Efficacy of Bone Marrow–Derived Stem Cells in Strengthening Osteoporotic Bone in a Rabbit Model

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ABSTRACT

Osteoporosis might be due to defects in mesenchymal stem cells (MSCs) that lead to reduced proliferation and osteoblast differentiation. We hypothesized that transplantation of MSCs into sites at risk for developing osteoporotic bone could improve bone structure and biomechanics. The aim of this study was to establish an osteoporosis rabbit model by ovariectomy (OVX), characterize the autologous MSCs from the OVX rabbits, and transplant the autologous MSCs into the OVX rabbits. MSCs harvested from bone marrow of normal and OVX rabbits were culture expanded and differentiated in osteogenic medium. Phenotypes were evaluated by collagen I immunostaining, von Kossa staining, and quantitative assays of bone-specific alkaline phosphatase (B-ALP) and osteocalcin (OCN). MSCs were transfected with green fluorescence protein (GFP) and implanted in the gluteus muscle to trace their fate in vivo. Cultured autologous MSCs from OVX rabbits were constructed in calcium alginate gels and then transplanted in the distal femurs. At 4 and 8 weeks after implantation, histomorphometrical and biomechanical analyses were performed on the samples. MSCs from OVX rabbits displayed higher B-ALP activity, but had similar OCN levels as compared to those from sham rabbits. After 8 weeks of implantation, more bone apposition was found in the MSC–alginate-treated group. Histomorphometry indicated increased trabecular thickness. Histology also illustrated improved microstructures with newly formed osteoids and enhanced trabecular thickness. In addition, biomechanical testing revealed stronger stiffness in the MSC–alginate treatment group. Therefore, this study implies that transplantation of MSCs can help to strengthen osteoporotic bone in rabbits.

INTRODUCTION

Osteoporosis is a disease characterized by abnormalities in the quantity and quality of bone tissue, which leads to impaired skeletal strength and increased susceptibility to fractures.1 Although the detailed pathologic mechanism remains unknown, the disproportion between osteoblasts and osteoclasts owing to a sharp decrease in hormone secretion plays an important role during the occurrence of osteoporosis.2,3 The imbalance of the remodeling parameters leads to low bone density and an increased risk of osteoporotic fractures, which are very common in the elderly, as indicated by lifetime risk.4–6

Mesenchymal stem cells (MSCs) from postmenopausal women differ from those from premenopausal women in having a slower growth rate and exhibiting a deficiency in the ability to differentiate along the osteogenic lineage.7–9 It is suggested that the lower bone density and reduced fracture healing capacity in osteoporotic humans might be due to defects in MSCs that lead to reduced proliferation and osteoblastic differentiation.10,11 The use of stem cells and tissue engineering technology has shown promising
results in treatment of conditions as varied as hematological malignancy to cartilage defects in joints. Current studies of bone tissue engineering concentrate mainly on the repair of segmental bone defects in animals. In osteoporosis, some of the treatment options include bone regeneration with growth factors such as recombinant human bone morphogenetic proteins or genetic alteration of cells to modify phenotypes. Although gene therapy may be one of the tools available to orthopedic surgeons to treat difficult bone healing problems in osteoporosis, the safety issues related to gene therapy are still uncertain, whereas the implantation of autologous cells is becoming useful for clinical practice.

We hypothesized that transplanting in vitro cultured bone marrow stromal cells with appropriate osteogenic phenotype into sites particularly at risk for developing osteoporotic bone could improve bone structure and enhance biomechanics. The study was conducted in 4 stages:

1. Establish an osteoporosis rabbit model using the ovariectomy (OVX) procedure;
2. Characterize the autologous MSCs from the osteoporosis rabbit model;
3. Determine MSCs survivability following implantation in a rabbit model; and
4. Explore the application of autologous MSCs for the treatment of the osteoporosis rabbit model.

These data suggest that a potential therapeutic strategy to treat osteoporosis is by transplantation of in vitro cultured autologous MSCs.

