Study of BMP-2 Gene Modified Goat Bone Marrow Stromal Cells in Promoting New Bone Formation

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Objective: *To evaluate the osteoinductive effects of adenoviral encoding human BMP-2 (AdBMP-2) gene modified goat bone marrow stromal cells (bMSCs) in nude mice.*
Methods: *Primary cultured goat bMSCs were transduced with 50 pfu/ml AdBMP-2 and AdLacZ. Gene transfer efficiency was detected by X-gal staining 3 days after AdLacZ gene transduction. AdBMP-2 or AdLacZ gene modified bMSCs were injected into nude mice intramuscularly (number of injection sites: AdBMP-2 = 9; AdLacZ = 9). Three samples in each group were obtained after 2 weeks, and the remaining six were harvested after 4 weeks. All samples were evaluated by histology. MicroCT, BMP-2 immunohistochemistry and X-gal staining were performed for 4-week samples.*
Results: *Gene transfer efficiency reached above 70%. Two weeks after injection into nude mice. AdBMP-2 samples demonstrated a combination of relatively mature bone formation and a lesser amount of cartilaginous tissue. In contrast, only fibroblastic-like tissue was detected in AdLacZ-transduced bMSCs samples. At week 4, all six AdBMP-2 sites had new bone formation, while in the AdLacZ group no obvious radiopaque area was detectable by microCT. Histologically, AdBMP-2 transduction induced a large bone mass with fatty marrow tissue. While the AdLacZ group showed mainly fibrotic tissue proliferation, X-gal staining confirmed the bMSCs origin in the AdLacZ group.*
Conclusion: *bMSCs modified with AdBMP-2 gene can promote new bone formation in a nude mice model and could be used for further bone regeneration in the oral and maxillofacial region.*
Key words: *bone marrow stromal cells, bone morphogenetic protein, gene therapy*

Repairing bony defects caused by trauma or surgical resection remains one of the main challenges for oral and maxillofacial surgeons. Autogenous bone grafting is associated with many complications[1]. Therefore, alternative biotechnological approaches are under investigation. Therapy with recombinant proteins such as bone morphogenetic proteins (BMPs) has shown potential as a clinically useful alternative to autogenous bone grafting[2]. However, supra-physiological doses of BMP-2 are required for osteoinduction in clinical trials[3]. When such high doses of BMP-2 are used, potential systemic side effects are of concern[4]. *Ex vivo* gene therapy has been investigated to transduce target cells with osteoinductive genes to enhance bone regeneration[5]. The method allows delivery of gene products to be local and target-oriented, which minimises systemic side effects and maximises local therapeutic effects[6].

The aims of the study were to evaluate BMP-2, a well-established osteoinductive molecule, via an *ex vivo* regional gene therapy approach using adenoviral gene transfer into large mammal (goat) bone marrow stromal

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This work was supported by National Natural Science Foundation of China (30400502), Science and Technology Commission of Shanghai Municipality (04dz05601, 05DJ14006, 055407034), Shanghai Rising-star Program (05QMX1426) and Shanghai Education Committee (03BC39, 04YQHB081, Y0203).

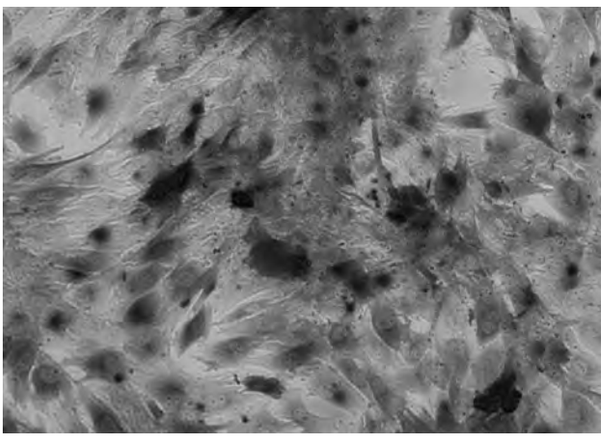

Fig 1 Gene transduction and gene transfer efficiency. X-gal staining 3 days after AdLacZ transduction of goat bMSCs. Positive cells are in blue (magnification 200x).

cells (bMSCs) in nude mice. The cell origin *in vivo* was traced by X-gal staining in AdLacZ control gene transduced bMSCs samples.

