

Fracture Healing Effects of Locally-Administered Adipose Tissue-Derived Cells

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Received: July 30, 2014

Revised: September 2, 2014

Accepted: September 24, 2014

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The authors have no financial conflicts of interest.

Purpose: Although the applications of adipose tissue-derived cells (ADCs) in regenerative medicine have been investigated, the role of ADCs in fracture healing remains unclear. In this study, we examined the fracture-healing effects and survival of transplanted ADCs using micro-computed tomography (CT) and bioluminescence imaging (BLI). **Materials and Methods:** Luciferase-expressing ADCs were suspended in solubilized basement membrane preparation (SBMP) and xenografted on defects in the right femur of nude mice (n=5). SBMP alone was grafted on a defect in the contralateral femur. Serial *in vivo* micro-CT and BLI were performed for 20 days. *Ex vivo* BLI images of both femurs were obtained. Differences in the Hounsfield unit (HU), HU_{ratio} , and luciferase activities were compared using Wilcoxon signed-rank tests and non-parametric longitudinal analyses ($p < 0.05$). **Results:** *In vivo* BLI revealed a signal drop on day 2, reconstitution on day 5, and continuous decrement thereafter. *Ex vivo* BLI revealed residual activity in the ADC-implanted and adjacent areas. No activity was detected in the contralateral femur. The overall increment rate of normalized HUs was higher for ADC-treated femurs than for SBMP-treated femurs. Cell migration to distant injury sites was not detected. **Conclusion:** Enhanced bone density in the implant area suggests that ADCs have fracture-healing effects.

Key Words: Fracture, stem cells, computed tomography, imaging

INTRODUCTION

Despite substantial advances in the field of orthopedics and related medicine, 5–10% of fractures that occur annually in the United States end in non-union.¹ Fracture healing is a complex process that is orchestrated by numerous factors, including cytokines, chemokines, osteoblasts, osteoclasts, fibroblasts, and inflammatory cells.^{2,3} Mesenchymal stem cells (MSCs) have been studied extensively for their ability to accelerate bone regeneration and healing.^{4,5} MSCs occur in various tissues, including bone marrow, adipose tissue, periosteum, fetal blood, and synovium.⁶⁻⁸ Although bone marrow-derived MSCs (BMMSCs) have been widely stud-

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ied, these cells have limited applications in fracture healing owing to low yields and invasive harvesting procedures.⁹ On the other hand, adipose tissue might be an ideal source of MSCs because of its abundance in the body. In addition, adipose tissue-derived MSCs (ADMSCs) may be easily harvested. Therefore, ADMSCs are being studied extensively for potential applications in the field of stem cell therapeutics.^{7,10}

MSCs from diverse tissues and organs have been introduced via systemic or local routes to treat fractures. Many studies have demonstrated that systemically introduced BMMSCs and ADMSCs migrate to the fracture site and promote bone healing.¹¹⁻¹⁴ However, other studies showed that only genetically modified cells that expressed bone morphogenetic protein-2 had significant bone regeneration effects, while ADMSCs alone failed to promote bone healing.^{10,15} Therefore, the exact role of MSCs in fracture healing remains controversial and requires further investigation.

Recent advances in imaging technologies such as micro-computed tomography (CT) have made it possible to assess the healing process of the skeletal system by measuring the Hounsfield unit (HU) of bone tissue. Bioluminescence imaging (BLI) has enabled us to monitor viable grafted cells expressing genetically engineered reporter genes such as green fluorescent protein (GFP) and luciferase.¹⁴ In our study, we harvested adipose tissue derived cells (ADCs) of transgenic mice and achieved stable levels of luciferase activity without degradation during the entire follow-up period.

The main purpose of this study was to evaluate the bone-healing effects and survival or proliferation rates of locally administered ADCs during the early phase of fracture healing. In addition, we attempted to evaluate the trafficking ability of locally administered ADCs to remote fracture sites using micro-CT and BLI.