MATERIALS AND METHODS

Stage 1: Development of the rabbit osteoporosis model

Six-month-old female New Zealand white rabbits were used for this study. The animals were fed in individual cages for 1 week prior to the OVX on a diet of normal pellets containing calcium (0.8%) and distilled water. They were fasted 24 h prior to operation. Hypnorm (fentanyl citrate 0.15 mg/mL; fluanisone 10 mg/mL) 0.3 kg/mg was injected subcutaneously to induce anesthesia, and Valium 0.2 mg/kg was infused via the auricular vein to maintain anesthesia. OVX was performed via a ventral incision from the umbilicus to the pubis of the rabbit. The ovarian vessels and fallopian tubes were ligated individually, and bilateral ovaries including periovarian fat pads were removed. The peritoneal incision was closed with simple interrupted sutures before skin closure.

Rabbits were randomized into 2 groups. Group I (n = 6) was subjected to sham surgery, and group II (n = 24) had bilateral OVX. Postoperatively, the animals were fasted for a further 12 h to minimize any gastrointestinal, urinary, or wound complications. Subsequently, they were allowed to move freely within their cage and were fed normal diets. Bone mineral density (BMD) of the distal femurs (10 mm from the distal end) was measured using a Norland XR-36 Bone Mineral Densitometer (Norland Corporation, Fort Atkinson, WI) before surgery and 6 months postsurgery to confirm the osteopenic status.

Stage 2: In vitro characteristics of bone marrow MSCs from normal and OVX animals

MSCs from the control group and OVX rabbits were isolated from bone marrow. Bone marrow was aspirated from the iliac crests of rabbits using a preheparinized flexible plastic tube affixed to a 10-mL syringe containing 1 mL of heparin solution (1000 U/mL). Under sterile conditions, the aspirate was transferred into a 50-mL centrifuge tube and centrifuged at 1000 rpm for 10 min. The pelleted cells were resuspended in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Grand Island, NY) containing 10% fetal bovine serum. Cultures were incubated in a humidified atmosphere of 95% air and 5% carbon dioxide (CO2) and the culture medium was changed twice a week. The cells were passaged and trypsinized at 80% confluency.

The MSCs were allowed to proliferate and differentiate in osteoinduction medium supplemented with 50 µg/mL ascorbic acid, 10 mM sodium-glycerophosphate, and 10 nM dexamethasone (Sigma, St. Louis, MO). Alkaline phosphatase (ALP) and osteocalcin (OCN) assays, and von Kossa and collagen type I staining were performed to identify the phenotypes.

Alkaline phosphatase assay

The first passaged cells were cultured in 96-well plates at 1 × 10^4 cells per well. Twenty µL of the culture supernatant were retrieved after 2, 4, and 6 days of culture and examined for ALP content using an enzyme-linked immunoassay (Metra alkaline phosphatase EIA kit, Quidel Corporation, San Diego, CA). The assay uses a monoclonal anti–bone-specific ALP antibody–coated strip to capture bone-specific ALP in the sample. All experiments were carried out in triplicate and reported as the mean values ± standard deviation.

Osteocalcin assay

The first passaged cells were cultured in 96-well plates at 1 × 10^4 cells per well. Twenty µL of the culture supernatant were retrieved after 2, 4, and 6 days of culture and examined for OCN content using a competitive immunoassay (Metra Osteocalcin EIA kit, Quidel Corporation). The assay uses OCN-coated strips, a mouse anti-OCN antibody, and a p-nitrophenyl phosphate substrate. All experiments were carried out in triplicate and reported as the mean values ± standard deviation.
von Kossa staining for calcium phosphate deposits

The first passaged cells were cultured in 6-well plates for von Kossa staining. At confluence, the medium was changed to osteoinduction medium and the cells were stained with von Kossa stain after 21 days of culture. Briefly, the monolayers were fixed with 4% formaldehyde buffered with 10 mM phosphate for 10 min. After rinsing with distilled water, 1% freshly prepared silver nitrate solution was added to the fixed cultures and left under ultraviolet light for 1 h. The cultures were rinsed 3 times with distilled water and incubated with freshly prepared 5% sodium thiosulphate solution for 5 min. The cultures were rinsed 3 times again with distilled water before viewing the stained cells under the light microscope. Calcium phosphate deposits were stained dark brown.

