

Mesenchymal Stem Cells with Increased Stromal Cell-Derived Factor 1 Expression Enhanced Fracture Healing

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Treatment of critical size bone defects pose a challenge in orthopedics. Stem cell therapy together with cytokines has the potential to improve bone repair as they cause the migration and homing of stem cells to the defect site. However, the engraftment, participation, and recruitment of other cells within the regenerating tissue are important. To enhance stem cell involvement, this study investigated overexpression of stem cells with stromal cell-derived factor 1 (SDF-1) using an adenovirus. We hypothesized that these engineered cells would effectively increase the migration of native cells to the site of fracture, enhancing bone repair. Before implantation, we showed that SDF-1 secreted by transfected cells increased the migration of nontransfected cells. In a rat defect bone model, bone marrow mesenchymal stem cells overexpressing SDF-1 showed significantly ($p=0.003$) more new bone formation within the gap and less bone mineral loss at the area adjacent to the defect site during the early bone healing stage. In conclusion, SDF-1 was shown to play an important role in accelerating fracture repair and contributing to bone repair in rat models, by recruiting more host stem cells to the defect site and encouraging osteogenic differentiation and production of bone.

Introduction

SKELETAL INJURIES and their complications are major causes of morbidity and mortality, and this problem is highlighted in patients with osteoporosis and related fractures.¹ Each year, ~7.9 million fractures occur in the United States, and of these, up to 10% result into delayed union or nonunion.^{2–5} In the United Kingdom, there are about 150,000 wrist, vertebral, and hip fractures yearly due to osteoporosis with an estimated health care cost of £17 billion per annum.⁶

Stem cell therapy has been used in the treatment of difficult cases, such as nonunion fractures and for treating large bone defects, and so far, results have been promising.⁷ For successful bone repair, an environment of biomechanical stability and biological vitality are very important. Bone repair can be impaired by insufficient vascularization, infection, mechanical instability, and systemic diseases.⁸ Mesenchymal stem cells (MSCs) under the correct conditions and signals can differentiate into tissues, such as bone cartilage, tendon, muscle, ligament, and marrow stroma.⁹ During the fracture healing process, MSCs migrate into the fracture site from blood, periosteum, bone marrow (BM), and other tissue niches. The MSCs then proliferate and differentiate into osteoblasts and chondrocytes.¹⁰ The osteoblasts from the cortex and

osteoprogenitor cells from the periosteum proliferate and differentiate to form immature bone. This immature bone eventually forms a bridge of mineralized woven bone between the fracture fragments.^{11–13}

One of the methods used to heal fractures is to percutaneously inject adult stem cells isolated from the BM preoperatively. The intention of this procedure is to create an osteogenic response, which would enhance the rate and amount of bone formation. Due to the age of an individual, aspiration site, and the techniques used, there is variability in the number of stem cells available for repair.^{10,14,15} Additionally, in the BM, it has been calculated only 1–10 cells per 100,000 cells are MSCs. Cellular movement has been identified as important in neovascularization, wound healing, and organ repair. Signaling molecules have been identified to play important roles in maintaining the mobilization, trafficking, and homing of stem cells.¹⁶

During organ regeneration, it has been suggested that local MSCs derived from the injured tissue and circulating MSCs work together in healing damaged organs. Stem cells sense the tissue injury, migrate to the site of the damage, and undergo differentiation,^{7,17} and this may explain the larger numbers of stem cells found in damaged tissues compared to normal healthy tissues, such as impaired sites in the brain

after hypoglossal nerve injury¹⁸ and cerebral injury.¹⁹ As a result of injury, the surviving cells may produce chemoattractants such as stromal cell-derived factor 1 (SDF-1) that may direct the migration of MSCs to the injury site.¹⁹ Chemokines are a group of small proteins (8–14 kDa) characterized as being able to direct the movement of receptor-presenting cells toward higher concentrations of chemokines in the environment.²⁰ The chemokine SDF-1 (also known as CXCL12) is produced in many organs by multiple BM stromal cell types and epithelial cells. It is known to play important roles in the migration of hematopoietic stem cells to the BM. The binding of SDF-1 to CXCR4 initiates a signaling pathway, which causes such responses as cell division and increased metabolic activity. The SDF-1 CXCR4 interaction is believed to hold stromal cells in the BM niche.^{16,21–23}

Enhancing fracture healing using stem cells would be beneficial, and the hypothesis of our study was that bone growth in fracture site is enhanced by the local delivery of MSCs overexpressing SDF-1 when compared to non-transduced MSCs in a rat model. The aim of the study was to improve fracture healing using stem cells by enhancing local SDF-1 protein levels in the fracture site.

