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| Original Article |

Healing of Tibial and Calvarial Bone Defect using *Runx-2*-transfected Adipose Stem Cells

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Abstract : The purpose of this study was to test the effect of *Runx-2*-transfected hASCs to heal the defect created on proximal tibiae and calvaria of immunosuppressed rats. Three kinds of hASCs (untransfected, *pECFP*-transfected ASCs or *Runx-2*-transfected ASCs) were cultured under osteogenic medium. Osteoblastic differentiation was measured by ALP staining on day 7 and osteoid matrix formation was observed by alizarin red staining on day 14 after osteogenic induction. Osteogenic potential in long bone defects were tested via 6 mm-sized circular defect on proximal tibiae of 9 immunosuppressed rats. Untransfected ASCs, *pECFP*-transfected ASCs or *Runx-2*-transfected ASCs embedded in fibrin scaffold were implanted in the defect (N=3 in each group). In order to assess the *in vivo* osteogenic capability of *Runx-2*-transfected ASC in intramembanous ossification, two critical size bone defects were created on parietal bone of 12 immunosuppressed rats. The defects were filled with fibrin scaffold containing *pECFP*-transfected ASCs, *Runx-2*-transfected ASCs or no cell (N=4 in each group). *Runx-2* transfected ASCs showed much stronger activity of ALP and greater formation of osteoid matrix compared with untransfected ASCs or *pECFP*-transfected ASCs 7 and 14 after osteo-induction, respectively. When the volume of regenerated bone was compared from gross examination and radiographs after 5 weeks in the proximal tibial defect model, the defects treated with *Runx-2*-transfected ASCs had the greatest area of healed bone compared with other groups. In the calvarial defect model, *Runx-2*-transfected ASCs had significantly increased area healed with bone ($p<0.05$) as well as better quality of regenerated bone compared with defects which was treated with untransfected ASCs from gross and micro-CT examination 8 weeks after implantation. The implanted human cells persisted in the newly regenerated bone in defects treated with *pECFP*-ASCs and *Runx-2*-transfected ASCs. In conclusion, *Runx-2*-transfection significantly increased the osteogenic potential of ASCs in the *in vivo* orthotopic models.

Key words: *bone defect, adipose stem cell, Runx-2, non-viral transfection*

1. Introduction

Bone has the capacity for self-repair without scarring. Most fractures heal spontaneously or with the help of surgical procedures.[1] Nevertheless, there are several clinical situations in which complete bone healing fails to occur.[2] Bone loss in patients with trauma or malignant tumor resection results in a critical-size bone defects (spanning > 2 cm).[3] The most commonly used surgical procedure to promote bone healing in these clinical situations is autogenous bone grafting.[4] While this method has been thought to be the “gold standard” for treating bone defects or nonunion, there is a limit in the amount of available bone from a patient as well as side effects such as pain at the harvest site.[5] Allograft bone from dead donors may be used for large bone defects. While allograft is unlimited in quantity, it is a dead bone that is less osteogenic than an autograft.[6] So allografts frequently fail when implanted into weight-bearing areas. In addition, it is associated with possible disease transmission and the risk of infection. [4]

For these challenging conditions, implantation of stem cells can offer a new opportunity for bone generation. Bone marrow-derived mesenchymal stem cells (BMMSCs) have been thought to be the best candidate for the purpose. However, available BMMSCs are also limited in an individual. For the dilemma, adipose tissue offers an abundant and easily accessible pool of stem cells.[7-9] Adipose stem cells (ASCs) obtained from

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lipoaspirates also have the multi-lineage potential to differentiate into adipogenic, chondrogenic, myogenic, and osteogenic cells.[8,10,11] Although ASCs have lower osteogenic potential than bone marrow stem cells,[12] ASCs still merit further attention and investigation as a source of stem cells for bone regeneration on account of their huge advantage in acquisition.