Materials and Methods

Culture of primary goat bMSCs

Adult male goats were obtained from the Ninth People's Hospital Animal Center, Shanghai, China. Approximately 5 ml bone marrow was obtained from the goat iliac crest by needle aspiration under general anaesthesia. The cells were centrifuged and suspended in Dulbecco's Modified Eagles's Medium (DMEM) (Gibco BRL, Grand Island, NY) containing 10% foetal bovine serum (FBS) (Hyclone, Logan, Utah), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/l L-glutamine (Sigma, St. Louis, MO). Haematopoietic cells that did not adhere to flasks were discarded. The adhered cells represented a population of primary bMSCs. Experiments were performed with cells from the second passage.

Gene transduction of bMSCs

bMSCs were cultured for 24 h to reach 80% confluence and transduced with a multiplicity of infection (MOI) of 50 pfu/cell AdLacZ and AdBMP-2. Cell morphology was evaluated microscopically (Leica DM 1RB, Germany). The gene transfer efficiency was determined by X-gal staining 3 days after transduction with AdLacZ by calculating the number of blue-stained cells among all the cells observed.

Animal experiments

Eighteen BALB/c nude mice of 5–6 weeks old were equally divided into AdBMP-2 and AdLacZ groups. Five million AdBMP-2- or AdLacZ-transduced bMSCs suspended in 100 μl PBS were injected into the left thigh muscle of each mouse under general anaesthesia (number of injection sites: AdBMP-2 = 9; AdLacZ = 9). Three mice in each group were sacrificed 2 weeks after injection, while the remaining six were sacrificed 4 weeks after injection. Two extra mice were injected with AdLacZ-transduced bMSCs for X-gal staining 4 weeks after injection.

MicroCT and histological analysis of ectopic bone formation

All 18 samples were fixed in 10% formalin, decalcified and parffin embedded, and samples collected 4 weeks after injection were scanned using high-resolution microCT (Scanco Medical, Basserdorf, Switzerland). Visualisation and reconstruction of the data was performed using the μCT Ray T3.3 and μCT Evaluation Program V5.0 provided by Scanco Medical. For histological analysis, the decalcified paraffin-embedded samples were sectioned at 5 μm thickness and stained with haematoxylin and eosin. X-gal staining was performed on cryosections of two samples collected from the extra two mice injected with AdLacZ-transduced bMSCs to determine the presence of LacZ *in vivo*.

Immunohistochemical analysis

The sections were deparaffinised and incubated with primary anti-BMP-2 antibody (1:200 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA). ABC complex (Vector Laboratories, Burlingame, CA) was applied to the sections following the incubation with biotinylated secondary antibody (Dako Corporation, Carpinteria, CA). AEC plus substrate in red colour (Dako) was used as a chromagen, and the sections were counterstained with light haematoxylin. The sections incubated with PBS substituted for the primary antibody were utilised as negative control.

Results

Gene transduction and gene transfer efficiency

An MOI of 50 pfu/cell produced optimal effects in transfer efficiency without excessive cell death in vitro. X-gal staining showed that over 70% bMSCs were stained blue 3 days after transduction with 50 pfu/cell AdLacZ (Fig 1).