Methodology

Cell culture

Rat bone marrow mesenchymal stem cells (rBMC) were harvested from 8 week young adult male Wistar rats. Rats were sacrificed by cervical dislocation and the BM cells were harvested by flushing the femora with Dulbecco's modified Eagle medium (DMEM), 10% fetal calf serum, 1% penicillin streptomycin (Sigma-Aldrich) in a 25-cm² flask after being pumped through the Ficoll gradient. The cells were cultured at 37°C and 5% CO₂. Media were changed after 4 days to remove the nonadherent cells and then continuously refreshed twice a week thereafter. After 10–14 days of primary culture, when the cells were 70–80% confluent, they were passaged using trypsin–ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich). Cells were then passaged about every 7–8 days. Passage 4 and 5 cells were used for the studies. Additionally, these cells were positively differentiated into adipocytes, chondrocytes, and osteoblasts using the trilineage differentiation protocol and characterized using FACS to show positive expression for CD29, CD44, CD73, CD90, CD105, and CD71 and negative for CD13, CD45, and CD34.^{24,25}

Preparation of recombinant adenovirus

The human SDF-1 cDNA (obtained from the National Yang-Ming University VYM Genome Research Centre) was digested by restriction endonucleases *NotI* and *XhoI* and then inserted into pShuttle-CMV (AdEasy XL Adenoviral vector system; Stratagene) to form pShuttle-CMV-SDF1. Human SDF-1 gene and the pShuttle-CMV vector were cut by restriction endonuclease *NotI* and *XhoI* to form sticky ends. Human SDF-1 cDNA was then cloned into the pShuttle-CMV vector through the matches of *NotI* and *XhoI* cutting sides using Taq DNA ligase. The incorporated shuttle vector was then linearized with *PmeI* restriction endonuclease and transformed into BJ5183-AD-1 competent cells. After identifying the recombinants, its copies were largely expanded in bulk using the recombinant-deficient XL 10-Gold strain.

Purified recombinant plasmid DNA was then used to transfect AD-293 cells, which were then used to transfect the rBMCs. The SDF-1 overexpressing rBMCs (rBMC-SDF-1) were engineered by infection of adenovirus carrying human SDF-1 gene at a multiplicity of infection (MOI) of 500. The infection rate was 70%.

Virus titer determination and cell infection

Thirty thousand rBMCs (Passage 5) were seeded in each well of 24-well plates and cultured at 37°C overnight. Cells were then infected by adenovirus expressing human SDF-1 α (Ad-SDF-1) with a MOI ranging from 0 to 500 (six replicates for each MOI group). After 2 days in the normal culture medium, the medium was changed to serum-free DMEM for 3 days. The concentration of secreted SDF-1 in the medium was detected by enzyme-linked immunosorbent assay (ELISA) of SDF-1, which was measured by absorbance of 450 nm wavelength (R&D Systems). To test the tolerance of the cells for adenovirus infection and the most efficient MOI, the cells were infected with Ad-LacZ separately and the β -galactosidase activity of cells was measured.

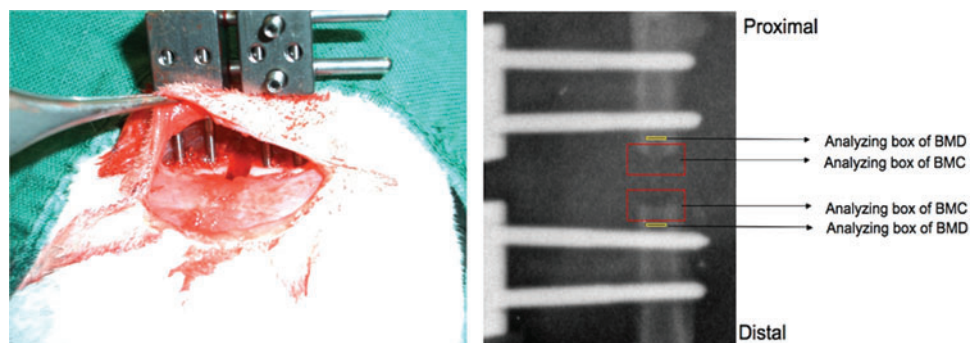
In vitro chemotaxis assay

For this *in vitro* study, a transwell chamber consisting of a polycarbonate membrane with 0.8 μ m porosity (Corning, Fisher Scientific) was used. Thirty thousand rBMCs (Passage 4) were seeded in 24-well plates at the bottom of the chamber and cultured at 37°C in an incubator overnight in the normal medium. Cells were infected with Ad-SDF-1 by various MOI of 0, 250, and 500 and cultured in the normal medium, in each separate well plate. On the fourth day after infection, 4500 cells were seeded on the upper surface of the transwell chamber and cultured at 37°C in an incubator. After 5 days, the upper chambers containing the untransfected rBMCs were placed into the well plates seeded with rBMCs. Cells that migrated to the opposite side of the membrane after 6 h were fixed, stained with toluidine blue, and counted.

Bone formation—fracture model

Eighteen adult female rats, weighing between 200 and 250 g, were anesthetized by inhalation of isoflurane and the left femur shaved and disinfected. A critical size of 3 mm gap in the middle of the femur was created during the surgery and stabilized by an external fixator (Fig. 1). The rats were divided into three groups with six rats in each group: (1) rBMC-SDF-1, (2) rBMC, and (3) control. In two groups, 300,000 rBMCs or rBMC-SDF-1 were seeded into a collagen type I sponge (4 \times 4 \times 7 mm) (Helistat; COLLA-TEC) and transplanted into the gap. In the control group, sponges without cells were used. The wound was then closed layer by layer and antibiotics and analgesics administered post-surgery. Rats were sacrificed 3 weeks later and the femora harvested. The osteotomy was stabilized by an external fixator attached to the two parts of the femur by 4 \times 1-mm-diameter titanium pins. A material test machine was used to check that the variability in stiffness between different fixators was less than 5%. A standard fixator stiffness was maintained by ensuring that the crossbeam of the fixator was a consistent distance from the femoral surface.

