



Adipose-derived stem cells transfected with *pEGFP-OSX* enhance bone formation during distraction osteogenesis*

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Abstract: This study was designed to investigate the effects of local delivery of adipose-derived stem cells (ADSCs) transfected with transcription factor osterix (OSX) on bone formation during distraction osteogenesis. New Zealand white rabbits ($n=54$) were randomly divided into three groups (18 rabbits per group). A directed cloning technique was used for the construction of recombinant plasmid *pEGFP-OSX*, where EGFP is the enhanced green fluorescence protein. After osteodistraction of the right mandible of all experimental rabbits, rabbits in group A were treated with ADSCs transfected with *pEGFP-OSX*, group B with ADSCs transfected with *pEGFP-N1*, and group C with physiological saline. Radiographic and histological examinations were processed after half of the animals within each group were humanely killed by injection of sodium pentothal at Week 2 or 6 after surgery. The distraction bone density was measured as its projectional bone mineral density (BMD). Three parameters were measured, namely, the thickness of new trabeculae (TNT), and the volumes of the newly generated cortical bone (NBV1) and the cancellous bone (NBV2) of the distracted regions. Good bone generation in the distraction areas was found in group A, which had the highest BMD, TNT, and NBV in the distraction zones among the groups. There was no significant difference in bone generation in the distraction areas between groups B and C. The results indicate that the transplantation of ADSCs transfected with *pEGFP-OSX* can effectively promote bone generation during distraction *in vivo*.

Key words: Adipose-derived stem cell (ADSC), Distraction osteogenesis, Transcription factor, Gene transfection
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1 Introduction

Gene-promoted bone tissue engineering provides a new treatment strategy for repairing bone defects caused by fractures, bone loss, or tumors (Breitbart *et al.*, 1999; Mason *et al.*, 2000; Edwards and Mason,

2006a; 2006b). Three elements are necessary: seed cells for osteogenesis, osteoinductive genes for osteoinduction, and specific scaffolds for osteoconduction. Bone marrow stromal cells (BMSCs) are the most universal source of adult stem cells and play an important role in the field of gene-enhanced tissue engineering in many seed cells (Pittenger *et al.*, 1999; Lange *et al.*, 2005; Liu *et al.*, 2006). Compared with other cells, BMSCs have intrinsic osteogenic properties, but they also have disadvantages: suitable quantities are difficult to obtain *in vitro*, and there is a

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limitation of age-related decline in the number of these cells (Oreffo *et al.*, 1998; Muschler *et al.*, 2001; Derubeis and Cancedda, 2004). In contrast, adipose-derived stem cells (ADSCs) are better able to differentiate into adipocytes but less able to differentiate into osteogenic cells. However, ADSCs have received much attention because they constitute an abundant and easily accessible resource of stem cells and have pluripotent lines able to differentiate into adipogenic, osteogenic, myogenic, and chondrogenic cells (Halvorsen *et al.*, 2001; Zuk *et al.*, 2001; Nathan *et al.*, 2003; Fraser *et al.*, 2006; Schaffler and Buchler, 2007).

Bone regeneration by transplantation of ADSCs must be preceded by *in vitro* osteogenic induction to osteoblasts during cell culture by the addition of dexamethasone, bone morphogenic proteins (BMPs), or other factors (Jaiswal *et al.*, 1997). However, these *in vitro* treatments cannot be sustained after implantation *in vivo*. Osteogenic differentiation of stem cells with the aid of gene transfection provides a possible solution to these limitations (Asahara *et al.*, 2000).

Osterix (OSX) is a transcription factor containing a zinc finger encoded by the *Sp7* gene, which is important in osteoblast differentiation and bone generation, activating downstream of *Runx2* to induce preosteoblasts to differentiate into functional osteoblasts (Nakashima *et al.*, 2002; Lee *et al.*, 2006; Nishio *et al.*, 2006). OSX is also a transcription factor essential for osteoblast differentiation because no bone generation occurs in *OSX*-null mice (Tai *et al.*, 2004). Overexpression of *OSX* gene has been shown to be sufficient to induce differentiation of ADSCs towards osteoblastic cells *in vitro* (Wu *et al.*, 2007).

