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Uncultured Autogenous Adipose-derived Regenerative Cells Promote Bone Formation During Distraction Osteogenesis in Rats

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Abstract

Background Adipose-derived stem cells have recently shown differentiation potential in multiple mesenchymal lineages in vitro and in vivo. These cells can be easily isolated in large amounts from autologous adipose tissue and used without culturing or differentiation induction, which may make them relatively easy to use for clinical purposes; however, their use has not been tested in a distraction osteogenesis model.

Question/purposes The question of this animal study in a rodent model of distraction osteogenesis was whether uncultured adipose-derived regenerative cells (ADRCs), which can easily be isolated in large amounts from autologous adipose tissue and contain several types of stem and regenerative cells, promote bone formation in distraction osteogenesis. We evaluated this using several tools: (1) radiographic analysis of bone density; (2) histological analysis of the callus that formed; (3) biomechanical testing; (4) DiI labeling (a method of membrane staining for postimplant celltracing); and (5) real-time polymerase chain reaction.

Methods Sixty rats were randomly assigned to three groups. Physiological saline (control group), Type I collagen gel (collagen group), or a mixture of ADRC and Type I collagen gel (ADRC group) was injected into the distracted callus immediately after distraction termination. To a rat femur an external fixator was applied at a rate of 0.8 mm/day for 8 days.

Results The bone density of the distracted callus in the ADRC group increased by 46% ($p = 0.003$, Cohen's $d = 10.2$, 95% confidence interval [CI] $\pm$ 0.180) compared with the control group at 6 weeks after injection. The fracture strength in the ADRC group increased by 66% ($p = 0.006$, Cohen's $d = 1.32$, 95% CI $\pm$ 0.180) compared with the control group at 6 weeks after injection. Real-time reverse transcription–polymerase chain reaction of the distracted callus from the ADRC group had higher levels of bone morphogenetic protein-2 (7.4 times higher), vascular endothelial growth factor A (6.8 times higher), and stromal cell-derived factor-1 (4.3 times higher). Cell labeling in the newly formed bone showed the ADRCs differentiated into osseous tissue at 3 weeks after injection.

Conclusions The injection of ADRCs promoted bone formation in the distracted callus and this mechanism involves both osteogenic differentiation and secretion of humoral factors such as bone morphogenetic protein-2 or vascular endothelial growth factor A that promotes osteogenesis or angiogenesis.

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Clinical Relevance The availability of an easily accessible cell source may greatly facilitate the development of new cell-based therapies for regenerative medicine applications in the distraction osteogenesis.

Introduction

Distraction osteogenesis as described by Ilizarov [5, 6] is widely used for limb lengthening and bone transport to reconstruct defects after trauma or tumor resection [21–23]. However, to achieve extensive bone regeneration, an external fixator must be applied for a considerable time, which may result in many complications [18]. To shorten the period of external fixation, which would also reduce costs and the frequency of complications, many approaches such as electrical stimulation [13], hyperbaric oxygen exposure [2], or low-intensity pulsed ultrasound stimulation [15] have been used to promote bone formation during the consolidation period. It has been reported that an injection of bone marrow stromal cells [8] or cytokines [17] into the distracted callus enhanced the bone formation and shortened the consolidation period.

Over the past three decades, bone marrow stromal cells (BMSCs) have been used as a common cell source for regenerative medicine research [1]. However, isolation of BMSCs frequently yields low numbers of stem cells and the isolation procedure is invasive for donors and patients. In contrast, adipose tissue has recently been identified as an alternative source of multipotent resident stem cells in humans [3, 31]. In humans, resident stem cells can be easily collected from white adipose tissue stores. These stem cells have the ability to differentiate into multiple mesenchymal lineages. Since Green and Meuth [4] first reported in 1974 that adipose tissue contains preadipocytes, a number of investigations have also described adipose tissue-derived stem/progenitor cells, or so-called adipose-derived stromal cells (ADSCs). Adipose-derived regenerative cells (ADRCs) are the nonbuoyant cellular fraction derived from the enzymatic digestion of adipose tissue. ADRCs contain several types of stem and regenerative cells, including ADSCs, vessel-forming cells such as endothelial and smooth muscle cells and their progenitors, and preadipocytes [30]. ADRCs can be easily isolated in large amounts from autologous adipose tissue and used without culturing or differentiation induction, which highlights the potential clinical application of these cells. Although they seem promising as a potential tool to improve the quality and quantity of bone in limb-lengthening procedures, their use has not been tested in a distraction osteogenesis model.

Our question is whether ADRCs can promote bone formation during distraction osteogenesis. To this end we determined whether transplantation of ADRCs mixed with collagen gel promotes callus formation after distraction osteogenesis in rats. We evaluated this using several tools: (1) radiographic analysis of bone density; (2) histological analysis of the callus that formed; (3) biomechanical testing; (4) DiI labeling (a method of membrane staining for postimplant cell tracing); and (5) real-time polymerase chain reaction.

Materials and Methods

Study Design

Experiments were conducted with the approval of the Kanazawa University Advanced Science Research Center. Sixty 10-week-old Wistar rats (Japan SLC Co, Shizuoka, Japan) were randomly assigned to three groups in which bone lengthening of the left femurs was performed. The control group (20 rats) received an injection of 0.1 mL physiologic saline into the distraction callus immediately after termination of distraction; the collagen group (20 rats) received an injection of 0.1 mL Type I collagen gel alone (Atelocollagen; KOKEN, Tokyo, Japan), whereas the ADRC group (20 rats) received an injection of 1×10^6 cells with 0.1 mL type collagen gel [19]. The rats were euthanized 2, 3, 4, and 6 weeks after injection whereupon the left femur was resected with the surrounding tissues. The specimens from these rats were evaluated using radiography, histologic analysis, three-point bending testing, real-time reverse transcription–polymerase chain reaction (RT-PCR), and cell labeling. There was no inflammatory response around the transplantation site, and all rats had been in good health during the experimental period.