FIG. 1. A 3-mm osteotomy created in the femur of a rat (left) and an analyzing box for bone mineral content (BMC) and bone mineral density (BMD) measurement (right). Color images available online at www.liebertpub.com/tea



A second experiment studied the direct administration of Ad-SDF-1. In the Ad-SDF-1 group, 1.2×10^8 Ad-SDF-1 virus particles were suspended in 200 μ L culture medium, which was soaked onto the collagen sponge. The other three groups were the same as the groups in the 3 weeks short-term experiment. In these groups, animals were kept alive for 6 weeks.

Analysis of bone formation

Bone mineral content (BMC) and bone mineral density (BMD) were measured in a consistent region by dual-energy X-ray absorptiometry (DEXA) scan (QDR-1000; Hologic) at the first, third, and where appropriate the sixth week after operation. An extra high-resolution program was used. A constant area was analyzed, which included the osteotomy with parts of the original bone and peripheral bone formed adjacent to the gap for the BMD. The results were generated by adding the measurements from the proximal and distal analyzing boxes (Fig. 1). Inhalation anesthesia was given to the rats before and during the scan. The rat's left leg was fixed by a clamp, which held the external fixator, in the same position during DEXA scanning at each time point. The area of new bone formation was measured using histomorphometry on hematoxylin and eosin-stained sections. After the rats were sacrificed, the left femur was retrieved. The specimens were fixed in 10% formal saline, dehydrated by a series of alcohol, and decalcified by EDTA. Decalcification was confirmed by radiography. After decalcification, specimens were dehydrated, treated with chloroform, and then embedded in wax. Samples were labeled on the longitudinal middle line of the femur before being embedded in wax to indicate the location of the sectioning area. A microtome was then used to create 7- μ m-thick sections. The sections were then quantified using an imaging analysis system.

The maintenance of the donor cells within the fracture site after 3 weeks was measured by fluorescence in situ hybridization (FISH) staining of the Y chromosome and is shown in ratio standardized by the rBMC group. FISH was performed by a commercial kit (Cambio), which was costained with 4',6-diamidino-2-phenylindole. The probe of the rat Y-chromosome was denatured and a prewarmed probe was added to the cell samples, followed by a detection solution. After the samples were washed by the detergent wash solution, they were viewed under a fluorescence microscope (KS-300; Zeiss).

Statistical analysis

Results were analyzed with one-way analysis of variance (ANOVA) test. p values ≤ 0.05 were considered significant.

For ANOVAs with significant F tests, a Tukey's *post hoc* procedure was performed to compare the significance between the two groups.

Results

SDF-1 infection

SDF-1 expression in rBMCs was estimated on the fifth day after the infection by Ad-SDF-1 of various MOI (Fig. 2). rBMCs infected with different MOIs of Ad-LacZ ranging from 0 to 500 was used to determine the tolerance of the cells to the adenovirus infection. The β -galactosidase activity of cells was tested. An increasing amount of positively blue cells was observed in the MOI groups higher than 175, with the highest number of blue cells at a MOI of 500.

Additionally, the concentration of the secreted SDF-1 in the medium, after the transfection, was detected by ELISA. The expression of SDF-1 was significantly upregulated ($p=0.007$) using a MOI 125 and reached the maximum at a MOI of 500 with an infection rate of 70%. No severe cell damage was observed for the different MOI of SDF-1.

In vitro chemotaxis assay

A transwell migration assay was performed to examine whether secreted SDF1 could successfully increase cell migration toward the infected cells in a dose-dependent manner. The rBMCs showed significant ($p=0.011$) and dose-dependent

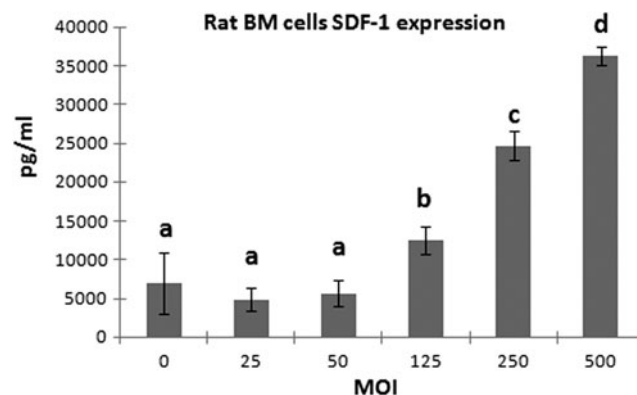


FIG. 2. Stromal cell-derived factor 1 (SDF-1) expression of rat bone marrow mesenchymal stem cells (rBMCs) 5 days after Ad-SDF-1 infection with different multiplicity of infection. Data points sharing different Tukey's letters are significantly different ($p < 0.05$).