Gene therapy in combination with cell implantation can provide a potential armamentarium for bone regeneration.[13] The gene therapy-based expression of factors that increase the osteogenic potential of ASCs offers a possible solution to limitations of ASCs. Expression vectors of secreted growth factor like bone morphogenic protein (BMP) have been successfully introduced into ASCs to increase their osteogenic activity, as has been attempted in mesenchymal stem cells (MSCs).[14-16] However, it is difficult to finely control osteogenic response arising from the diffusion of BMPs away from the implanted sites. The use of bone-specific transcription factors that are not secreted outside of cells to direct ASCs can provide an alternative to the use of secreted proteins.[17,18]

Runx-2 is a member of the Runt domain family of transcription factors that encode proteins homologous to Drosophila runt which is crucial for proper embryonic development.[19,20] Runx-2 binds to specific promoters and regulates transcription of numerous genes that are necessary for osteoblast development.[21] Alterations in Runx2 expression levels are associated with skeletal diseases such as cleidocranial dysplasia.[22] Considering that Runx-2 is essential for osteogenic differentiation of uncommitted progenitor cells, the transfection of *Runx-2* can possibly enhance the osteogenic potential of ASCs in bone tissue engineering. Because viral transfection methods pose a risk to patient such as immunological reactions and mutagenesis, viral transfection methods cannot be safely indicated for non-lethal conditions such as bone defect. Non-viral transfection, which has improved efficiency recently, offers an alternative in a gene therapy for bone regeneration. In the previous study, we tested the hypothesis that electroporation-mediated transfer of *Runx-2* enhanced *in vitro* and *in vivo* ostegenesis from ASCs. ASCs were transfected with *Runx-2* using electroporation. Overexpression of *Runx-2* significantly increased the gene and protein expression of osteogenic differentiation markers (alkaline phosphatase [*ALP*], osteocalcin [*OCN*], type I collagen [*COL1A1*], and bone sialoprotein [*BSP*]) in ASC. *Runx-2*-transfected ASC-PLGA scaffold hybrids promoted ectopic bone formation in nude mice after 6 weeks of *in vivo* implantation in subcutaneous tissue.[23] However, the evidence of enhanced orthotopic bone formation is necessary to consider the *Runx-2*-transfected ASCs for clinical application.[23] The purpose of this study was to test the effect of *Runx-2*-transfected ASCs to heal the bone defect created on proximal tibiae and calvaria of immunosuppressed rats.

2. Materials and Methods

2.1 Cell isolation and Cultivation

The ASCs were isolated from lipoaspirates generated during elective liposuction of three patients (mean age, 41 years; range, 32–48 years). Cell isolation and cultivation was done according to a protocol established on the authors' previous study.[23]

2.2 Non-viral Transfection of ASCs using Microporation

Subconfluent ASCs were harvested and washed with DPBS. Cells were resuspended in resuspension buffer R (Invitrogen) at a density of 3×10^7 cells/mL and mixed with 0.5 mg of *pECFP* plasmid [coding for green fluorescent protein (GFP)] or *pRunx-2* (coding for GFP and Runx-2) plasmid as established in the author's previous study.[23] Then electroporation was performed with the Microporator (Invitrogen) using programs recommended by the manufacturer (1400 voltage, 20 ms, two pulses). After electroporation, cells were plated on a 12-well plate containing antibiotics-free growth medium and placed at 37°C in a 5% CO_2.

2.3 Osteogenic Differentiation

To induce differentiation, the transfected ASCs were cultured with a specific induction media (osteogenic medium [OM] consisting of α-MEM solution containing 10% FBS, 100 nM dexamethasone, 50 μM L-ascorbate-2-phosphate, 10 mM glycerophosphate, and 1% penicillin and streptomycin). The cells were incubated in OM for up to 2 weeks at 37°C in a 5% CO_2 in a 12-well plate at a density of 3×10^5 cells per well. The medium was changed every third day. The analyses were performed on days 7 and 14 to test the osteogenic differentiation of ASCs. To quantify ALP enzymatic activity, the transfected ASCs were cultured for 7 days under osteogenic differentiation condition. The differentiated cells on plates were washed twice with PBS and fixed in 4% paraformaldehyde for 10 min. Following three washes with PBS, cells were permeabilized for 30 min with 0.1% TritonX-100 in PBS. The cells were stained with nitro blue tetrazolium (Sigma-Aldrich, St. Louis, MO) and 5-bromo-4-chroro-3-indolyl phosphate (Sigma-Aldrich). To measure calcium deposition in the extracellular matrix, the differentiated cells for 14 days were washed twice with PBS and fixed in 4% paraformaldehyde for 10 min. Following three washes with PBS, cells were then stained with 2% alizarin red solution (Junsei Chemical, Tokyo, Japan) for 10 min.