MATERIALS AND METHODS

Mesenchymal stem cells isolation and culture

All experiments were approved by the Institutional animal care and use committee of the Stanford University. To obtain steady reporter gene signals, we isolated ADCs from transgenic mice carrying the β -actin promoter gene and expressing GFP and firefly luciferase, instead of transfecting reporter genes into harvested ADCs. Subcutaneous adipose tissue was obtained from the region between the lower anterior abdominal wall and the inguinal area of transgenic mice, washed in phosphate buffered saline several times, and finely

minced for 5 min. The tissues were then digested with 0.075% collagenase (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 1 h. Neutralized cells were centrifuged, and mature adipocytes and fibro-vascular fractions were discarded. Pelleted stromal cells were then passed through a 100- μ m cell strainer before plating. The cells were cultured in Dulbecco's modified Eagle medium, containing 10% fetal bovine serum, 100 IU/mL penicillin, 100 IU/mL streptomycin, and 0.25 μ g/mL amphotericin at 37°C in an atmosphere of 5% CO₂. The passage of ADCs used in this study was 2 or 3. A preliminary *in vitro* BLI of cultured ADCs was performed to confirm luciferase expression before implantation into animal models.

Surgery and local administration of ADC in the fracture model

Five nude mice (NU/NU), aged 8–10 weeks, were purchased from the Charles River Laboratory (Wilmington, MA, USA). The animals were placed in a supine position and administered isoflurane (an inhalational anesthetic). A surgical incision was made along the lateral side of the bilateral thighs to expose the femoral shafts. A hole with 0.5 mm diameter was drilled in the femora using a small trephine bit, under saline irrigation.

The harvested reporter-expressing ADCs (6×10^5) were suspended in solubilized basement membrane preparation (SBMP) (Matrigel™, BD Biosciences, San Jose, CA, USA) and inserted into the hole in the right femur shaft. Subsequently, the muscles and skin were sutured. In addition, SBMP alone was administered into a hole of the same size in the contralateral femur, using the same protocol.

In vivo and *ex vivo* BLI

Approximately 3 mg of luciferin (30 mg/mL solution of D-luciferin; Biosynth International, IL, USA) was administered via intraperitoneal injection. BLI was performed 10 min after the administration of luciferin using a cooled charge-coupled device camera (IVIS; Xenogen, CA, USA). The acquisition time was 5 min, and five mice were imaged simultaneously in the prone and bilateral decubitus positions. BLI was performed on days 0, 2, 5, 7, 9, 12, 14, 16, and 20 after the operation and administration of ADCs. All BLI data from mice with implanted ADC were obtained from left decubitus images. The BLI data were quantified by drawing a 3.0-cm sized region of interest (ROI) to include the entire BLI signal detected on day 0. An ROI of the same size was used to measure BLI signals during the follow-up period to avoid loss of BLI activity from the areas near the

hole in the femur (Fig. 1). A time versus activity curve was obtained from this data to evaluate changes in luciferase activity with time. Bioluminescence activity was expressed in terms of average radiance (photon/cm²/s/steradian).

After the last CT follow-up, the animals were administered an intraperitoneal injection of D-luciferin and sacrificed after 10 min. ADC-treated and control femora were harvested, and the soft tissue overlying the femoral defect was excised. BLI data were recorded for the bones, with the entrance to the hole facing the camera.

Micro-CT

CT images of both femurs were obtained using a dedicated small animal CT scanner (eXplore RS micro-CT system, GE Medical Systems, Raleigh, NC, USA). The animals were placed on the scanner table in a prone position under inhalation anesthesia and exposed to a cone beam (80 kVp, 450 μ A) for 400 ms. Image acquisition and reconstruction were performed using the eXplore Evolver and eXplore Reconstruction Interface software, respectively. Micro-CT images were obtained on days 0, 3, 5, 7, 12, 14, 16, and 20 after the operation and implantation of ADCs. The resolution of the reconstructed image (voxel) was 0.45 \times 0.45 \times 0.45 mm³ (Fig. 2). The change in bone density in the femoral defect

was evaluated by measuring the HU of the bone in a 0.2285 mm³-sized cylindrical volume of interest (VOI). The HU data obtained from the VOIs of bilateral femoral holes were plotted versus time. To minimize bias due to differences in the initial baseline HUs between the control and ADC-treated sites, we used another parameter, the HU_{ratio}. The HU_{ratio} was calculated by dividing the HU at each time point with the baseline HU. The HUs and HU_{ratio} of both the left and right femurs during the follow-up period were compared to evaluate the bone-healing effects of ADCs.