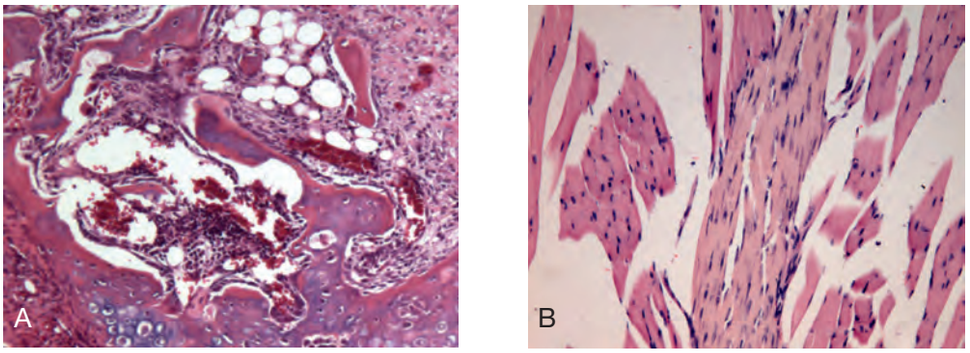

Fig 2 Histological analysis of week 2 samples *in vivo*.
(A) Osseous tissue is shown with cartilaginous tissue in the muscle injected with AdBMP-2-transduced bMSC, (magnification 200x).
(B) Fibroblastic-like tissue is mainly shown in the muscle injected with AdLacZ-transduced bMSC (magnification 200x).

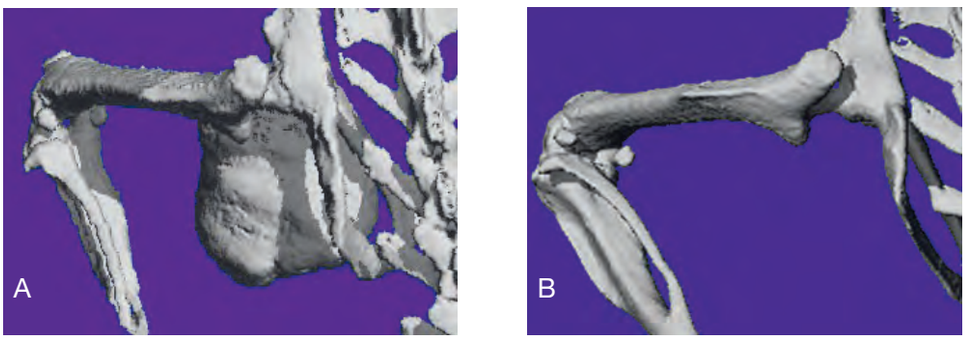

Fig 3 MicroCT evidence of bone formation in vivo 4 weeks after injection.
(A) Bony nodule is seen in the place injected with AdBMP-2-transduced bMSC.
(B) No bone formation is seen in the place injected with AdLacZ-transduced bMSC.

Analysis of intramuscular bone formation

At week 2, AdBMP-2 samples showed a large amount of relatively mature bone formation along with fatty marrow and a little of cartilaginous tissue (Fig 2A). In contrast, clusters of fibroblastic-like cells were showed in AdLacZ-transduced bMSCs samples (Fig 2B).
The three-dimensional microCT and histological analyses were used to evaluate the bone formation *in vivo* with AdBMP-2 and AdLacZ-transduced bMSCs for the week 4 samples. MicroCT analysis demonstrated an intramuscular high-density mass in AdBMP-2 samples (Fig 3A), which was not observed in AdLacZ samples (Fig 3B). Correspondingly, all the six AdBMP-2 samples showed bone masses histologically. Areas of mature cortical bone formation were also seen interspersed among fatty marrow tissue with a little cartilaginous tissue (Fig 4A). However, in the AdLacZ-transduced bMSC samples, histological analysis showed mainly fibroblastic tissue (Fig 4B).

Although there was weakly endogenous BMP-2 expression in newly formed bone (data not shown), BMP-2 immunohistochemistry displayed very strong staining in the samples of AdBMP-2-transduced bMSCs 4 weeks after injection (Fig 5A). In addition, it was also shown by X-gal staining that 4 weeks after injection, the AdLacZ-transduced bMSCs maintained the expression of LacZ (Fig 5B).