2.4 Surgery and Transplantation Procedure

The animal experiments conducted in this study were approved by the Animal Research and Care Committee of our institution. 9-week-old male Sprague-Dawley rats were used in this study. The animals were anesthetized with zoletil (40 mg/kg) and xylazine (10 mg/kg).

2.4.1 Long bone defect model

Nine-week-old Sprague Dawley (SD) rats were used for tibia partial defects. A total of 9 animals were assigned to 3 groups and fibrin was used as scaffolding material. A 6 mm (diameter) × 2 mm (depth) circular defect was created on left proximal tibiae of rats using a surgical microdrill fitted with a 2-mm drill point. The wound was thoroughly irrigated with warmed saline to remove residual bone dust. The harvested cells (1×10^6 cells) was resuspended with 25 μl fibrin and mixed with 25 μl thrombin for gel formation in microtube. And then, the cell-fibrin composites were placed into the circular defect, respectively. There were 3 animals in each of the three following groups: (1) ASC only (2) fibrin-ASC/pECFP (3) fibrin-ASC/pRunx2. After cell implantation, the muscle and skin were closed with a surgical black silk suture. The rats received daily injections of cyclosporin A to suppress immune responses in rats. After 5 weeks, the rats were sacrificed by carbon dioxide. The diameter of defects was measured to assess the degree of healing.

2.4.2 Calvarial defect model

A full-thickness calvarial bone defects of 4-mm in diameter was created without dura perforation using a surgical microdrill fitted with a trephine burr. The wound was thoroughly irrigated with warmed saline to remove residual bone dust. A total of 12 animals were randomly assigned to 3 groups: group 1, the fibrin-only; group 2, fibrin-ASC/pECFP; group 3, fibrin-ASC/pRunx2. The harvested cells (1×10^6 cells) was resuspended with 10 μl fibrin and mixed with 10 ml thrombin for gel formation in plastic mold of 4-mm in diameter. And then, the cell-fibrin composites were placed into the calvarial defects. After implantation of the cell-fibrin composite, the defect region was covered with polycaprolactone membrane, and the skin was closed with a surgical black silk suture. The rats were received daily injections of cyclosporin A to suppress immune responses. After 8 weeks, the rats were sacrificed by carbon dioxide. Calvarial bones were dissected out and underwent gross and histological analysis as well as micro computed tomography (micro-CT). CT images were obtained with a micro-CT system (SkyScan-1172; Skyscan, Kontich, Belgium). The bone formation area was measured using an image analysis system (KS400; Zeiss, Munich, Germany) coupled to a light microscope.

2.4.3 Macroscopic observation, histological and immunohistochemical analysis

Following macroscopic examination, the calvarial bone was dissected and embedded in an Optimal Cutting Temperature (O.C.T.) compound (aqueous embedding medium within a mold), frozen in a metal pan over a bath of liquid nitrogen. All frozen tissue blocks were cryo-sectioned to a nominal thickness of 10 μm. Goldner's trichrome staining was performed for analysis of bone formation. For immuno-tracking of the transplanted human ASCs in the repaired tissue, the sections were blocked with PBS containing 10% normal goat serum (Vector laboratories, Burlingame, CA) and 0.3% Triton X-100 at room temperature for 1h after 1% SDS antigen retrieval. Following an anti-human nuclei antibody (1/200 diluted in PBS containing 0.3% Triton X-100; Millipore, Billerica, MA) was applied overnight at 4°C. The slides were incubated with biotinylated anti-mouse secondary antibody (1/200 diluted in PBS; Vector Laboratories) for 1h. The fluorescein (DTAF)-conjugated streptavidin (1/400 diluted in PBS; Jackson ImmunoResearch Lab Inc., West Grove, PA) were used for visualization.