As an internal bone tissue engineering technique, distraction osteogenesis (DO) provides an ideal model for the study of bone regeneration. Therefore, we hypothesized that the local delivery of ADSCs transfected with OSX would increase the numbers of osteoblasts in distracted areas and promote osteogenesis in distraction regions.

2 Materials and methods

2.1 Animals

Fifty-four six-month-old New Zealand male rabbits were purchased from the Experimental Ani-

mal Center of Shandong University, China. Animals were housed in pathogen-free conditions and all studies performed were in accordance with institutional guidelines at the Animal Care Veterinary Service Facility of Shandong University, China.

2.2 Surgery of distraction osteogenesis

All rabbits received osteotomy and distraction of the right mandibles and were then randomly divided into three groups with 18 rabbits in each group. The rabbit model has been reported in many previous studies dealing with mandibular distraction (Qi *et al.*, 2006; Hu *et al.*, 2007; Lai *et al.*, 2011). Briefly, the rabbits were given penicillin with at a dose of 15 U/kg prior to operation; anesthesia was achieved by intravenous administration of pentobarbital (1 mg/kg) and subcutaneous injection of local anesthesia [1% (w/v) lidocaine]. Surgical procedures were conducted according to Lai *et al.* (2011). The distractors (Fig. 1) were purchased from Xi'an Zhongbang Titanium Biological Materials Co., Ltd. (China). After the distraction surgery, the distractor was fixed in the mandible (Fig. 2). The rabbits were fed on a soft diet for the following three weeks. Their teeth were polished to prevent overgrowth during mandibular distraction.



Fig. 1 Custom-made internal distractor purchased from Xi'an Zhongbang Titanium Biological Materials Co., Ltd., China

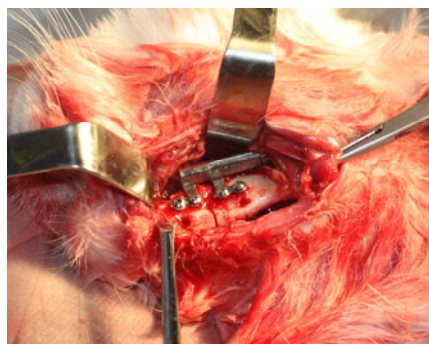


Fig. 2 Custom-made distraction device fixed *in situ*

2.3 Isolation and culture of ADSCs

A sterile surgical procedure was performed to obtain 10 ml inguinal adipose tissue from the rabbits. To remove contaminating debris, tissues were excised and washed 3–4 times with sterile phosphate-buffered saline (PBS), and then were minced into small pieces and incubated with 0.2% (w/v) type I collagenase (Sigma, USA) in PBS at 37 °C on a shaker for 2 h. After centrifugation of the resultant soup, the pellet with adherent stromal cells was placed in Ficoll solution (Biosera) and centrifuged again. The stromal vascular fraction (SVF) in the second layer was transferred into a tube and washed with PBS. The pellet with SVF was resuspended in culture medium that contained 1% (w/v) penicillin/streptomycin (Biosera) and 10% (w/v) fetal bovine serum (GIBCO). The adherent cells were cultured and non-adherent cells were discarded. The medium was changed every 4 d and cells were harvested after about 30 d of culture.

The recombinant plasmid *pEGFP-OSX* (EGFP is the enhanced green fluorescence protein) was constructed by Shanghai Genechem Co., Ltd. (China) using a directed cloning technique. ADSCs were transfected with *pEGFP-OSX* using Lipofectamine™ 2000 (Invitrogen, USA) when they grew to 80% confluence, and the transfection efficiency was determined using fluorescence microscopy (Leica, Wetzlar, Germany) on Day 2.

2.4 Therapy

After 6 d of latency, the fractured bones were distracted by 0.8 mm twice a day for 10 d. Immediately after the distraction phase, the distraction areas of the animals in group A received an injection of about 1×10^7 *OSX*-transfected ADSCs suspended in 0.2 ml physiological saline. Rabbits in group B were injected on the same schedule with 0.2 ml of ADSCs transfected with *pEGFP-N1*, and rabbits in group C received an injection of 0.2 ml of physiological saline only. Animals were sacrificed at Week 2 (half of the animals in each group) or at Week 6 (the remaining animals in each group) after surgery.