Statistical analysis

Statistical differences in the BLI signals during the follow-up period were assessed using the Mann-Whitney U test. The HUs and HU_{ratio} of the ADC- and SBMP-treated (control) sites were compared and evaluated using the Wilcoxon signed-rank test. To analyze whether the differences between the HU_{ratio} of the SBMP- and ADC-treated sites increased significantly at each time point, a non-parametric longitudinal analysis was performed. Differences were considered statistically significant if $p < 0.05$. All statistical evaluations were performed using the SAS statistical software (version 9.2; SAS Institute Inc., Cary, NC, USA).

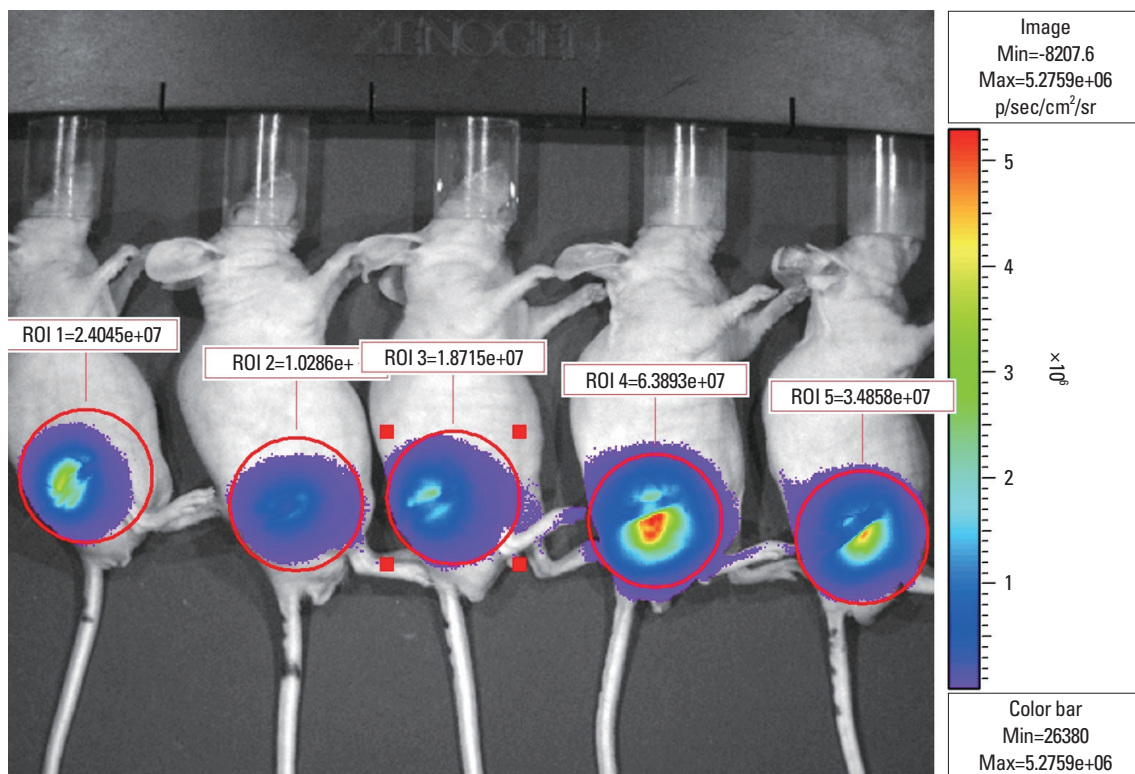


Fig. 1. Bioluminescence imaging of adipose tissue-derived cell-implanted fracture model. A region of interest (ROI) that included nearly the entire signal was drawn on the image recorded in the left lateral decubitus position.