Collagen type I stain

The first passaged MSCs were seeded at 10⁴/cm² onto glass culture chambers. When the cells were 80% confluent, the monolayer cells were fixed with 10% formalin overnight. The cells were incubated with a primary antibody (goat anti-rat) to collagen type I for 2 h at room temperature. The cells were then incubated with the second antibody (biotinylated anti-mouse IgG) overnight at 4°C. The cells were rinsed with phosphate-buffered saline solution and avidin/biotinylated chromogen was added for 10 min at room temperature. A brown precipitate was formed indicating positively stained collagen type I.

Green fluorescence protein transfection of MSCs

To trace the fate of MSCs in vivo, we labeled MSCs with green fluorescence protein (GFP) by using the CalPhos Mammalian Transfection Kit (K’205101, Clontech Laboratories, Mountain View, CA). In brief, MSCs were plated at 4 x 10⁵ cells in 35-mm culture plates the day before transfection. Two h prior to transfection, 1 mL of the transfection solution were added dropwise under slow vortexing. The transfection solution was incubated at room temperature for 20 min. Two hundred μL of the transfection solution were added dropwise to the culture plates and returned to the CO₂ incubator for 6 h. After incubation, the calcium phosphate-containing medium was replaced with 2 mL of fresh complete osteoinduction medium and incubated at 37°C for 48 h. The GFP transfected cells were loaded onto culture chambers, cultured until 80% confluent, and harvested for in vivo study. For intramuscular implantation, 2 rabbits received GFP-transfected MSC implants (2 implants per animal). Prior to injection of the implants, the animals were immobilized and placed in a ventral position. The backs of the animals were shaved and disinfected with povidone-iodine. Two small incisions were made on each side of the vertebral column. Lateral to the incisions, an intramuscular pocket was created using blunt dissection with scissors between the space of glutius maximus muscle and glutus medius muscle. Two million GFP-transfected MSCs mixed in alginate gel were transplanted in the intramuscular pocket. After placement of the implants, the skin was closed over the implants with Vicryl 5-0 sutures. Specimens were harvested at 4 weeks post-implantation and were decalcified and sectioned at routine. GFP-marked cells were detected by direct fluorescence with laser confocal microscopy fluorescence.

Stage 4: Transplantation of autologous MSCs into the distal femur

To explore the restoration effect by MSC transplantation, 12 OVX rabbits were used for this experiment. We processed 5 x 10⁶ cultured autologous MSCs with 1 aliquot of calcium alginate gel, and injected them into the cancellous space of the right distal femur. One aliquot of calcium alginate gel alone was injected in the left femur as a control. Briefly, the calcium alginate constructs were prepared as follows. First, 2 wt% sodium alginate (A0682, Sigma) was dissolved in a solution of 0.15 M sodium chloride and 25 mM HEPES buffer at pH 7.0 and sterilized by autoclaving. Next, the monolayer cells were trypsinized and resuspended in the sterile alginate solution. Finally, the cells in alginate solution were loaded into a 10-mL syringe equipped with a 22-gauge needle, added dropwise into 1 mL of 100 mM CaCl₂ solution, and allowed to cure for 10 min at room temperature before implantation. Upon contact with CaCl₂, the alginate in the solution polymerizes and entraps the cultured cells in suspension. One aliquot of calcium alginic cell sample was fixed with 2% glutaraldehyde for 24 h and observed under a scanning electron microscope to study the distribution of transplanted cells.

Histologic, histomorphometrical, and biomechanical assessment

Rabbits were humanely killed at 4 or 8 weeks post-implantation by CO₂ euthanasia. Femurs were dissected and soft x-ray detection (SOFTEX Inc., Tokyo, Japan), histomorphometry and biomechanical testing were performed. The harvested femurs were fixed with 10% formalin overnight. For osteoid staining, the specimens were first fixed with xylanuric chloride before decalcification and then stained with H & E. Ten-μm-thick undecalcified sections of the distal femurs in OVX rabbits were processed with a Polycut machine and stained with H & E. Histomorphometric analyses were performed with the use of an Image Analysis System (Olympus Micro Image 4.0, Tokyo, Japan). The parameters measured included the bone volume/total volume, trabecular bone thickness, cortical bone thickness, and the ratio of nodes to free ends. Five sections of each
specimen were measured and the results were analyzed with Student’s *t*-test.