2.5 Radiographic examination

Lateral radiographs of specimens were taken at

two and six weeks after distraction. The radiation dosage (SIEMENS, Germany) was set at 50 kV and 16 mA, with a 0.06-ms exposure time. The distraction bone density was measured as the projectional bone mineral density (BMD).

The distraction gap of each specimen was evenly divided into three parts along the axial plane, and nine evenly distributed points were marked in the distraction gap, three in each part. Their values were standardized according to the mean of two marker points in incisor area of the mandible. The value of each point (Y) was calculated according to $Y=255 \times Z/X$, where X stands for the mean of the actual brightness at two marker points and Z stands for the actual brightness of each point. The means of nine points were used as the measure of distraction bone density.

2.6 Histological examination

After the animals were euthanized, gross features of their distraction regions were examined. Specimens were decalcified in a solution of 13% (w/v) ethylenediaminetetraacetic acid (EDTA) buffer (pH 7.2) after fixation. The samples were cut into four pieces along the axial plane longitudinally, and then all pieces were embedded in paraffin. Sections of 5- μ m thickness were prepared for hematoxylin and eosin (H&E) staining. Histological analysis was performed in a prospective, randomized, and blinded manner by two experienced pathologists, similar to the methods of Ashinoff *et al.* (2004) and Hu *et al.* (2007). Three parameters, including the thickness of new trabeculae (TNT; μ m), and the volumes of the newly generated cortical bone (NBV1) and the cancellous bone (NBV2) of the distracted regions, were measured in 10 discontinuous slices of each specimen (Ashinoff *et al.*, 2004; Hu *et al.*, 2007). The means of TNT, NBV1, and NBV2 for groups A, B, and C were subjected to statistical analysis.

2.7 Statistical analysis

Data are presented as mean \pm standard deviation (SD). Statistical significance was determined by one-way analysis of variance (ANOVA). Variables were considered significant with $P < 0.05$. All analyses were performed with SPSS 16.0 for windows.

3 Results

3.1 Gross appearance

All the surgeries were completed successfully and all experimental animals survived without serious complications, such as the non-union of bones and the loss of distracters. Twenty days after consolidation, four rabbits suffered from shin infections, but because the newly reformed bone was strong enough to withstand occlusal force, these animals were not excluded from the experiments. The three groups all exhibited typical changes of unilateral mandibular lengthening, overgrowth of the lower incisors, and obvious cross-bite. There were no obvious differences in DO wounds, gross bone volume, or appearance of the distraction zones among groups A, B, and C.

3.2 Transfected cell evaluation

ADSCs transfected with *pEGFP-OSX* showed transient expression of EGFP under fluorescence microscopy. The transfection efficiency decreased gradually from about 47% at Day 2, 42% at Day 6, 31% at Day 13, 18% at Day 20, to 13% at Day 27.

3.3 Radiographic results

Fig. 3 shows representative lateral radiographs of distracted mandibles at Weeks 2 and 6 after the end of distraction. There were significant differences among groups A, B, and C in terms of gray density analysis. The radiodensity of the distraction areas in group A was higher than that in groups B and C at Weeks 2 and 6 after the distraction procedure.