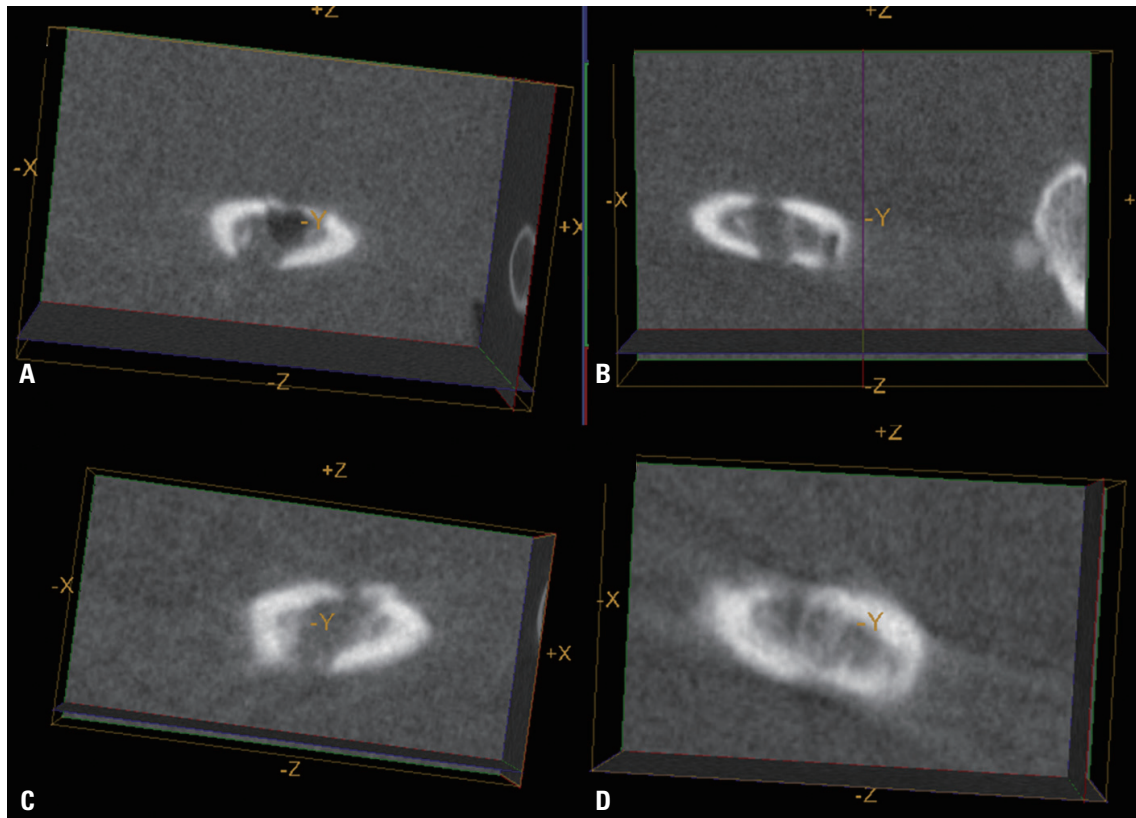


Fig. 2. Micro-computed tomography (CT) of bilateral femurs with holes (diameter, 0.5 mm). Baseline micro-CT images of control (A) and adipose tissue-derived cell (ADC)-implanted sides (B) showed relatively low bone densities in the holes. Final micro-CT images on day 20 showed increased bone densities in both the control (C) and ADC-implanted sides (D).

RESULTS

Second or third passage ADCs harvested from transgenic mice expressing two reporter genes—GFP and firefly luciferase—showed relevant bioluminescence activities in a preliminary *in vitro* BLI. Post transplantation, initial *in vivo* BLI of the five femur fracture models revealed strong bioluminescence activities; however, an abrupt decrease in activity was noted on day 2, followed by an increase on day 5. However, the Mann-Whitney U test showed no significant differences in the activities between days 0 and 5 ($p=0.841$). After day 5, bioluminescence activity decreased continuously with time. However, the time versus activity curve revealed a relatively rapid decrease in the activity until day 9 and a slow decrease thereafter, until day 14. This was followed by a steady level of activity in the remaining 6 days (Fig. 3). There were no significant differences in the bioluminescence activities between days 9 and 20. During the follow-up period, no significant increase in the BLI signal could be detected, which might be indicative of proliferation of implanted ADCs.

