMSCs and empty vector-transduced BMSCs were cultured in maintenance media (non-differentiating) for different times. **(A)** Representative image of semiquantitative reverse transcriptase polymerase chain reaction analyses of Osx expression. **(B)** Levels of Osx were normalized with those of the loading control glyceraldehyde-3-phosphate dehydrogenase in 3 independent experiments. ^a $p < 0.05$ vs. 1-week time point.

A

B

FIG. 3.

Osterix (Osx) promotes bone healing in the skull assay. **(A)** Representative images of x-ray radiograph of calvarial defects 5 weeks after bone marrow–derived mesenchymal stem cell (BMSC) implantation. Defect treatments, from left to right: group 1, unfilled defect; group 2, collagen alone; group 3, collagen + primary BMSCs; group 4, collagen + clonal BMSCs; group 5, collagen + RCAS vector-transduced BMSCs; group 6, collagen + RCAS-Osx-transduced BMSCs. **(B)** Percentage of healing. The extent of mineralization filling the calvarial defects was monitored using densitometry 5 weeks after implantation, and then the percentage of healing was calculated in all groups as described in the Materials and Methods section. ^a $p < 0.05$ vs. group 1 or group 2; ^b $p < 0.05$ vs. group 3, 4, and 5.

FIG. 4.

Histological analyses of implanted calvarial bone defects. (A) Calvarial specimens were stained with hematoxylin and eosin. 1. Group 1, unfilled defect; 2. group 2, collagen alone; 3. group 3, collagen + primary bone marrow-derived mesenchymal stem cells (BMSCs); 4. group 4, collagen + clonal BMSCs; 5. group 5, collagen + RCAS vector-transduced BMSCs; 6. group 6, collagen + RCAS-OSX-transduced BMSCs. Photographs were taken at 40 $\times$. The arrowheads designate the surgical margins. (B) Areas of new bone formation were calculated as percentages with respect to total area and showed underneath representative pictures. ^a $p < 0.05$ vs. group 1 or group 2; ^b $p < 0.05$ vs. group 3, 4, and 5.

FIG. 5.

Immunohistochemical staining for bone sialoprotein (BSP; original magnification, 200×) and immunolocalization of green fluorescent protein (GFP) expression (original magnification, 400×) within newly formed bone areas. **(A)** Expression of BSP in the sections was detected using immunohistochemical staining using a BSP monoclonal antibody (1:25; Chemicon International, Temecula, CA) followed by counterstaining with hematoxylin. Calvarial specimens were from the following groups: 1. unfilled defect; 2. collagen alone; 3 collagen + primary bone marrow-derived mesenchymal stem cells (BMSCs); 4. collagen + clonal BMSCs; 5. collagen + RACS vector-transduced BMSCs; 6. collagen + RCAS-OSX-transduced BMSCs. BSP expression (reddish staining) is localized to osteoblasts and osteocytes within newly formed bone. **(B)** Immunohistochemistry for GFP expression was performed to analyze the fate of the implanted BMSCs within areas of newly formed bone. Counterstaining with hematoxylin was performed to detect the nuclei of the cells in the newly formed bone areas. Negative staining was found in host bone (HB, 1) and clonal BMSC implant without GFP viral infection (2). GFP expression was detected in the specimens from defects filled with collagen + empty RCAS-transduced BMSCs (3), and defects filled with collagen + RCAS-OSX-transduced BMSCs (4), which were labeled with GFP viral co-infection. Signals for GFP staining were localized in osteoblasts and osteocytes within newly formed bone (NB), periosteum, and fibrous connective tissue (FT) surrounding new bone. BM, bone marrow. Color images available online at www.liebertpub.com/ten.

Table 1**Bone Sialoprotein (BSP) Expression in Calvarial Defect***

Group	HB	NFB	FT
RCAS VECTOR	++	++	–
RCAS-Osx	++	+++	–

* Qualitative analyses of the location and intensity of BSP staining in the nascent tissues of the defect. Estimates included the regions of newly formed bone (NFB), adjacent host bone (HB), and fibrous connective tissue (FT). Staining was graded as negative (–), moderate (++) and intense (+++). RCAS, replication-competent, subgroup-A avian leukosis viral vector; Osx, osterix.