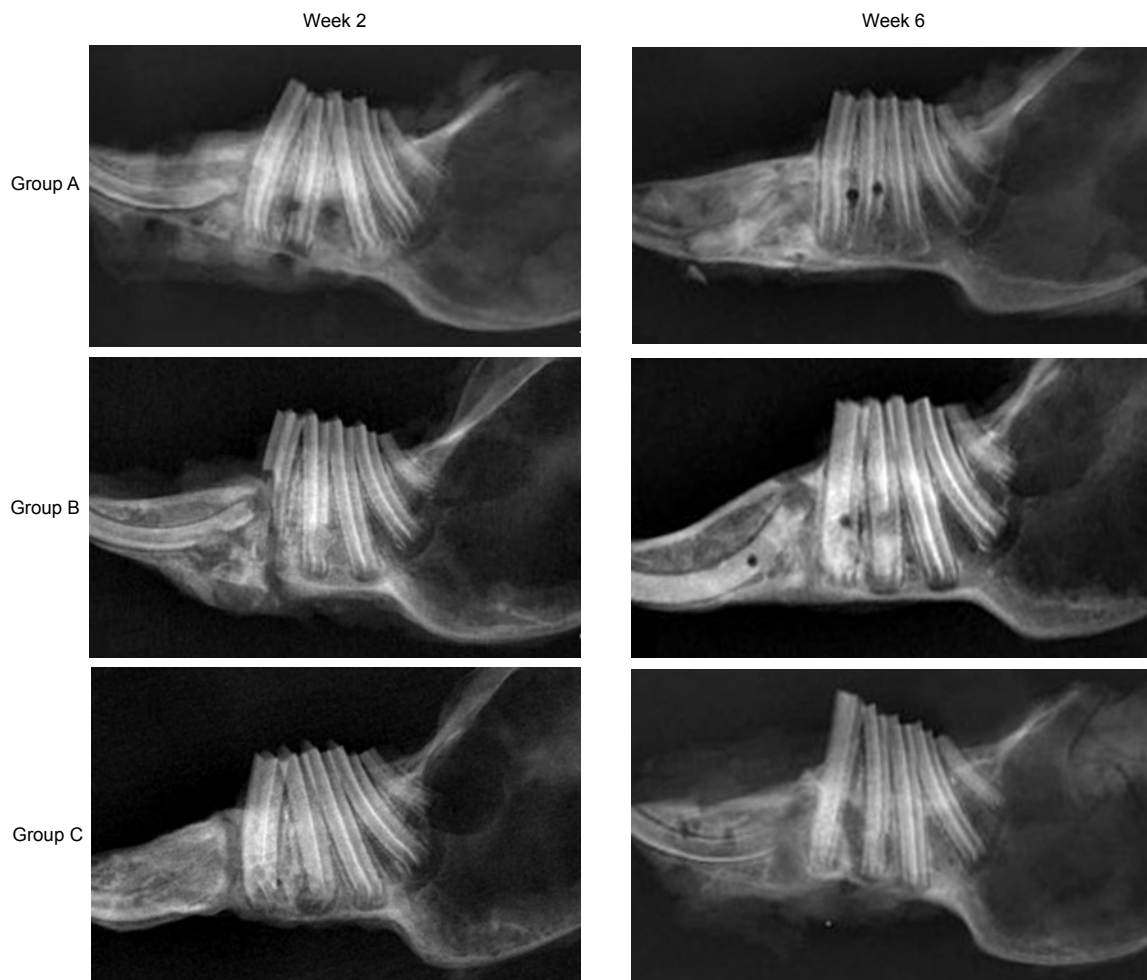


Fig. 3 Lateral radiographs of distracted mandibles in three groups at Weeks 2 and 6 after the end of distraction

Statistical analysis of the gray density (Fig. 4) also supported these results. The radiodensity of the distracted bone in group A was higher than that in groups B and C ($P<0.01$). There was no difference in radiodensity between groups B and C ($P>0.05$).

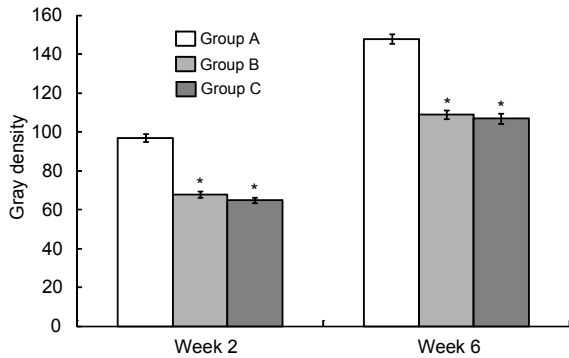


Fig. 4 Radiodensity of distracted gaps of different groups at Weeks 2 and 6

* $P<0.01$ vs. group A. Data are expressed as mean \pm SD ($n=9$)

3.4 Histological findings

Light microscopy showed that bone cells in the distracted areas were stretched along in the direction of the distraction. At the second week after the distraction procedure, the two fragments of mandibles in all groups were filled with newly formed bone trabeculae. Similar results were seen in groups B and C, but much denser and thicker bone trabeculae were observed in the distracted areas in group A (Fig. 5a) than in group B (Fig. 5b) and group C (Fig. 5c).