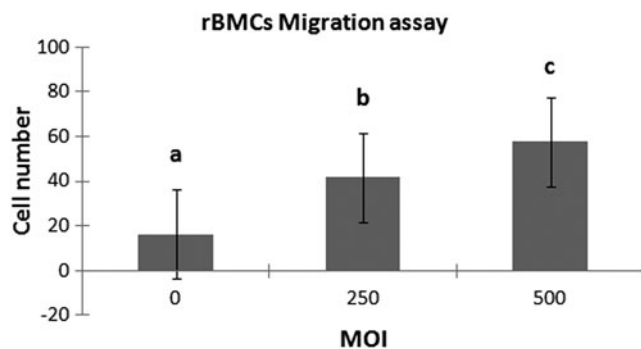


FIG. 3. Cell migration due to the secreted SDF-1 in Ad-SDF-1-infected rBMCs. Data points sharing different Tukey's letters are significantly different ($p < 0.05$).

chemoattractive activity to Ad-SDF-1-infected stem cells at the bottom of the chamber (Fig. 3). Furthermore, two times more cells migrated to the opposite side of the membrane in the MOI 250 group and even larger numbers with the MOI 500 group.

Bone mineralization

Bone mineral content. It has been suggested that SDF1/CXCR4 signaling is a principal axis in the retention, migration, and repopulation of stem cells during homeostasis and injury. We therefore investigated whether stem cells over-expressing SDF1 would enhance bone formation at a defect site. BMC of the osteotomy area was measured following

DEXA scanning. The rBMC-SDF-1 group showed significantly increased BMC than both the control and rBMC groups ($p = 0.003$ and $p = 0.0029$, respectively) (Fig. 4), and this could be due to the maintenance of the donor cells in the osteotomy, which was measured by FISH after 3 weeks. The rBMC-SDF-1 group showed about 2.5-fold more donor cells than the rBMC group in the fracture site.

BMC and BMD were measured every 3 weeks during the experiment. The rBMC-SDF-1 group showed an almost four times BMC increase when compared with the control group ($p = 0.008$), and a higher increase when compared with the rBMC group ($p = 0.088$) (Fig. 5). After 3 weeks (from the fourth week to the sixth week), all groups showed a decrease in BMC (Fig. 5). However, the rBMC-SDF-1 group demonstrated the least decrease compared with the control group ($p = 0.08$). The change after 6 weeks showed that only the rBMC-SDF-1 group had an increased BMC compared with the control group ($p = 0.003$) (Fig. 5).

Bone mineral density. The BMD change in the peripheral bone adjacent to the osteotomy gap was also measured at 3 and 6 weeks. Both the rBMC-SDF-1 and Ad-SDF-1 groups showed increased BMD in the first 3 weeks while the control and rBMC groups showed reduced density (Fig. 6). During the second 3 weeks, all groups showed further BMD loss. Although there was no significant difference in BMD change among all groups after 6 weeks (Fig. 6), the rBMC-SDF-1 group had the least BMD loss compared with the control group ($p = 0.167$) and the rBMC group ($p = 0.140$).