2.5 Statistical Testing

All quantitative data are expressed as the group means and standard deviations. Statistical analysis was performed with the Mann-Whitney U-test using SPSS software (SPSS; Chicago, IL). Significance was set at a $p<0.05$.

3. Results

3.1 Osteoblastic Differentiation and Osteoid Matrix Formation of ASCs

The degree of osteoblastic differentiation was measured by ALP staining on day 7 and osteoid formation was observed by alizarin red staining on day 14. *Runx-2-* transfected ASCs showed much stronger activity of ALP and greater formation of osteoid matrix compared with controls (untreated ASCs or *pECFP*-transfected ASCs, Fig 1)

3.2 Enhancement of Bone Formation on the Long Defect in Proximal Tibiae using Runx-2 -transfected ASCs

Osteogenic potential in long bone defects were tested via circular defect created on proximal tibiae. We abstained from creating complete segmental defect in order to avoid the need of internal or external fixation. Untransfected ASCs only, *pECFP*-transfected ASCs or *pRunx-2* transfected ASCs

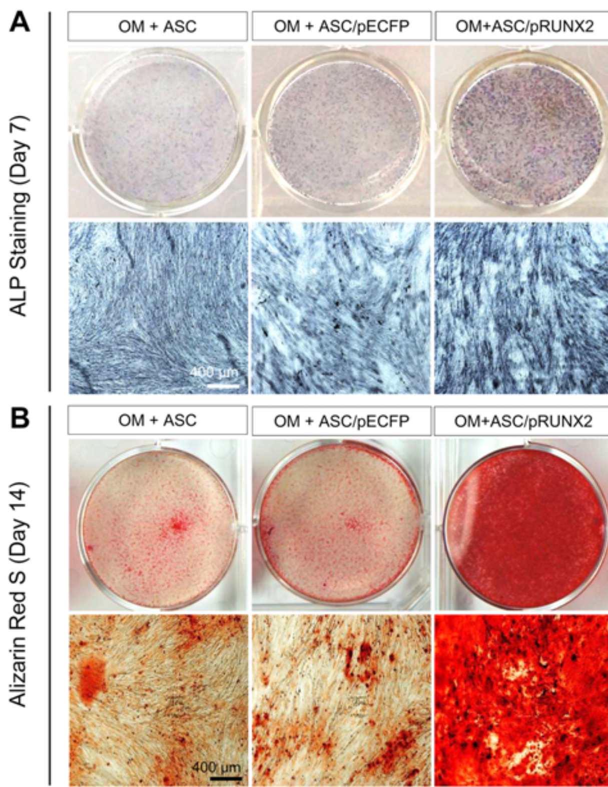

Figure 1. Osteoblastic differentiation and osteoid matrix formation as observed by ALP staining (A) and alizarin red staining on day 14 (B).

