Transcription factor osterix modified bone marrow mesenchymal stem cells enhance callus formation during distraction osteogenesis

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This study was designed to investigate the effects of local delivery of bone marrow mesenchymal stem cells (BMMSCs) with or without osterix (OSX) gene transfected on bone regeneration in the distracted zone using a rabbit model of mandibular distraction. Fifty-four New Zealand white rabbits underwent osteodistraction of the left mandible and were then randomly divided into group A, group B, and group C (n = 18 for each group). At the end of distraction BMMSCs transfected with OSX, autologous BMMSCs and physiological saline were injected into the distraction gaps in groups A, B, and C, respectively. Nine animals from each group were humanely killed at 2 and 6 weeks after completion of distraction. The distracted mandibles were harvested and processed for radiographic, histological, and immunohistochemical examination. Excellent bone formation in the distracted callus was observed in group A and group B; the former showed better bone formation and highest bone mineral density (BMD), thickness of new trabeculae (TNT, mm) and volumes of the newly formed bone area (NBV) in the distraction zones. Group C animals showed poor bone formation in the distracted callus when compared with groups A and B. Positive immunostaining of bone sialoprotein (BSP) was observed in the distracted callus in all groups; however, BSP expression was much stronger in group A than in groups B and C. The results of this study suggest transplantation of BMMSCs can promote bone formation in DO; OSX-mediated ex vivo gene therapy was more effective during bone deposition and callus formation in distraction osteogenesis. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;111:412-419)

Distraction osteogenesis (DO) is a well-established technique, originally developed in orthopedic surgery for correction of limb length discrepancies and later also used to treat hereditary malformations in the craniofacial region, including Nager syndrome, Pierre-Robin syndrome, temporomandibular joint ankylosis, postoncologic ablation, posttraumatic growth retardation, and midface and zygomatic hypoplasias.

Although DO is believed to be superior to traditional bone augmentation methods, the long treatment period and the potential of fibrous union or nonunion under some circumstances remain major limitations that hamper further clinical application of DO.

Many attempts have been made to improve the technique to accelerate osteogenesis in the distraction gap. Such improvements would provide the opportunity to shorten the bone consolidation period and hence minimize complications such as the development of nonunion, infection, or fracture.

Gene therapy based on bone growth genes has been introduced in experimental animals as a novel approach and successfully promoted the new bone formation during distraction osteogenesis. Despite promising results, the clinical feasibility of these growth factor-based gene therapy approaches may be hampered by complex release kinetics and unregulated, ectopic bone formation caused by paracrine signaling to neighboring nonosseous tissues. The delivery of downstream transcriptional activators has been extensively explored to address these limitations.

Osterix (OSX) is a zinc-finger-containing transcription factor of the sp family that is critical in osteoblast...

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differentiation and bone formation.\textsuperscript{21-23} OSX acts downstream of Runx2/Cbfa1 to induce differentiation of preosteoblasts into fully functional osteoblasts.\textsuperscript{23} In OSX-null mice, no bone formation occurs. OSX regulates the expression of related osteoblastic genes such as osteocalcin (OCN), osteopontin (OPN), collagen type I (COL I), and bone sialoprotein (BSP), and overexpression of OSX has been shown to be sufficient to guide differentiation of murine embryonic stem cells toward the osteoblastic lineage in vitro.\textsuperscript{24}

To date, OSX gene therapy for distraction osteogenesis has not been reported, so we hypothesized that local delivery of bone marrow mesenchymal stem cells (BMMSCs) transfected with osterix (OSX) would allow efficient transduction of bone-generation cells in distraction zone and accelerate osteogenesis in mandibular DO.

**MATERIALS AND METHODS**

**Animals**

Fifty-four skeletally mature male New Zealand white rabbits were used in this study. The body weights ranged from 3.0 to 3.5 kg at the beginning of the experiment. The experimental protocol was approved by the Animal Care Committee at Shandong University.

**Osteotomy and distraction procedures**

Fifty-four New Zealand white rabbits underwent osteodistraction of the left mandible and were then randomly divided into 3 groups of 18 each. The rabbit model has been used in many previous studies that deal with distraction osteogenesis of the mandible.\textsuperscript{25,26}

Briefly, the animals were given a preoperative dose of antibiotic (penicillin 15 \(\mu\)g/kg); anesthesia for all experimental procedures was achieved by intravenous injection of pentobarbital (1 mg/kg) and subcutaneous local anesthesia (1% lidocaine). Surgical procedures were performed while the animals were under general anesthesia. The operative area was shaved and disinfected with iodophor, and a 2-cm incision was made along the inferior border of the left mandible. The periosteum and the masseter muscle were incised and carefully elevated. Under constant saline irrigation, the osteotomy was performed on the buccal (outer side) through the anterior part of the mandible (just anterior to the first molar) using a diamond disk. A custom-made distractor was placed and fixed to the mandible with 4 self-tapping screws (Fig. 1). The masseter muscle was repositioned with sutures, and the surgical wounds were closed in layers.