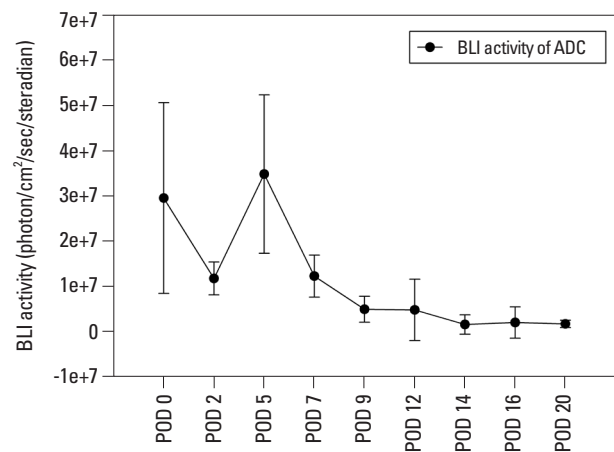


Fig. 3. The time vs. activity curve for firefly luciferase-expressing ADCs showed rapid decrement on post-operative day (POD) 7, followed by slow decrement until POD 12, before reaching a plateau. BLI, bioluminescence imaging; ADCs, adipose tissue-derived cells.

To determine whether the locally administered ADCs migrated to remote fracture sites, serial *in vivo* and *ex vivo* BLI were performed. No bioluminescence activity was detected in the left femur (control side) in both the *ex vivo* and *in vivo* studies, although *ex vivo* BLI of the right femora of all five mice showed persistent luciferase activities to various de-

grees. In two mice, the focal luciferase signal was confined to the hole in the femur; however, in the other three mice, the BLI signal could be detected in the femoral hole as well as the adjacent femoral shaft (Fig. 4).