At the sixth week, the distraction gaps of the mandible were full of newly generated bone in all three groups. There was no difference between groups B and C, but group A (Fig. 5d) displayed more and denser bone trabeculae than the other two groups (Figs. 5e and 5f). The values of TNT, NBV1, and NBV2 were significantly greater in group A than in groups B and C ($P<0.05$) at both time points (Table 1). There was no significant difference between groups B and C ($P>0.05$).

4 Discussion

The foundation of this study was the successful distraction of fractured bones, which depended on

having a suitable animal model. Large-animal models are convenient for distraction surgeries, but lack molecular reagents and the cost is rather high. Although a mouse or rat model may avoid the defects of large animals, such models have other disadvantages: the size of the rat mandible is rather small, the rat has poor tolerance to anesthesia, and there are technical difficulties in using an extremely small distractor compared to those of large-animal models. Our study overcame all such obstacles as that rabbits were suitable for studies of large numbers of animals, and the feeding of rabbits was convenient and at moderate cost. Moreover, there was also sufficient space, in terms of mandibular bone, for surgery.

Previous studies found that BMSCs and ADSCs transfected with *OSX* or cultured in osteogenic medium differentiated into functional osteoblasts which changed greatly in shape and size (Gao *et al.*, 2004; Hu *et al.*, 2007; Lai *et al.*, 2011). These osteoblasts originating from ADSCs or BMSCs secreted extracellular matrix and calcified, expressed high levels of alkaline phosphatase (ALP), and eventually formed well-defined mineralized nodules, analogous to ADSCs growing in osteogenic medium. However, the NIH3T3 cell line transfected with *OSX* did not show an improved ability for osteoblastic differentiation or mineralization (Kim *et al.*, 2006), which indicated that osteogenic differentiation was specific to only certain multipotent stem cells. In our study, ADSCs transfected with *pEGFP-OSX* in an *in vivo* environment promoted osteogenesis greatly during distraction compared with the other two groups. This suggested that ADSCs transfected with *pEGFP-OSX* maintained the ability for osteoblastic differentiation in an *in vivo* environment. These results also agreed with those of our previous study (Lai *et al.*, 2011). In that experiment, we used autologous BMSCs as the seed cells. Bone generation *in vivo* was achieved by transplanting BMSCs modified by *OSX* into the distraction gaps at the end of the distraction, and excellent bone generation in the distraction gaps was observed. This showed that the transplantation of *OSX* modified BMSCs can promote bone generation in distraction osteogenesis (Lai *et al.*, 2011).

Adipose tissue provides an abundant source of ADSCs for isolation and use in tissue repair and regeneration (Mason *et al.*, 2000; Lange *et al.*, 2005; Liu *et al.*, 2006; Qi *et al.*, 2006). ADSCs are easy to