The animal was injected with a prophylactic antibiotic, penicillin 15 \(\mu\)g/kg intramuscularly (IM) twice per day for 6 days. All animals received conventional care, feeding, and ambulation in the rabbit holding cages. All the surgical procedures and postoperation treatment were approved by Animal Experimentation Ethics Committee of the Shandong University. After 6 days of latency, unilateral mandibular distraction was activated at a rate of 0.4 mm/12 hours for 6 days, to produce a distraction gap of approximately 4.8 mm. After distraction was completed, the distractor was left in situ. The rabbits were fed a soft diet for the first 4 weeks. Their teeth were clipped to prevent overgrowth during hemimandible osteodistraction.

**Cell cultures and transfection**

Autologous BMMSCs were removed from the left tibia and isolated by density gradient centrifugation,\textsuperscript{27} suspended in alpha-minimum essential medium with 15% fetal bovine serum (Gibco BRL, Gaithersburg, MD), and 100 mg/mL penicillin-streptomycin (Gibco BRL), and incubated at 37\(^\circ\)C with 5% humid CO\textsubscript{2}. Cells that reached 80% confluence were suspended for passage. The successive passage was cultured for about 8 days and cells of passage 3 were used for the gene transfection in vitro and transplantation in vivo.

The recombinant plasmid pEGFP-OSX were constructed by directed cloning technique by Shanghai Genechem Co., Ltd. (Shanghai, China). When BMMSCs were grown to 80% confluence, they were transfected with pEGFP-OSX using Lipofecta-mineTM2000 (Life Tech, Invitrogen, Carlsbad, CA), and were observed under fluorescent microscopy (Leica, Wetzlar, Germany) to determine the transfection efficiency at day 2.

**Therapy**

Immediately after distraction, the rabbits in group A received distraction gap injection with \(1 \times 10^7\) OSX transfected autologous BMMSCs suspended in 0.2 mL physiological saline, whereas group B rabbits received injection of the same dosage of autologous BMMSCs.
and the animals of group C received only injection of 0.2 mL of physiological saline.

Half of the animals randomly chosen from each group were humanely killed with sodium pentothal injections at the end of weeks 2 and 6 throughout the consolidation stage respectively. The mandibular samples were harvested for laboratory analysis and all the measurements were conducted blindly.

**Radiographic examinations**

The mandibles were harvested and the lateral radiographs of the specimens were taken. The x-ray unit (Siemens, München, Germany) was set under a standard condition of 50 kV, 16 mA, with a 0.06-ms exposure time. The density of distraction callus was then measured to represent its projectional bone mineral density (BMD).

**Histological examination**

The samples were decalcified in a solution of 13% ethylenediaminetetraacetic acid buffer (pH 7.2) immediately after radiographic examinations. The specimens were sectioned longitudinally along the axial plane and embedded in paraffin, then cut with a microtome (Leica) into 5-μm sections for hematoxylin and eosin (H&E) staining. Histomorphometric analysis was performed by 2 experienced pathologists in a blinded manner and similar to the method of Ashinoff et al.\textsuperscript{14} and Hu et al.\textsuperscript{15}

The thickness of new trabeculae (TNT, mm) and bone volumes of the newly formed cortical bone area (NBV1, %) and the cancellous bone area (NBV2, %) of the distraction zones were measured. The mean of TNT, NBV1, and NBV2 was calculated for each group and then subjected to statistical analysis.

**Immunohistochemical examination**

Expression of bone sialoprotein (BSP) in the sections was detected using immunohistochemical staining for BSP followed by counterstaining 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) using methods of Phillips et al.\textsuperscript{17}

**Statistical analysis**

All data are expressed as means ± standard errors. Statistical differences among 3 groups were analyzed by 1-way analysis of variance (ANOVA) and post hoc multiple comparison tests were performed when significance was obtained. \(P\) less than .05 was considered statistically significant.

**RESULTS**

**Gross appearance of the distraction zone**

The surgical procedures were well tolerated by all experimental animals and their skin wounds healed well. Osteogenesis was achieved between the 2 bone fragments in 3 groups, with no cases of nonunion observed. Obvious crossbite and overgrowth of the lower incisors developed in all rabbits after unilateral mandibular lengthening and the lower incisors shifted to the nonsurgical side. However, no obvious difference in the shape of the distracted areas was found among the 3 groups.