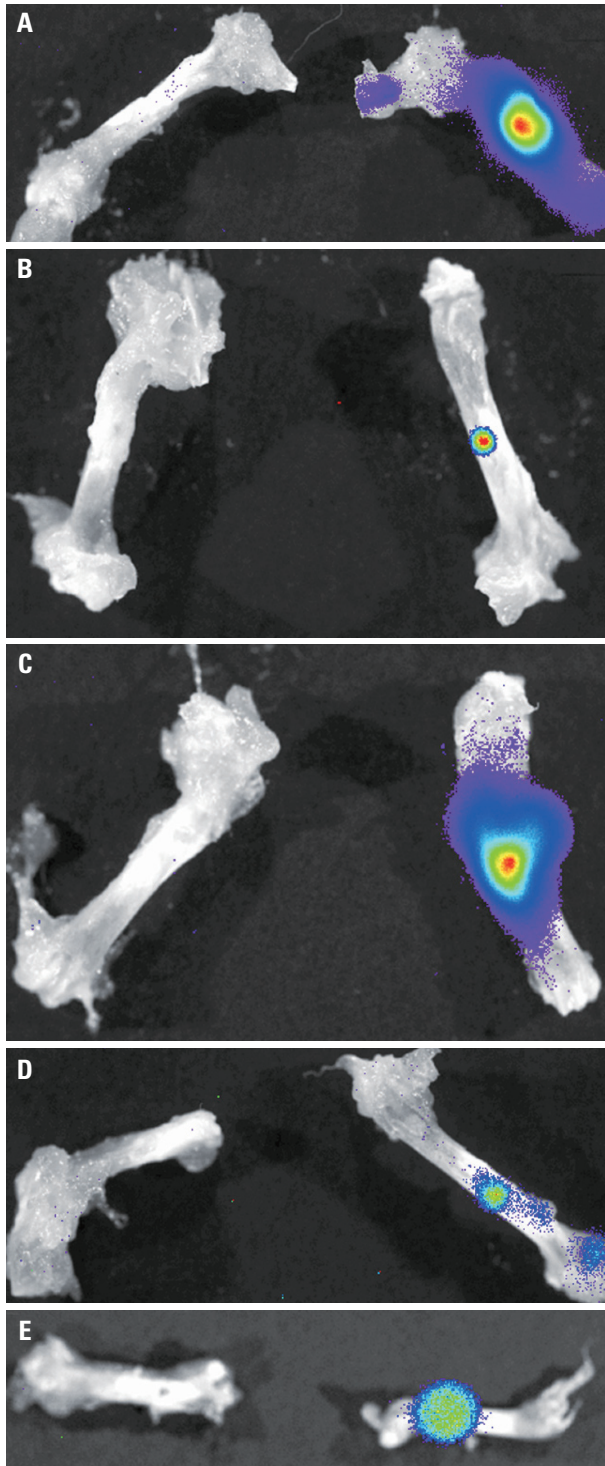


Fig. 4. *Ex vivo* BLI revealed no luciferase activity in the left femur. However, in three out of five mice, luciferase activity was detected in the primary site on the right femur as well as in adjacent areas (A, C, and D), while in the remaining two, activity was detected only in the primary sites (B and E). BLI, bioluminescence imaging.

Micro-CT imaging of the femoral holes revealed higher HU values for the SBMP-treated side than for the ADC-treated side from baseline to day 16; serial HUs increased steadily with time. However, there was no significant difference in the HUs between the two sides at each time point (Fig. 5A). The addition of the ADC pellet to SBMP may have increased the semi-liquid volume of the injectate in the ADC-SBMP group, compared with the SBMP-only group. This increased liquid volume might have washed away the hematoma and calcifications in the bone defect, resulting in a lower HU on the ADC-treated side. Therefore, to minimize bias caused by the difference in baseline HUs between the two groups, HU_{ratio} , which represents the relative change of HUs at follow-up time points with respect to baseline HUs, was calculated and compared in the same manner. The HU_{ratio} graph revealed an increasing difference in the HU_{ratio} between the two groups (Fig. 5B), and a higher increment ratio in the ADC-treated group than in the control group. We also compared the differences between the HU_{ratio} of the two groups (inter-group gap) on days 16 and 20. The gap on day 20 was significantly larger than that on day 16 and earlier time points. These results were confirmed by performing a non-parametric longitudinal analysis ($p < 0.001$). These results imply that the ADC-treated side showed accelerated bone healing with time.

DISCUSSION

Although fractures are fairly common, treatment is not always successful because of various factors such as injuries to surrounding soft tissues, poor blood supply, and osteoporosis.¹⁶ Therefore, fracture treatment in complicated cases such as in the elderly and in patients of osteoporosis and immune suppression, has been difficult despite advances in surgery and regenerative medicine.² Among the various new therapeutic approaches that have been proposed for the treatment of fractures, stem cells are promising candidates that activate bone healing. In particular, MSCs are known to be involved in the bone healing process. It has been suggested that MSCs are recruited to the injury site, where they proliferate and differentiate into osteoblasts.⁵ Initially, most studies investigated the bone-healing properties of BM-MSCs. However, these cells have limited applications as therapeutic agents because of their invasive harvesting procedures and relatively low yields.¹⁷ Therefore, ADMSCs were investigated as an alternative to BMMSCs owing to