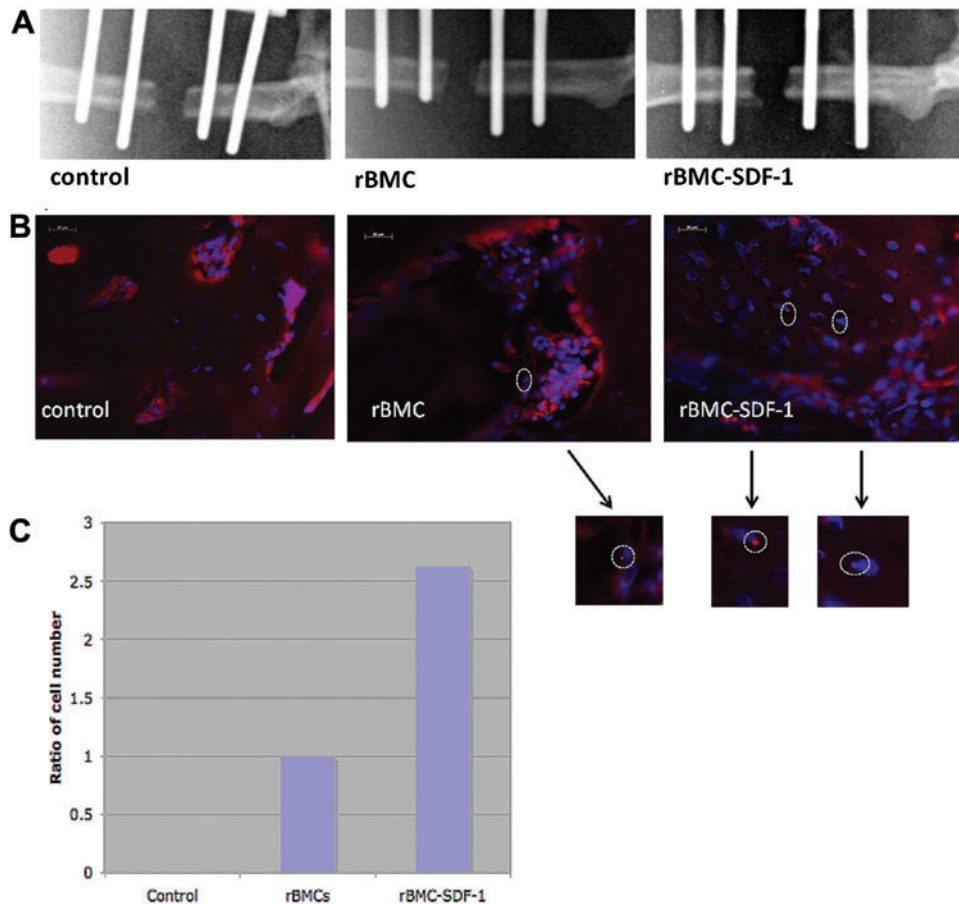


FIG. 4. Radiographs of osteotomy site after 3 weeks (A), fluorescence in situ hybridization (FISH) staining showing the donor cells (400 \times). The male Y chromosomes staining are shown in red and the nuclei are shown in blue (B) and the ratio of cell numbers with the FISH staining for the different controls (C). Color images available online at www.liebertpub.com/tea

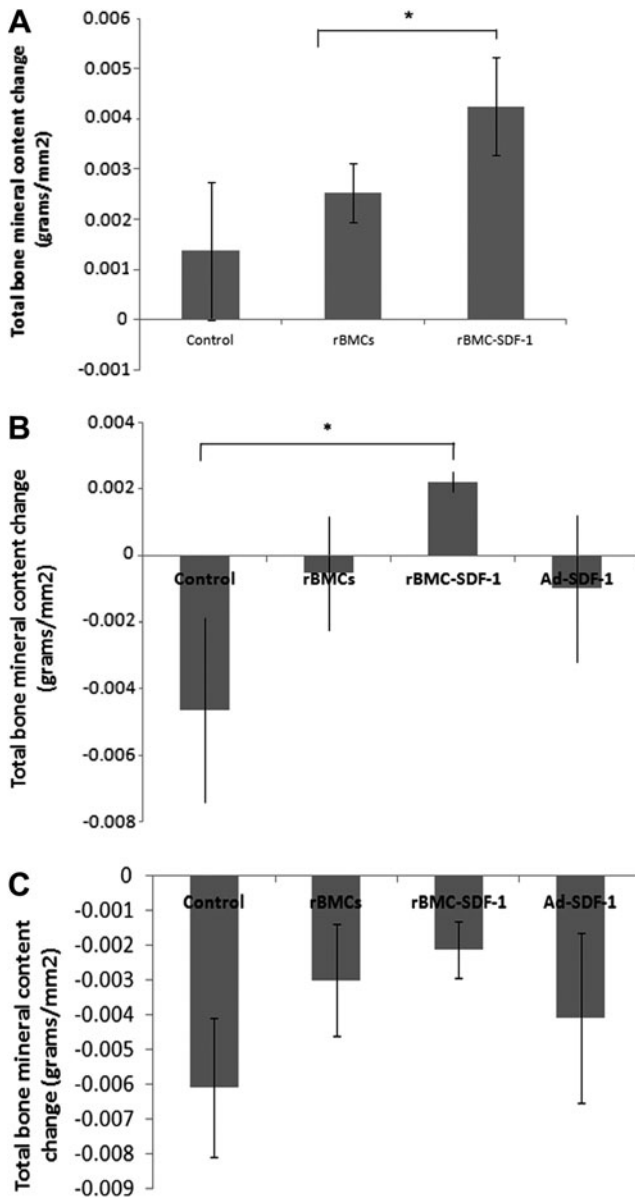


FIG. 5. BMC change within the osteotomy site after 3 weeks (A), BMC change after 6 weeks (B), and BMC change from the fourth week to the sixth week (C) (*significant difference $p \leq 0.05$).

New bone formation

Histology of the osteotomy site after 3 weeks showed new bone formation in all three groups with the rBMC-SDF-1 group showing greatest amounts of new bone formation. The rBMC-SDF-1 group produced significantly more new bone than the rBMC group ($p=0.02$), but no significant difference was seen when compared with the control group ($p=0.08$). No significant difference was found when the control and rBMC groups were compared ($p=0.8$). New bone formation in most defects in the control group was only found on one side of the fracture, whereas in the other two groups, bone formation was more uniform (Fig. 7).

New bone formation after 6 weeks showed a similar trend to the new bone formation observed in the 3-week experiment. Highest amounts of new bone had formed in the rBMC-