**Transfected cell evaluation**

Transient expression of EGFP was detected in MSCs transfected with pEGFP-OSX by fluorescent microscopy. The initial transfection efficiency was about 48% at day 2 (Fig. 2), and the percentage of transfected cells decreased to 40% at day 7, 33% at day 14, 19% at day 21, and 14% at day 28.
Radiographic observation

Fig. 3 shows a representative lateral radiograph of a distracted mandible at 2 weeks after the end of distraction. Gray density analysis confirms statistically significant differences among group A, B, and C. The radiodensity of the callus in group A appeared to be greater when compared with that in group B, and group B appeared to be higher than group C at 2 and 6 weeks after the end of distraction. Gray density analysis also confirmed these results, and statistical analysis showed that the radiodensity of the distraction callus in group C was significantly lower than group B, and group B was lower than group A at both time points ($P < .05$) (Fig. 4).

Histological observation

Bone regeneration in the distraction gaps was predominantly accomplished by intramembranous ossification under light microscopy. At 2 weeks after distraction, the new bone trabeculae formation began bridging between the 2 fragments in the 3 groups. However, more thick, homogeneous and dense trabecules were seen in the distraction gaps in group A (Fig. 5, A) than group B (Fig. 5, B) and C (Fig. 5, C).

At 6 weeks, the gaps were filled with newly formed bone in all groups. However, group A (Fig. 5, D) showed the greatest amount and densest regenerated bone trabeculae. Group B (Fig. 5, E) had more mature and thick bone trabeculae than group C (Fig. 5, F).

Values for the newly formed cortical bone area (NBV1), newly formed cancellous bone area (NBV2), and the thickness of new trabeculae (TNT) were significantly higher in group A than group B, and in group B more than group C ($P < .05$) at both time points (Table I).

Immunohistochemical examination

At 2 weeks after the end of distraction, immunohistochemistry was performed to determine BSP expression levels. As shown in Fig. 6, areas of the fibrous connective tissue within the gaps exhibited expression for BSP and were mainly detected in the cellular components of fibroblastlike cells, preosteoblasts, and osteoblasts in all 3 groups. Cells in group A (Fig. 6, A) showed greater amount and more intense staining for BSP within the gaps than group B (Fig. 6, B), and group B more than group C (Fig. 6, C).

Discussion

In contrast to osteoinductive growth and differentiation factor-based therapies, most attempts to use transcription factors with an intracellular mode of action have involved the use of a genetic engineering approach. LMP-1 is an intracellular protein that has been shown to induce secretion of soluble factors such as BMP-2, BMP-4, BMP-6, and BMP-7. Both transient and long-term expression of Runx2 in primary BMSCs enhances in vivo mineral deposition compared with unmodified BMSCs. Recently, Tu et al. observed that retroviral gene delivery of Osterix enhances the mineralization capacity of primary BMSCs in vitro, suggesting that this strategy may lead to stimulation of bone formation in vivo. In this study, OSX ex vivo gene therapy was used to accelerate callus formation in a rabbit model for mandibular osteodistraction. Radiographic, histologic, and immunohistochemical examinations showed greater newly formed bone and earlier mineralization in the distracted callus treated with MSCs transfected by OSX gene compared with unmodified MSC transplantation. In the areas of newly formed bone and fibrous connective tissue, active regulation of BSP expression by OSX was observed. All these results were consistent with the previous in vitro experiment of Tu et al. In that study, 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. Histological examination of the implants demonstrated that the OSX-transduced group exhibited amounts of newly formed bone that was 5 times as high as in a group transduced with the empty vector.

A current study confirmed the contribution of MSCs to enhancing bone regeneration during mandibular distraction osteogenesis, but MSCs may progressively lead to a decrease in their proliferation and loss of their...
osteogenic potential after long-term ex vivo expansion. 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, which may be another approach for callus stimulation in osteodistraction because it may not only guarantee enough soluble osteoinductive factors, but also offer more bone-forming cells for osteoregeneration. Further, a genetic approach such as ex vivo gene delivery may offer other advantages, such as the ability to stably deliver genes and to use a combinatory approach for a synergistic effect, for example, in combination with local delivery of osteoinductive factors. Moreover, the use of BMSCs is associated with the formation of cartilage-like tissue at the distraction gap, which is a known side effect of distraction osteogenesis, and such a phenomenon may be partially avoided by the stimulation of osteoblastic differentiation of BMSCs.
thermore, ex vivo gene transfer in a cell-based delivery vehicle may be safer in a clinical setting than direct injection of viral particles in vivo. But long-term of expression of exogenous growth factors in local gene therapy during DO is unnecessary, and transient expression of the target gene in situ is enough for bone regeneration. So, in our study, OSX was used to enhance proliferation and osteogenic potential of BMSCs both in vitro and in vivo with the liposome-mediated transfection technique.