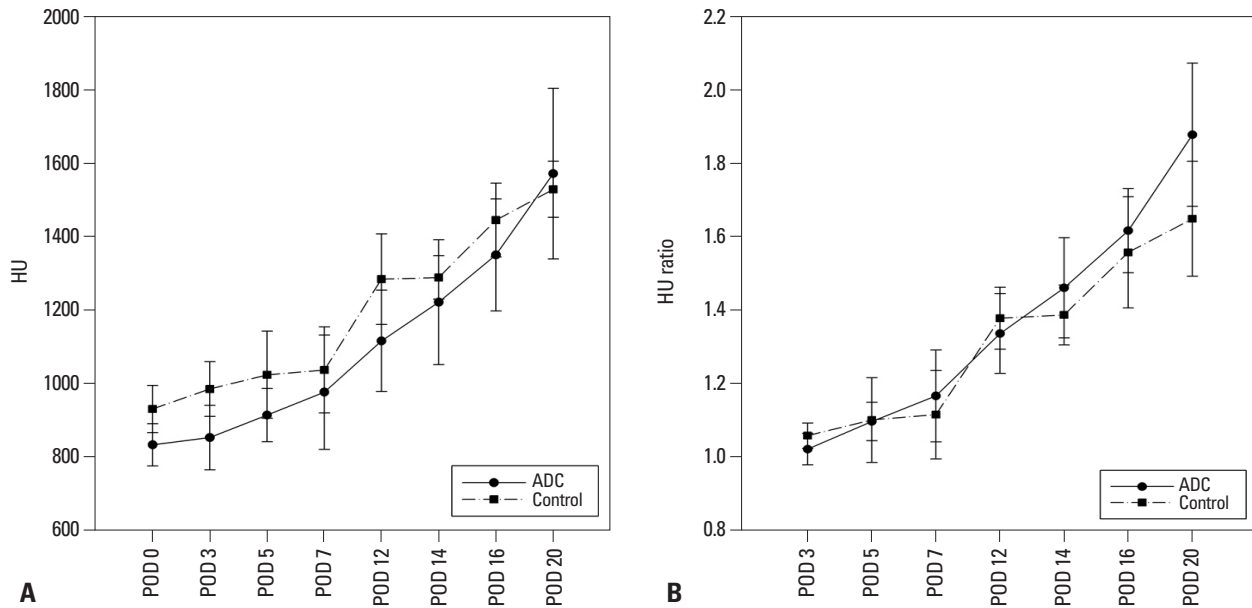


Fig. 5. (A) There was no significant difference in the Hounsfield units (HUs) of the right and left femurs until day 20. (B) However, there was a significant difference in the HU_{ratio} (normalized to the baseline values) of the right and left femurs on day 20. POD, post-operative day; ADC, adipose tissue-derived cell.

their abundance and easy harvesting procedures.¹⁸ However, the therapeutic potential of ADMSCs for treating fractures has not been studied extensively.

Recently, advanced imaging techniques such as BLI and micro-CT have made it possible to serially monitor implanted MSCs and changes in bone density without the need to sacrifice the animals. In our study, we used ADCs harvested from transgenic mice carrying a ubiquitous promoter gene to avoid low yield of reporter gene upon transfection using lipofectamine or lentivirus. Consequently, we could precisely evaluate the survival and proliferative behavior of implanted MSCs in a living animal model. BLI in the early phase of follow-up revealed a rapid drop in cell population on day 2, and subsequent reconstitution on day 5. This result was similar to that obtained in our previous study involving the systemic injection of reporter ADCs in fracture models, where the signal dropped 3 days after intravenous injection, increased on day 6, and then decreased to a plateau thereafter.¹⁴ These results suggest that the stem cells undergo rapid cell demise in the initial hostile environment around the fracture, followed by a brief surge, and a gradual decline thereafter. This result also suggests that too many cells cannot survive in the targeted fracture area, and an appropriate number of cells must be transplanted in each case to ensure survival. Shoji, et al.⁹ reported successful treatment of fracture with ADMSCs using a dosage equal to one sixth of the dose used in our study. Further studies are necessary to optimize the dosage of ADMSCs.

Many studies have reported that transplanted stem cells proliferate, differentiate into osteoblasts, and promote bone healing. In our study, however, *in vivo* BLI showed no evidence of proliferation of ADCs in the implant site.¹⁹ Therefore, the healing effect of locally administered ADCs may be attributed to cytokine production and humoral effects, rather than direct cellular proliferation and differentiation into osteoblasts and stromal cells. However, *ex vivo* BLI revealed persistent signals in the implanted area, albeit slightly lower than that in the initial phase, suggesting that ADCs do survive in the fracture site and promote fracture healing. It is possible that the implanted cells proliferate and survive for longer periods, but the signal may have been lost because of the limited optical penetration of bioluminescence signal through scarred soft tissue. Further investigations are required to confirm this hypothesis.