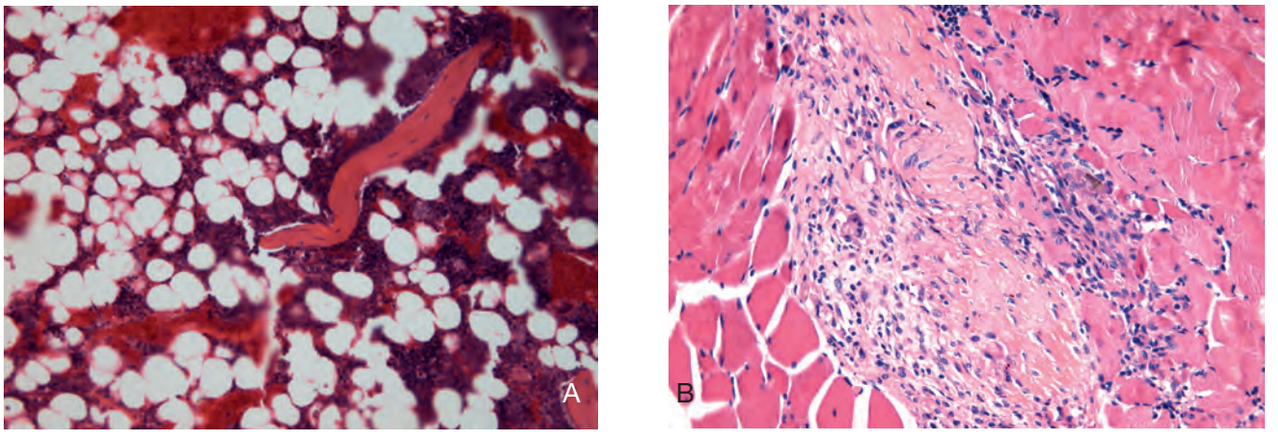

Fig 4 Histologic analysis of week 4 samples *in vivo*.
(A) AdBMP-2 sample shows mature bone with fatty marrow. (B) AdLacZ sample mainly shows fibroblastic tissue (magnification 200x).

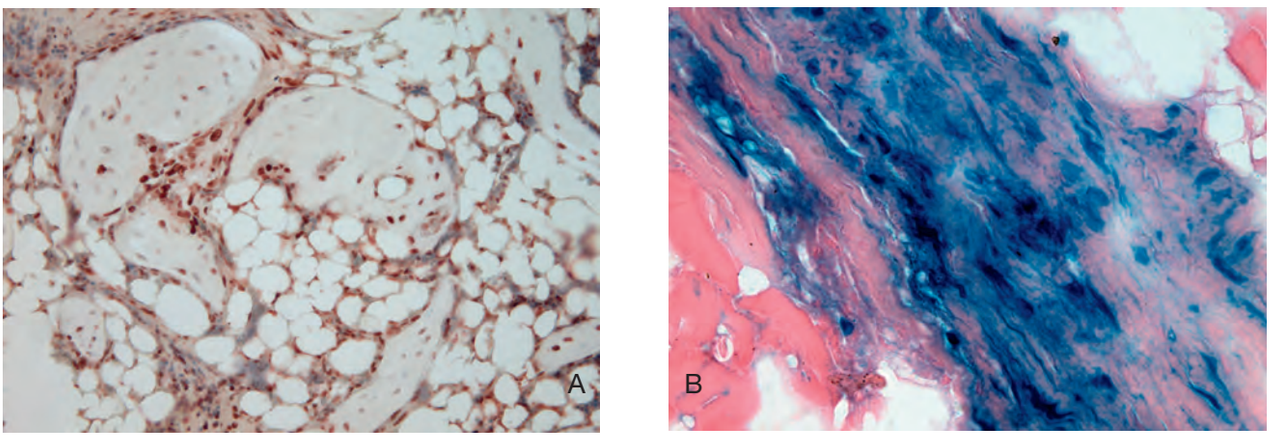

Fig 5 Verification of expressions of BMP-2 and LacZ in the week 4 samples.
(A) Immunohistochemistry demonstrates a strong BMP-2 expression in both the bone and surrounding fibroblastic tissue in AdBMP-2 sample 4 weeks after injection (magnification 200x).
(B) LacZ was shown in X-gal stained frozen section of AdLacZ samples 4 weeks after injection (magnification 400x).