**Indentation tests of femurs**

For the indentation tests, the samples were cut and ground to a depth of 1.5 mm to create a surface for testing. Both right and left femurs were loaded to failure in an Instron 5543 mechanical testing machine (Instron, Canton, MA). The indenter diameter used was 1 mm and the loading rate was 1 mm/min. Ultimate strength was calculated from the formula $4P_i/\pi d^2$, where $P_i$ is the ultimate indentation load and $d$ is the diameter of the indenter.

**RESULTS**

**Establishment of the rabbit osteoporosis model**

There were no surgical complications and no macroscopic signs of infection. Interestingly, OVX resulted in changes to the rabbits’ fat metabolism, followed by depletion of estrogen. The average weight of the OVX rabbits was $4.6 \pm 0.3$ kg at 6 months postsurgery compared with the sham rabbits, which weighed $4.1 \pm 0.3$ kg ($p < 0.05$). The baseline BMD for the OVX group was $339 \pm 12$ mg/cm$^2$ ($n = 14$) and $303 \pm 4$ mg/cm$^2$ ($n = 4$) for the sham group. The OVX rabbits experienced constant bone loss 3 months following surgery, whereas those in the sham group had significant bone mass increase with growth. At 6 months postsurgery, the BMD for the OVX rabbits was $318 \pm 7$ mg/cm$^2$ versus $323 \pm 5$ mg/cm$^2$ for the sham rabbits. Soft x-ray analysis also showed less dense and loose microarchitecture (data not shown) in the OVX group compared to that of the sham group. Histomorphometrical results demonstrated significantly less bone mass volume and thinner trabecular thickness in the OVX group (Table 1).

**Cell culture**

Initially, there were difficulties in achieving MSC proliferation from osteoporotic rabbits. The colony-forming rate was low and the growth was inhibited by sparse cell-to-cell contact. Consequently, repeated aspirations were performed (as a form of stimulus) to acquire significantly

<p>| Table 1. Histomorphometrical Analysis of Undecalcified Sections in Sham and OVX Rabbits |
|---------------------------------|-----------|----------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>BV/TV(%)</th>
<th>Th. Tr (μm)</th>
<th>Node/Free ends ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVX</td>
<td>8</td>
<td>22 ± 9*</td>
<td>123 ± 15*</td>
<td>0.53 ± 0.09*</td>
</tr>
<tr>
<td>Sham</td>
<td>4</td>
<td>51 ± 12</td>
<td>156 ± 31</td>
<td>0.98 ± 0.17</td>
</tr>
</tbody>
</table>

Data are expressed as mean values ± SE.

*Significant difference between OVX and sham group; $p < 0.01$.

![FIG. 1. In vitro characteristics of bone marrow–derived stem cells. (A) Primary MSCs attain confluency at 21 days of culture. Original magnification, $\times 40$. (B) von Kossa staining of BMSCs, 2-week culture in osteogenic medium. Original magnification, $\times 40$. (C) Collagen I immunostaining of BMSCs, 2-week culture. Original magnification, $\times 200$. (D) Scanning electron microscopic image of exterior surface of MSC–alginate constructs. Spheroid cells positioned around an interconnected pore orifice. Original magnification, $\times 300$. (Color images available online at www.liebertpub.com/ten.)](Color images available online at www.liebertpub.com/ten.)
higher populations. Primary MSCs from osteoporotic rabbits were confluent after 21 days of culture (Fig. 1A).

**Characterization of bone marrow–derived stem cells**

Nodule formation was observed in cells cultured in OM medium and was positive for the von Kossa stain (Fig. 1B) and collagen type I (Fig. 1C). To quantify osteoblast differentiation, ALP and OCN activity were measured after 2, 4, and 6 days of subculture. As shown in Figure 2, there was significantly higher ALP expression in osteoporotic MSCs compared to normal MSCs (Fig. 2A; *p* < 0.05). However, initially the OCN levels in osteoporotic MSCs were lower than normal MSCs, but restored to a normal level with time during *in vitro* culture (Fig. 2B; *p* > 0.05). SEM imaging of BMSC–alginate constructs showed deposition of aggregates of spherical cells throughout the scaffold (Fig. 1D).