Because our previous study revealed trafficking of intravenously administered stem cells to fracture sites, we investigated the trafficking ability of transplanted ADCs to a remote injury area i.e., the hole in the contralateral femur. The results revealed that there was no detectable signal in the left femur, as assessed by *ex vivo* and *in vivo* BLI. *Ex vivo* images revealed luciferase activities in relatively distant areas on the right femur in addition to areas near the ADC-implanted hole. This might be attributed to SBMP-assisted cell proliferation. In addition, it is possible that locally administered ADCs behave differently *in vivo*, and might be able to migrate to nearby areas through blood capillaries. The time ver-

sus activity curves and the *ex vivo* BLI images suggest that the detection of activity in areas adjacent to the hole in the right femur may be attributed to local migration of implanted cells rather than proliferation. If the implanted cells had proliferated, bioluminescence activity would have increased at more than one instance during the follow-up period.

Using high-resolution micro-CT, we evaluated the differences in early bone changes between the ADC-treated and control sides. Although the final HU value of the ADC-treated side was higher than that of the control side, there was no significant difference between the serial HUs of the ADC-treated and control femora; this may be attributed to the lower baseline HU value of the ADC-treated side than the control side (Fig. 5A). Next, we compared the HU_{ratio} of both sides to minimize bias arising from the different baseline values. We found that the HU_{ratio} of the ADC-treated side was higher than that of the control side from day 7 onwards, and the rate of increment on the ADC-treated side was higher than that on the control side, suggesting that ADC-SBMP treatment promotes bone healing. These results contradict those obtained in a study by Lyons, et al.,²⁰ which suggested that MSCs might adversely affect healing by creating a barrier to host response. Because of the low levels of bioluminescence activity on day 20, we did not continue the micro-CT analysis for a longer follow-up period. Instead, we analyzed luciferase activity using *ex vivo* BLI. Therefore, complementary research that includes long-term monitoring of ADCs is necessary. It may be noted here that a study by Shoji, et al.⁹ revealed that ADMSCs might have long-term healing effects on fractured bones.

There are several limitations to our study. Most importantly, the follow-up period was too short to evaluate the bone union status of the fracture models. However, the main aim of our study was to evaluate the behavior of locally administered ADCs in a living animal and to monitor early changes in bone density using micro-CT. Therefore, when the *in vivo* BLI revealed diminished activity, we had to sacrifice the animals to determine whether the ADCs had survived in the femoral holes. A second limitation was that we could not perform an immunohistochemical analysis of the specimens owing to a delay in tissue preservation to perform *ex vivo* BLI. However, many previous studies have investigated the histological characteristics of MSCs. Therefore, we reasoned that demonstrating the presence of surviving ADCs by *ex vivo* BLI might be more useful for future studies. Because of the limited sensitivity of BLI, follow-up for a longer period was not possible in our study. However, future

improvements in the sensitivity of BLI and reporter gene technology might enable the monitoring of locally administered ADCs for longer time intervals.

In summary, BLI data revealed that the levels of locally administered ADCs fluctuated in the early postoperative phase and reached a plateau thereafter, without providing definite evidence of cell proliferation or migration to remote sites of injury. Despite the relatively short follow-up period, micro-CT revealed that the implanted ADCs enhanced the rate of fracture healing during the study period, as suggested by the incremental patterns of the HUs and HU_{ratio} . Our results suggest that ADCs play a role in fracture healing; this effect might be mediated by cytokines or other humoral effects.

ACKNOWLEDGEMENTS

We gratefully acknowledge Dr. Contag (Stanford University, USA) for providing the transgenic mice.

This work was supported by academic promotion grant of the Korean Society of Nuclear Medicine (2010).

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