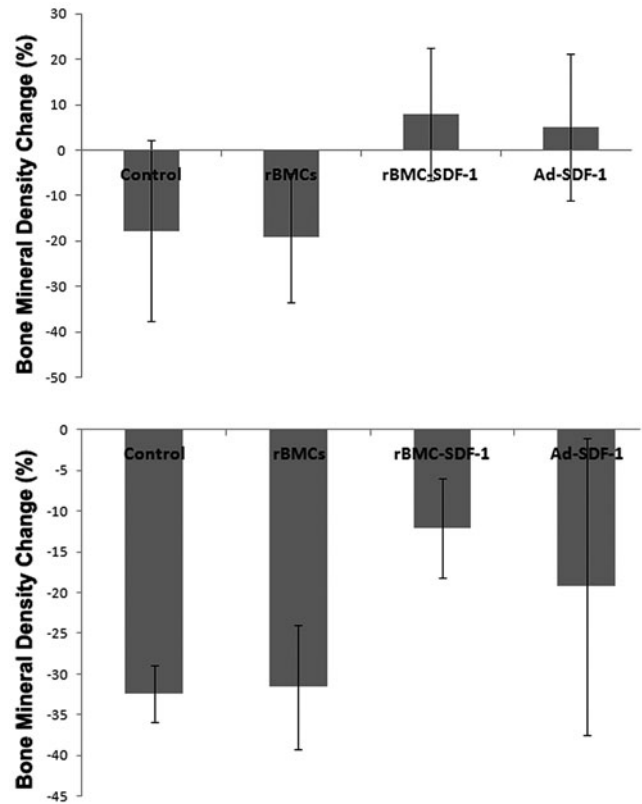


FIG. 6. BMD change from the first week to the third week (top) BMD change of the original bone area adjacent to the osteotomy gap after 6 weeks (mean \pm standard deviation) (bottom).

SDF-1 group when compared with all other groups ($p=0.029$ with control group). Additionally, increased bone formation was measured in the Ad-SDF-1 group when compared with the control group, but values obtained were lower than that found in defects containing the rBMC-SDF-1 and with those in the rBMC group. However, no significant differences were found when these groups were compared (Fig. 8).

Discussion

In this study, we have demonstrated that MSCs transfected with the SDF1 gene secreted greater levels of this protein and that *in vivo* this leads to increased MSC migration. These cells when incorporated into the fracture site led to enhanced fracture healing. This may be associated with the retention of MSCs in the fracture site and mobilization of nontransfected cells into this site. Cellular movement and relocalization are crucial for many important physiological properties, such as embryonic development, neovascularization and angiogenesis, immunologic responses, wound healing, and organ repair. Both local MSCs from the injured tissue and circulating MSCs collaborate in the healing of organs during organ regeneration, and this cell movement is regulated by chemotaxis, which causes directional migration through signaling molecules called chemokines.^{7,26} Recruitment of stem cells to areas of bone damage is therefore an important modality for the repair and remodeling process.²⁷ The potential of MSCs for bone repair have been investigated extensively in various studies because these cells play important roles in skeletal homeostasis.^{28,29}

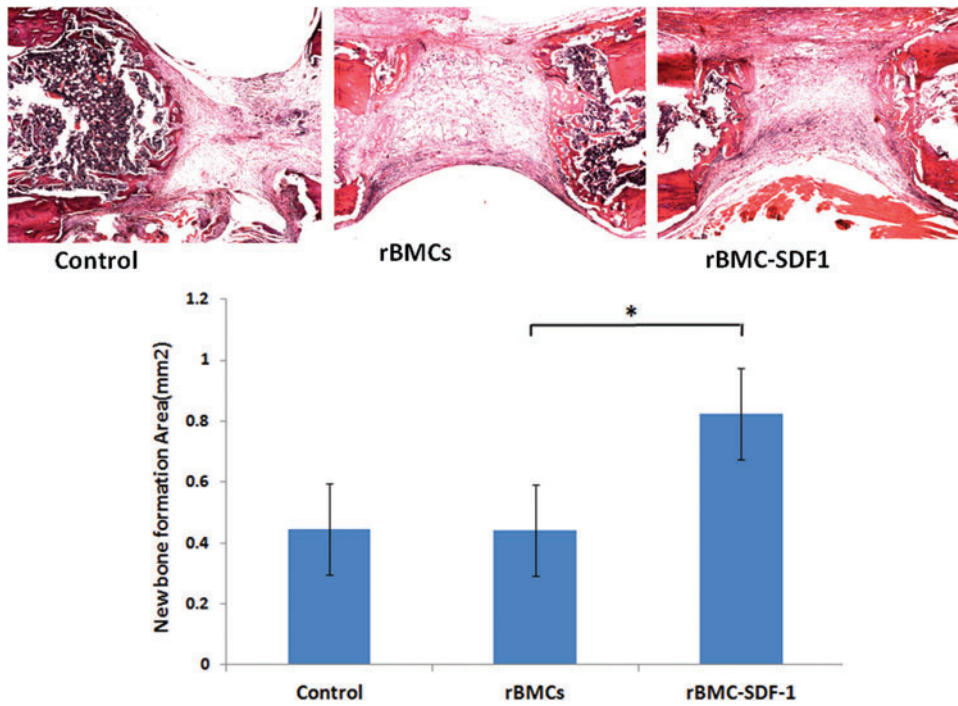


FIG. 7. Hematoxylin and eosin (H&E) staining of new bone formation (200×) (*top*) and new bone area in the osteotomy (*bottom*) after 3 weeks (*significant difference $p \leq 0.05$). Color images available online at www.liebertpub.com/tea