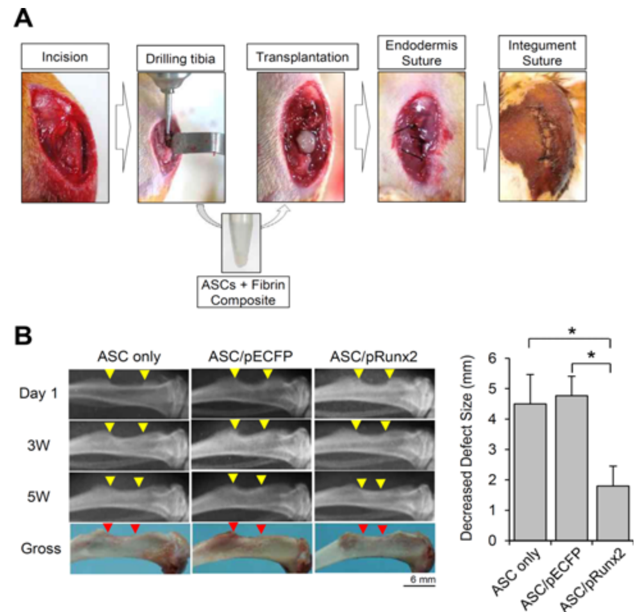

Figure 2. Bone regeneration from long bone defect created on proximal tibial defects using *Runx-2-* transfected ASC. The surgical procedure of creating proximal tibial defect (A). The radiographic observation (Day 1, 21, 35) and gross findings of the defects after 5 weeks of implantation (B), N=3, $*P<0.05$. ASC/pECFP : *pECFP*-transfected ASCs, ASC/pRunx2: *Runx-2*-transfected ASCs.

embedded in fibrin scaffold were implanted in the defect. After 5 weeks, the volume of regenerated bone among different groups was compared from gross examination and radiographs. While bone regeneration was observed from all groups, rats treated with *Runx-2*-transfected ASCs had the smallest remaining defect compared with other groups ($p<0.05$, Fig 2).

3.3 Enhancement of Bone Formation on Calvarial Defect using *Runx-2-* transfected ASCs

In order to assess the *in vivo* osteogenic capability of *Runx-2*-transfected ASCs in intramembanous ossification, two critical size- bone defects were created on parietal bone of rats. The defects were filled with fibrin scaffold containing *pECFP*-transfected ASCs, *Runx-2*-transfected ASCs or no cell. Eight weeks after implantation, rats were sacrificed and evaluated by micro-CT and histology. Gross examination revealed durable bone formation in defects treated with *Runx-2*-transfected ASCs. Defects treated *pECFP*-transfected ASCs showed new bone formation in the periphery with focal area of fibrotic tissue centrally. On the other hand, defect treated with fibrin scaffold were filled with fibrotic or membranous tissue only. *Runx-2*-

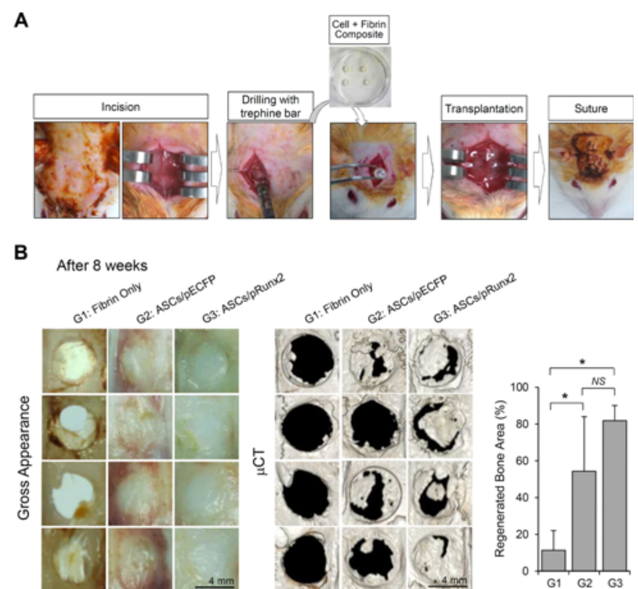

Figure 3. Regeneration of calvarial defects from immunosuppressed rats. Surgical procedure (A). Gross and micro-CT appearance (B), N=4, $*P<0.05$. ASC/pECFP : *pECFP*-transfected ASCs, ASC/pRunx2: *Runx-2*-transfected ASCs.

transfected ASCs had significantly increased area healed with bone ($p<0.05$) compared with the control defect which was

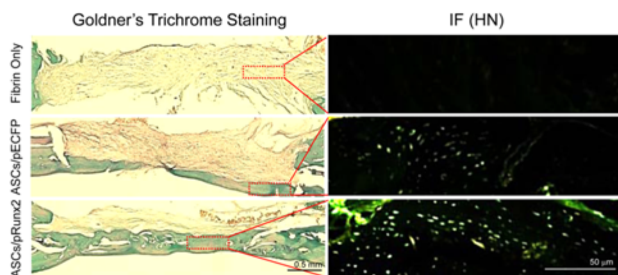

Figure 4. Histological findings from Goldner's trichrome staining (A) and immunochemistry for human nuclear antigen from regenerated calvarial bone (B). ASC/pECFP : *pECFP*-transfected ASCs, ASC/pRunx2: *Runx-2*-transfected ASCs, HN: human nuclear antigen.

treated with untransfected ASCs from gross and micro-CT examination (Fig 3).