**Detection of implanted GFP-transduced cells**

The ectopic bone formation from GFP-transduced MSCs in alginate gels was evaluated in an intramuscular location created between gluteus muscles. After 8 weeks of implantation, green fluorescence was detected positively within the implant. H & E staining also confirmed the formation of trabeculae-like tissues with bone marrow, indicating the presence and differentiation of transplanted MSCs (Fig. 3).

**Evaluation of tissue engineered MSC–alginate constructs in osteoporotic femurs**

In the *in vivo* study, samples were harvested at 8 weeks postimplantation. No inflammation or fracture was present. Gross examination of the morphology of harvested femurs showed no inflammation or necrosis. Novel bone tissue formation could be seen in the MSC–alginate-treated femur; in contrast, the control femur presented loose bone structure (sagittal section, Fig. 4A–D). Histomorphometry of undecalcified sections revealed that the bone mass volume increased by 50% and thicker trabecular structures (trabecular thickness of treatment group 165 ± 32 μm versus control group 132 ± 22 μm) could be observed around the injection sites in MSC–alginate-treated sites (Table 2). This is evident of the osteogenic effect induced by the tissue engineering constructs. H & E stains showed denser microstructures with formation of novel osteoid bodies and enhanced trabecular thickness of the MSC–alginate-treated femurs compared to the alginate-only control femurs (Fig. 5). Table 3 shows the results from the indentation tests. The ultimate load and stress values obtained for the MSC–alginate-treated group were similar to those of the sham group (*p* > 0.05), whereas values in the alginate-only control group decreased to two thirds compared to the MSC–alginate-treated group (*p* < 0.05).

**DISCUSSION**

Autologous cell transplantation or genetically modified MSCs expanded *in vitro* is a potential strategy to augment bone formation in elderly subjects suffering from osteoporosis.\textsuperscript{16,17} This study presents the use of rabbits for an osteoporosis model and the subsequent implantation of autologous MSCs in alginate hydrogels for the treatment of...
FIG. 3. Transplantation of GFP-transduced BMSCs in muscle pocket. (A) Fluorescence image of implants with GFP-transfected BMSCs, 4 weeks postsurgery. Original magnification × 20. (B) H & E staining of the same section of the specimen. Typical trabecular bone formation lines with osteoblast zones. Original magnification × 40. (Color images available online at www.liebertpub.com/ten.)

FIG. 4. Gross views of harvested osteoporotic distal femurs, 8 weeks postsurgery. Soft-s photos. (A, B) Circles indicate the injection area. There is significantly higher radiopaque intensity in the treatment group. (C, D) Sagittal sections. (C) MSC–alginate treatment. New bone tissue formation can be observed in the MSC-alginate–treated femur. (D) Alginate only. There remains sparse trabecular structure as compared to the MSC-alginate–treated femur. The arrow indicates the injection point. (Color images available online at www.liebertpub.com/ten.)
osteoporosis. Utilizing rabbits as a model has been established for the study of bone defects, inflammatory arthritis, and even local osteopenia. Experimental models of estrogen-deficiency osteopenia are now regarded as among the most accurate for adult human diseases. Rodent OVX models appear to undergo the same bone remodeling pattern as found in human cancellous bone, and the periosteal and endocortical surfaces of adult rat cortical bone functionally behave similarly to those same surfaces in adult humans. However, the adult rat continues to be inappropriate for use in studying Haversian remodeling of cortical bone.

One of the advantages of using rabbits to establish an osteoporosis model is that they have moderate bone size with sufficient bone volume for harvesting bone marrow as compared to rodents. Also, rabbits are the smallest animals where Harvesian structures are found within the bone. Rabbits attain sexual maturity between 20 and 24 weeks with skeletal maturity at 32 weeks, and regular bone resorption and formation are well established at this stage. Similar to sheep, rabbits display seasonal estrogen-deficiency bone loss. Porcine or canine models show little or poor bone loss after OVX; therefore, they are inappropriate for the study of estrogen-deficiency osteopenia. Although recent data demonstrate that some strains of adult mice develop estrogen-deficiency bone loss after OVX, no transgenic or knockout mice have been generated that mimic typical adult human osteoporosis. In our study, we indicated that all rabbits achieved osteoporosis 6 months post-OVX. Despite initial difficulties such as lower population frequency and proliferation rates of cultured MSCs from OVX rabbits, with repeated aspirations we successfully restored the proliferation and differentiation potentials of MSCs from the osteoporotic rabbits. Physiologically, this difficulty could be due to aging and estrogen depletion, where red bone marrow of the OVX rabbits is replaced by adipose-rich yellow marrow.