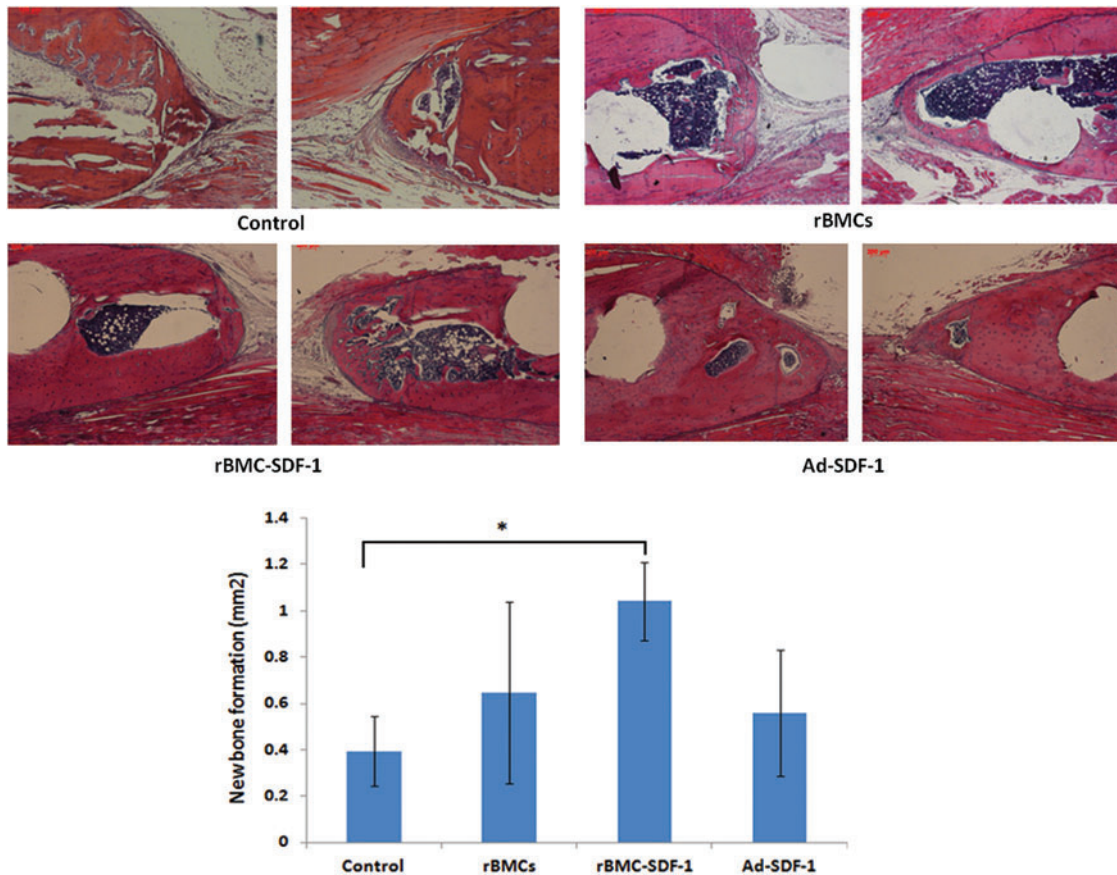


FIG. 8. H&E staining of new bone formation (200×) (*top*) and new bone area in the osteotomy (*bottom*) after 6 weeks (*significant difference $p \leq 0.05$). Color images available online at www.liebertpub.com/tea

We investigated the chemotaxis ability of stem cells *in vitro* using a Boyden chamber. The number of stem cells that migrated to the opposite side of the membrane increased in a dose-dependent manner. A higher SDF-1 concentration was secreted by the cells with a MOI of 500 and therefore caused more cells to migrate toward it. A similar study by Wynn *et al.*²³ found that maximum migration occurred at an SDF-1 concentration of 30 ng/mL and a neutralizing anti-CXCR4 antibody inhibited MSC migration by ~46%. This shows that MSCs express functionally active CXCR4 receptors, which cause the migration of MSCs to the BM. However, it was found that the CXCR4 receptor is present at low levels on the cell surface of MSCs, and to improve the engraftment of MSCs to BM and bone, an increase in its functional expression may be required.²³

After 3 weeks, the rats transplanted with stem cells expressing SDF1 (rBMC-SDF-1 group) had increased new bone formation, BMC, and density. rBMC-SDF-1 not only increased new bone formation but also showed significantly higher BMC after 3 weeks compared to the control group. Additionally, more donor cells were found in the compact new bone in the rBMC-SDF-1 group compared to the control groups. This bone healing progress may be due to the enhanced local SDF-1/CXCR4 interaction that recruited more host stem cells into the fracture site. Similar studies have shown that SDF1 recruits MSCs to the periosteum of the injured bone and therefore promote endochondral bone repair.²⁸ The CXCR4/SDF1 signaling pathway has been reported to play a critical role in bone healing as it affects cell migration and differentiation to the defect site as well as affecting the numerous cellular processes involved in bone healing, such as chondrogenesis and osteogenesis, bone remodeling, and vascularization.³⁰ Our previous study also showed that SDF-1 could enhance the osteoblastic differentiation of human MSCs, which may additionally contribute to new bone formation. When cells were cultured with SDF-1 and osteoinductive medium, they showed a significantly higher alkaline phosphatase (ALP) activity compared to cells that were cultured in the osteoinductive medium alone, indicating an enhanced osteogenic differentiation.³¹