The histological findings observed by trichrome staining also demonstrated significantly better quality of new bone formation including full restoration of bone width and robust formation of trabeculae in defects treated with *Runx-2*-transfected ASCs. New bone formation was also observed from *pECFP*-transfected ASCs, but the bone width was thinner and the defects were mostly filled with fibrous tissue. The defects treated with fibrin only were filled with fibrous tissue without any evidence of new bone formation. The detection of human nuclear antigen was performed to see if the implanted human cells persist in the newly regenerated bone. Human cells were observed in numbers in both defects treated with *p*ECFP-ASCs and *Runx-2*-transfected ASCs while defects treated with fibrin scaffold only showed no detectable cells (Fig 4).

4. Discussion

The overall results demonstrated successful *in vivo* osteogensis with *Runx-2*- transfected ASCs. A successful repair of critical size calvarial and long bone defect which were better than untransfected ASCs were demonstrated.

The transfection of *Runx-2* by electroporation is known to be of short duration. Runx-2 triggers expression of major bone matrix proteins at an early stage of differentiation. Previous results showed that the short duration of Runx-2 expression in electroporation-mediated transfection still worked to form the bony tissue while persistent high expression of Runx-2 rather cause increased bone resorption.[23] Several studies investigated viral transduction of *Runx-2* for bone regeneration. Zhao *et al*.[20] transfected *AdRunx2* to mouse MSCs and observed that Runx-2 protein expression was highest on the 1st day after transfection and declined to an undetectable level after the 15th day. However, *Runx-2*-dependent mineralization persisted and MSCs expressing Runx-2 formed substantially more bone than the control MSCs when implanted in subcutaneous tissue or the calvarial defects of mouse. When primary rat bone marrow stromal cells transduced with *Runx-2* or control (no *Runx-2* insert) retroviral vector were seeded onto 3D-fused deposition-modeled polycaprolactone scaffolds, *Runx-2*-transduced cells produced biologically-equivalent mineralized matrices at nearly 2-fold higher rates than control cells.[24] *AdRunx-2* transfection model in relatively short expression of Runx2 led to enhanced bone formation from adult stem cells.[17]

The clinical implication from our study lies in the demonstration of enhanced osteogenesis from both long bone (tibial defect) and flat bone (calvaria defect) from *Runx-2*-transfected ASCs using non-viral gene transfer. This technique can be applied for tissue engineering purposes without concern for immunological reactions or mutagenesis which is associated with the use of viral vectors.

Calvarial defect models are golden standard model to assess in vivo osteogenesis. However, it evaluates intramembranous bone formation in the nonweight-bearing area, and does not provide an information on whether it also promotes healing in long bone defects in which endochondral bone formation plays an important role. Hence, proximal tibial defect model was used to assess the bone-forming effect of the transfected ASCs in weight bearing bone. While this study demonstrated successful bone regeneration using *Runx-2*-transfected ASCs in rodents, a further experimentation in large animals will be necessary to explore the possibility of clinical application. While large long bone defects caused from trauma or malignant tumor resection are primary targets of bone regeneration using *Runx-2*-transfected ASCs, intractable bone diseases such as osteonecrosis of femoral head can be also indicted for this method of bone regeneration.

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Conflict of Interest: Jong Min Lee, Eun Ah Kim, and Gun-Il Im declare that they have no conflict of interest.

Ethical Statement: The animal experiments conducted in this study were approved by the Animal Research and Care

Committee of Dongguk University Isan Hospital.

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