Many transcription factors including Dlx-3, Runx2, Smads, Dlx-5, MSX-2, AP-1, and Osterix are expressed during bone formation and fracture healing. Among these, Runx2 and Osterix have been extensively characterized for their role in regulating the commitment of multipotent MSCs toward the osteoblastic lineage. Runx2 is an essential transcriptional regulator of chondrocyte hypertrophy, osteoblast differentiation, and bone formation. Many studies have demonstrated that overexpression of Runx2 up-regulates osteoblast-specific gene expression and induces mineralization in a cell-type-dependent manner. But transgenic mice overexpressing Cbfa1 in osteoblasts under the control of the collagen I promoter have been shown to exhibit severe osteopenia and fragile bones owing to inhibition of the late stage of osteoblast maturation. Osterix acts downstream of Runx2 to induce the differentiation of osteoprogenitors into mature osteoblasts and overexpression of OSX did not increase Cbfa1/Runx2 expression but promoted bone regeneration; therefore, the BMSCs transfected with OSX will probably provide a better gene therapy approach than Cbfa1/Runx2 in bone regeneration and wound repairing.

In conclusion, ex vivo gene therapy based on bone marrow MSCs genetically engineered to express OSX can effectively promote callus formation and therefore reduce the consolidation period during distraction osteogenesis. Our results provide a strong rationale for the development of ex vivo therapies using OSX to facilitate clinical distraction osteogenesis treatment, especially for the patients whose osteogenic potentials are compromised by diseases or aging, osteoporosis, postoncologic irradiation, and severe trauma.
REFERENCES

1. Kojimoto H, Yasui N, Goto T, Matsuda S, Shimomura Y. Bone lengthening in rabbits by callus distraction. The role of perios-


3. McCarthy JG, Stelnicki EJ, Mehrara BJ, Longaker MT. Distrac-


5. McCarthy JG, Katzen JH, Hopper R, Grayson BH. The first

6. Alesyniene R, Thomsen JS, Eckardt H, Bundgaard KG, Lind M, Hvid I. Parathyroid hormone PTH(1-34) increases the volume, mineral content, and mechanical properties of regenerated min-

7. Alesyniene R, Thomsen JS, Eckardt H, Bundgaard KG, Lind M, Hvid I. Three-dimensional microstructural properties of regen-
erated mineralizing tissue after PTH (1-34) treatment in a rabbit


9. Kim JY, Cho BC. Effect of calcium sulfate pellets on early bone
demineralization in distraction osteogenesis for craniofacial micro-

10. Watanabe Y, Matsuhashi T, Bhandari M, Zdero R, Schemitsch


12. Kawamoto K., Kim WC., Tsuchida Y., Tsuji Y., Fujioka M.,


14. Ashinoff RL., Cetrulo CL Jr., Galiano RD., Dobryansky M., Bhatt

15. Watanabe Y., Matsushita Y., Bhandari M., Zdero R., Schemitsch


17. Phillips JE., Gersbach CA., Garcia AJ.,

18. Chung CH., Tsui S., Chen X., Li Z., others.

19. Viggesswarapu M., Boden SD., Liu Y., Hair GA., Louis-Ugbo J.,

20. Tu Q., Verlende P., Chen J., Osterix enhances proliferation and


22. Nishio Y., Dong Y., Paris M., O’Keeffe RJ., Schwarz EM., Drissi H.,


24. Tai G., Polak JM., Bishop AE., Christodoulou I., Buttery LDK.,

25. Guerrissi J., Ferrentino G., Margulies D., Fiz D.,

26. Stewart KJ., Lyvov GO., White SA., Bonar SF., Walsh WR., Smart

27. Scrivani S., Raju A., Licata L., Patel M., Rojavin Y., Wasielewski

28. Byers BA., Guldberg RE., Garcia AJ.,

29. Byers BA., Guldberg RE., Garcia AJ.,

30. Byers BA., Guldberg RE., Garcia AJ.,

31. Zhao Z., Zhao M., Xiao G., Franceschi RT.,

32. Tu Q., Valverde P., Chen J.,

33. Owen M.,

34. Perrin RO., et al. Parallel, cell-specific expression of the
cDNA encoding for the intracellular protein LMP-1. J Bone Joint

35. Hutmacher DW., Garcia AJ.,

36. Hutmacher DW., Garcia AJ.,

37. Bianco P., Riminucci M., Gronthos S., Robey PG.,


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