Discussion

bMSCs, being multipotent, can be easily obtained and manipulated, and thus are frequently utilised in promoting bone regeneration for combination with gene therapy and for use in tissue engineering[7]. However, few studies have utilised large mammal bMSCs for *ex vivo* regional gene therapy to regenerate skeletal tissue. It is reported that the osteogenicity of bMSCs declines from rodents to large mammals[8]. And there is evidence that the concentration of cells response to growth factors decreases as the species size increases, and osteoinductive factors may be effective in smaller animals but not in larger mammals[9]. Therefore, bone tissue engineering with ex vivo gene therapy appears to be much more challenging with large mammal bMSCs, and its potential clinical applications need to be verified[10].

The success of gene therapy is dependent on efficient delivery of the target gene[11]. Various vectors can be used to achieve the purpose. A retrovirus requires target cell division and has possible insertional mutagenesis. In fact, short-term expression may not only be adequate to start the cascade of events leading to bone formation, but may also be advantageous for preventing the excessive growth of skeletal tissue that may potentially occur due to long-term BMP expression[12]. Among the transient

expression vectors, adenoviral vectors can transfect both dividing and non-dividing cells with a high transfer efficiency. Therefore, this vector has been widely used in regional gene therapy.

Previous studies have shown that bMSCs are less efficient for adenoviral *ex vivo* gene transfer. One report demonstrated that at an MOI of 250–500 pfu/cell 30–40% bMSCs were transduced, and the target protein was expressed at the highest level 1 day after transduction, and declined below the limits of detection by 15 days[13]. In the present study, goat bMSCs were successfully transduced at a relatively high efficiency of approximately 70% for AdLacZ and AdBMP-2 at an MOI of only 50 pfu/cell. In addition, the transduced bMSCs expressed the target proteins at least for 4 weeks after *in vivo* injection, as verified by either X-gal staining for LacZ or partially by specific BMP-2 immunohistochemistry for BMP-2.

Direct intramuscular injection of adenoviral-transduced bMSCs into nude mice is a widely accepted model for initial evaluation of osteoinductive properties of growth factors or stimulants[14]. In the present study, a simple model without use of a carrier or scaffold was chosen for an initial study. AdBMP-2-transduced bMSCs were injected intramuscularly in nude mice to evaluate the direct osteogenic potential of BMP-2 gene within 4 weeks before pursuing further tissue engineering applications in the goat model. As expected, at week 2, AdBMP-2 samples demonstrated a large amount of relatively mature bone formation along with fatty marrow growing and a little of cartilaginous tissue. In contrast, clusters of fibroblastic-like cells were mainly detected in AdLacZ-transduced bMSC samples. At week 4, AdBMP-2 samples showed large volumes of bone mass by microCT, whereas bone mass was not detected in AdLacZ samples. The histological appearance of the AdBMP-2 samples was characterised by islands of mature lamellar bone formation among extensive amounts of fatty marrow tissue. However, in the AdLacZ-transduced bMSC samples, fibroblastic tissue was mainly shown. The results suggested that AdBMP-2-transduced bMSCs formed bone through an endochondral bone formation process[15]. In eliciting this anabolic response, it is presumed that the genetically modified bMSCs had released BMP-2 for a certain period of time and were ultimately induced to differentiate into osteoblastic cells in vivo through the paracrine and autocrine effects of BMP-2.

In summary, the adult goat bMSCs could be efficiently transduced with AdBMP-2 and AdLacZ. AdBMP-2-transduced bMSCs could form new bone after intramuscular injection in nude mice within 4 weeks. Therefore they might be useful for future bone regeneration applications in oral and maxillofacial regions.

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