A number of in vivo animal studies have demonstrated that calcium cross-linked sodium alginate hydrogels are suitable carriers for MSCs and various other cell types. Hydrogels have desired properties such as high porosity and injectability. The alginate can be conveniently and accurately injected at the site, and solidify within 30 min of injection. In our in vivo studies, there were significantly enhanced radiologic features and histomorphometrical results in autologous MSC–alginate-treated femurs. Biomechanical testing showed improved ultimate load and stress levels in the experimental group as compared to the control group. In our in vitro studies, we had a low MSC population, which resulted in difficulty in cell culture. Also, our phenotype studies showed that the MSCs still presented osteoblast differentiation including mineralization and bone-specific matrix production. Our functional studies revealed higher ALP levels as compared to normal animals, which may be due to biofeedback. Interestingly, OCN levels in the OVX MSCs were lower than normal, but still showed a tendency to increase over time.

![Undecalcified section of distal femur. H & E staining.](Color images available online at www.liebertpub.com/ten.)

**Table 2. Histomorphometry of Undecalcified Sections in MSC–Alginate and Control Groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>BV/TV(%)</th>
<th>Th. Tr (µm)</th>
<th>Nodes/free ends ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>32.3 ± 9.8*</td>
<td>165 ± 32*</td>
<td>0.92 ± 0.28*</td>
</tr>
<tr>
<td>Control</td>
<td>21.2 ± 7.8</td>
<td>132 ± 22</td>
<td>0.55 ± 0.19</td>
</tr>
</tbody>
</table>

Data are expressed as mean values ± SE.

*Significant difference between treatment and control group; p < 0.05.

**Table 3. Indentation Testing in Sham, OVX Control, and MSC–Alginate Groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Ultimate load (N)</th>
<th>Ultimate strength (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>8</td>
<td>68.9 ± 15.9*</td>
<td>87.8 ± 20.3†</td>
</tr>
<tr>
<td>OVX-control</td>
<td>8</td>
<td>45.7 ± 12.7</td>
<td>58.3 ± 16.2</td>
</tr>
<tr>
<td>OVX-treatment</td>
<td>8</td>
<td>61.3 ± 21.8*</td>
<td>78.1 ± 22.9*</td>
</tr>
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</table>

* † p < 0.05, OVX treatment versus OVX control. p > 0.05, OVX treatment versus sham.
be a result of changes in the differentiation potential of MSCs, which is in turn regulated by various hormones and growth factors. The shift from osteoblast differentiation to adipocyte differentiation with advancing age may be involved in the pathophysiology of senile and postmenopausal osteoporosis. In a study of long-term cultures of human BMSCs obtained from young and old donors, it was found that cells obtained from elderly donors showed decreased proliferation potential and accelerated senescence compared with cells from younger donors.

Although we were able to obtain a sufficient amount of bone marrow stromal cells by in vitro multiplication, we did not compare the differences in proliferation and differentiation rates of MSCs obtained from OVX and aged rabbits. It would be important to characterize and compare the replication capacity and osteogenic potential of MSCs obtained from ovariectomized and aged rabbits to determine whether autologous implantation of MSCs can be achieved successfully in older rabbits. Future studies focusing on the combination of MSCs and hormone growth factor replacement–containing scaffolds, modification of autologous MSCs therapy by genetic engineering, and growth factor composition would be very beneficial in the treatment of osteoporosis.

In summary, autologous MSCs can be transplanted into osteoporotic rabbits, and MSC–alginate-treated femurs showed significant improvement in bone histomorphometric structure and mechanical strength. These results implied that cell therapy–based tissue engineering implantation of MSCs into sites at high risk may prevent osteoporotic bone fracture from occurring as well as improve bone fracture healing in osteoporosis patients.

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