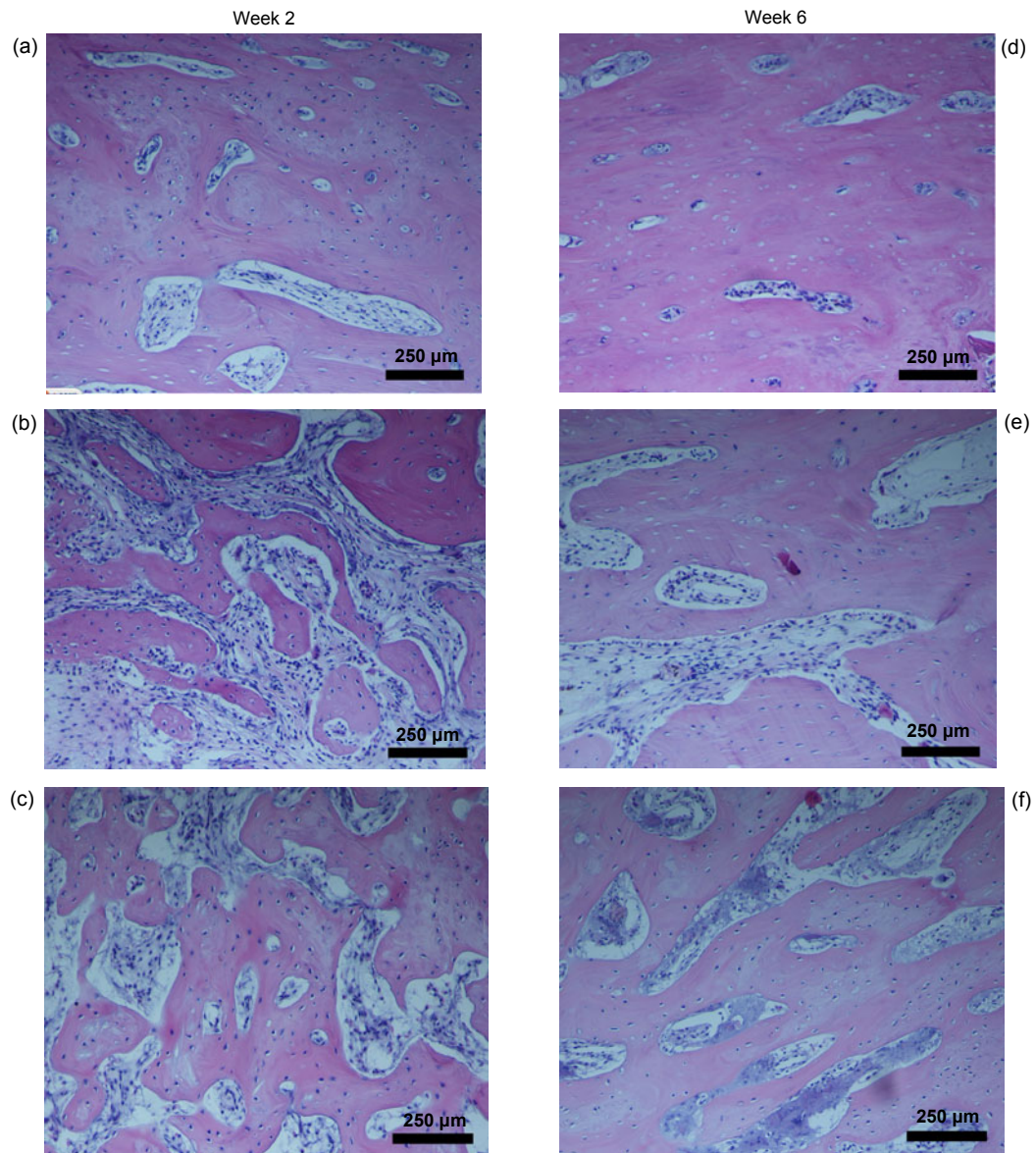


Fig. 5 Histological behavior of the distracted bone at Weeks 2 and 6 in rabbit mandibles (H&E staining)
(a, d) Group A; (b, e) Groups B; (c, f) Group C

Table 1 Thickness of new trabeculae (TNT) and the volumes of the newly generated cortical bone (NBV1) and the cancellous bone (NBV2) in different groups

Group	TNT [#] (μm)		NBV1 [#] (%)		NBV2 [#] (%)	
	Week 2	Week 6	Week 2	Week 6	Week 2	Week 6
A	38.79±3.09	55.11±2.28	62.02±3.04	95.92±2.75	43.54±2.89	67.65±4.14
B	24.76±3.13*	35.61±2.40*	45.33±2.54*	83.46±2.32*	25.65±3.57*	41.80±2.81*
C	23.89±1.86*	34.61±3.62*	44.83±5.33*	83.24±3.16*	25.45±3.12*	42.08±3.26*
<i>F</i>	82.803	149.892	58.604	62.038	94.485	166.839
<i>P</i>	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

[#] Data are expressed as mean±SD (*n*=9). * *P*<0.01 vs. group A

maintain and proliferate and their harvesting procedure is less aggressive than that of stem cells derived from bone marrow procedures, giving them a quantitative advantage (Banfi *et al.*, 2001a; 2001b; Liu *et al.*, 2012). However, the osteogenic ability of BMSCs is greater than that of ADSCs because BMSCs have intrinsic osteogenic properties, while adipose-derived cells need outside environmental factors to stimulate differentiation into osteogenic cells.