The control group, in which no cells were transplanted into the defect site, showed an increase in new bone formation following histological analysis, but a reduced BMC after 3 weeks. On the other hand, the rBMC group, which consisted of only stem cells transplanted into the defect site, showed similar new bone area but higher BMC compared to the control group. This implies that BMCs initiate faster bone regeneration. Both rBMC and rBMC-SDF-1 groups had a higher BMC and a more compact new bone showing enhancement of bone mineralization by the transplanted stem cells. Granero-Molto *et al.*³² has shown that implanted MSCs migrated to a fracture site, and this migratory capacity of stem cells may be driven by CXCR4. Similar to this study, they also reported that mice transplanted with MSCs at a fracture site displayed a significant increase in total volume, as well as total bone, soft tissue, new bone and callus volumes, and callus mineralization content, in comparison to the control group. The MSC transplant improved the fracture healing by increasing the material toughness of the callus and causing it to be less brittle.³²

During endochondral bone repair, SDF-1 expression is upregulated by progenitor cells in the periosteum and this

results in recruitment of MSCs.²⁸ Additionally, other growth factors could direct the formation of bone in the early stages of bone repair. Vascular endothelial growth factor has been shown to be upregulated in the initial stages of fracture healing, improving angiogenesis, leading to conversion of soft cartilaginous callus to hard bony callus.^{33–35} This could explain the increased new bone formation seen at the defect site, although no cells were transplanted there. Additionally, SDF1 could also enhance the early osteogenic differentiation of stem cells, mediated by a bone morphogenic protein (BMP) signaling pathway. A study by Hosogane *et al.*³⁶ showed that blocking of the SDF-1/CXCR4 signal axis or adding SDF-1 protein to MSCs significantly affected BMP2-induced ALP activity and osteocalcin synthesis. Bone nodule mineralization decreased as well when the SDF-1 signaling was disrupted. They also showed that blocking the SDF-1 signaling inhibited the expression of Runt-related factor-2 and osterix, two important regulators of osteogenesis.³⁶

The key drivers to bone healing are cytokines, platelets, and growth factors, of which BMPs have been recognized as critical players. BMP2 and transforming growth factor (TGF) β are required for normal fracture healing, and in their absence, MSCs at the repair site do not differentiate toward the osteogenic lineage, leading to a failed healing response of the bone. It has been shown that levels of BMP receptors present on the callus cells increase early in the repair process. However around day 10–20, the levels of BMP receptors begin to decrease as the callus cells differentiate and robust bone formation occurs. Similar results have also been observed with TGF β as callus formation increases.^{27,37–40} During the 6 week experiment, all groups showed an increase in BMC during the first 3 weeks followed by its reduction during the second 3 weeks, which is in contrast to a typical bone healing procedure in a critical size bone defect model. By enhancing the local SDF-1 level, the rBMC-SDF-1 group showed significantly higher BMC and more new bone formation than the control and rBMC group at both 3 and 6 weeks postoperatively. Interestingly, although control animals had a higher BMC at 3 weeks and they lost that gain at 6 weeks, the rBMC-SDF-1 group gained the most and lost the least amount of BMC. The large standard errors in the rBMC group at 6 weeks could be due to less bone formation in one of the samples in this group. It is also important to note that the expression period of adenoviral vector is about 3–4 weeks,⁴¹ so future studies could possibly look at upregulating the local SDF1 levels by multiple administrations of adenovirally infected MSCs.

In summary, this study demonstrated that SDF1 plays an important role in fracture repair. SDF1 recruited MSCs toward the defect site, initiating bone repair. However, several questions need to be answered, before this approach can be used in clinical settings. In light of the previous data on the effect of stem cells on fracture healing, it has been shown in this study that there was a trend, which was not significant. However, the effect of SDF-1 transduced BM cells was significant. This may indicate the very positive effect SDF1 has and fracture models can produce different results depending on a number of variables. In this study, we have used controls of non-transduced stem cells and defects where no stem cells were used. As the untransfected cells had no effect, this model is probably extreme but still shows the effect of recombinant rBMC. Moreover, in this study, plasmid-alone transfected or

scrambled SDF-1 DNA was not utilized. However, due to the fact the transfected cells *in vitro* overexpressed SDF-1 and had a positive effect *in vivo*, we therefore believe that this was due to SDF-1 production and was not associated with the transfection technique. Although we have shown that transfected MSCs overexpress SDF-1 *in vitro*, we did not show that these cells continued to produce SDF-1 when implanted *in vivo*. It would have been interesting to compare the *in vivo* levels of SDF-1 from the four groups, using immunohistochemistry and/or qPCR for SDF-1 on the tissues. We have, however, shown the effects these transfected cells had on fracture healing, and our results suggest that this is due to these cells overexpressing SDF-1.

One of the limitations of this study was we measured bone using an area algorithm, whereas micro-CT would give volume. Micro-CT could possibly have been a more accurate measure of bone formation. Another limitation of this study includes the use of adenovirus. Even if the adenoviral vectors have been established to be replication defective and offer many advantages in gene therapy, they could trigger strong immune responses in humans. These vectors could also cause local damage and inflammation.^{25,42} Hence, if this approach was used for the treatment of deficient bone, a more improved approach to deliver SDF-1 must be evaluated.

Disclosure Statement

None of the authors received payments or services, either directly or indirectly from a third party in support of any aspect of this work. No author has had any other relationships or has engaged with any other activities that could be perceived to influence or have the potential to influence what is written in this work.

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