Little is known about the fate of transplanted ADSCs in recipient animals and the factors that induce ADSC migration and differentiation *in vivo*. Studies suggested that exposure of ADSCs to the bone microenvironment or to transforming growth factor- β -1 (TGF- β 1) before transplantation promoted their survival rates in bone tissues. Also, ADSCs, when exposed to the bone microenvironment, showed high levels of ALP activity. In our study, simply implanting ADSCs into the distraction gaps had no effect, suggesting that unmanipulated ADSCs have a lower ability to migrate and differentiate in bone tissues of the recipient animals. This may be because the microenvironment of the distraction areas did not enhance the survival or differentiation of ADSCs. Thus, pretreatment with TGF- β 1 and early exposure of ADSCs to the bone microenvironment to promote cell migration and the differentiation and survival of the cells, are all methods that can be used to enhance osteogenesis.

In the present study, we used scaffold-free allograft ADSCs transfected with *OSX* in a rabbit model of DO. Our results showed that the recipient rabbits responded well to the injection of allogeneic ADSCs without immunological reaction. This was possibly due to the anti-inflammatory response and low immunogenicity of ADSCs (Uccelli *et al.*, 2007).

It has been reported that the immunosuppressive effects of ADSCs are mediated by the secretion of soluble factors such as 3-dioxygenase (IDO), indoleamine 2, interleukin (IL)-10, prostaglandins, nitric oxide, hepatocyte growth factor, and TGF- β (Dazzi *et al.*, 2007). Here, the injection of ADSCs transfected with the *pEGFP-OSX* gene has shown clear evidence of promotion of osteogenesis, especially six weeks after the distraction procedure. Statistical analyses of radiological and histological results showed that bone generation was significantly improved during that

time. Therefore, their immunosuppressive effects and differentiation potential make ADSCs promising cells for bone tissue repair (Dazzi *et al.*, 2007).

This *in vivo* study indicates that the application of ADSCs transfected with *pEGFP-OSX* can improve bone regeneration during DO. Given that it was a short term observation, we believe that studies with larger numbers of other kinds of animal models conducted over longer periods of time can provide more evidence of the effectiveness of this method in future strategies for bone repair and reconstruction. Considering that adipose tissue is easy to harvest and highly abundant, it may become a more promising alternative to bone marrow as a source of stem cells (Zuk *et al.*, 2002).

5 Conclusions

The results of this study suggest that the transplantation of ADSCs transfected with *pEGFP-OSX* can effectively promote bone deposition and callus formation during distraction.

Compliance with ethics guidelines

Qing-guo LAI, Shao-long SUN, Xiao-hong ZHOU, Chen-ping ZHANG, Kui-feng YUAN, Zhong-jun YANG, Sheng-lei LUO, Xiao-peng TANG, and Jiang-bo CI declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要:

本文题目: *pEGFP-OSX* 修饰的脂肪干细胞促进牵张成骨中新骨形成

Adipose-derived stem cells transfected with *pEGFP-OSX* enhance bone formation during distraction osteogenesis

研究目的: 观察牵张间隙植入 *pEGFP-OSX* 修饰的脂肪干细胞对牵张成骨中新骨形成的作用。

创新要点: 提出来源广泛容易获取的脂肪干细胞作为种子细胞, 被基因 *OSX* 修饰后, 在体内环境下可能促进成骨。

研究方法: 通过 X 射线检查、组织学检查等手段观察成骨效果, 进行统计学分析。

重要结论: 通过短期的动物试验发现, *pEGFP-OSX* 修饰的脂肪干细胞在体内环境下促进牵张成骨中新骨形成 (见表 1 和图 3-5)。

关键词组: 脂肪干细胞; 牵张成骨; 转录因子; 基因转染