ADRC Isolation

Isolation of ADRCs was performed by modifying a previously reported method [32]. Briefly, adipose tissue (1.5 g) was harvested from the right inguinal region of a rat and washed with phosphate-buffered saline (PBS; Wako, Osaka, Japan). The tissue was cut into strips over a period of 5 minutes. Collagenase (Wako) was dissolved in PBS to a final concentration of 0.12% in 20 mL and used to digest adipose tissue for 45 minutes in a 37 °C water bath. The mixture was shaken every 15 minutes during the digestion period. Immediately after the reaction was completed, 20 mL Dulbecco's modified Eagle's medium (Wako) was added to neutralize the collagenase activity before filtration of the resulting solution. The filtrate was centrifuged at 1300 rpm for 6 minutes at 25 °C, and the supernatant was removed. The ARDC pellet was subsequently mixed with 0.1 mL collagen gel before implantation. The mean number of cells was 1×10^6 in this pellet.

Surgical Procedure

Surgery was performed as reported previously [20, 28] using aseptic techniques in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication no. 85-23, revised 1996). Anesthesia was achieved with 6.5 mg sodium pentobarbital (Somnopentyl®; Intervet, Tokyo, Japan) administered intraperitoneally. Each animal was placed in a lateral position on the operating table. A lateral longitudinal skin incision was created over the left femur followed by incision and subsequent separation of the quadriceps femoris and hamstrings. After predrilling with a Kirschner wire (1.0 mm diameter), an external fixator (Meira, Nagoya, Japan) was fixed with four self-tapping pins (1.6 mm diameter; Japan Medicalnext, Osaka, Japan). Osteotomy was performed between the second and third pins using a manual saw under irrigation with physiological saline. The fascia and the skin were sutured using absorbable surgical sutures. The rats were allowed free movement in their cages after anesthesia. Seven days after the operation, femoral lengthening was initiated at a rate of 0.8 mm/day for 8 days.

Radiographic Evaluation

Radiographs of the distracted femur were taken using a soft x-ray apparatus at 2, 3, 4, and 6 weeks after injection ($n = 20$ for each group). The distracted callus was evaluated by comparing the density on an AP radiograph with an aluminum step wedge (20 steps, 1 mm/step) placed on the same film. The quantity of distracted callus was determined using Scion Image Beta 4.03 image analysis software (Scion, Frederick, MD, USA). To measure the bone density of the distracted area, the entire image was interpreted with respect to the thickness of the aluminum plate by comparing it with the luminosity gradient obtained from the aluminum wedge. The bone density is reported in terms of the aluminum thickness (mm Al).

Histological Examination

We performed a histological examination to evaluate callus formation qualitatively. Two rats in each group were euthanized 4 weeks after injection. The lengthened femurs were fixed in 4% paraformaldehyde in PBS, pH 7.4. All specimens were decalcified in 10% ethylenediaminetetraacetic acid solution, embedded in paraffin, sectioned in the coronal plane, and stained with hematoxylin and eosin.

Biomechanical Analysis

Seven rats in each treatment group were used for biomechanical evaluation at Week 6 after injection. Lengthened femurs were prepared and external fixators and pins were removed before the bending test. Femurs were loaded in a three-point bending test using Legacy 4482 (Instron, Kanagawa, Japan). The fracture strength was determined as the maximum load supported by each femur.

DiI Labeling

To confirm survival potential and location of transplanted cells, adipose-derived regenerative cells were labeled with DiI (Vybrant® DiI Cell Labeling Solution; Life Technologies, Carlsbad, CA, USA) and transplanted. DiI binds to cellular thiols and has long-term stability, which enables the tracing of DiI-labeled transplanted cells in the host tissue. Two and 3 weeks after injection of labeled cells, a frozen section was prepared using Kawamoto's film method [7] in the coronal plane. The sections were stained with hematoxylin and eosin after the survival of the transplanted cells was determined in the unstained samples ($n = 5$ for each group).

Analysis of mRNA Expression

Real-time RT-PCR was performed on the distracted callus 2 weeks after transplantation using the methods outlined ($n = 8$ for each group). The expression of bone morphogenetic protein-2 (BMP-2), vascular endothelial growth factor A (VEGFA), stromal cell-derived factor-1 (SDF-1) and interleukin-6 (IL-6) was analyzed.

Total mRNA Isolation

RNA was extracted using a NucleoSpin® RNA II kit (Takara Bio, Otsu, Japan). Each sample was disrupted and homogenized using a syringe. Thereafter, treatment was performed according to the manufacturer's protocol. The absorbance of the resulting total RNA was measured at 260 nm, and the concentration was calculated.

cDNA Synthesis

cDNA was synthesized using High Capacity RNA-to-cDNA Master Mix® (Applied Biosystems, Foster City, CA, USA). Treatment was performed according to the manufacturer's protocol, and adjustments were made to yield a total volume of 20 μL. The following conditions

were used with a GeneAmp® PCR System 9700 (Applied Biosystems) to synthesize cDNA: 25 °C for 10 minutes, 42 °C for 60 minutes, 85 °C for 5 minutes, and a 4°C hold.

Real-time RT-PCR

To 1 μL of the resulting cDNA sample, 5 μL TaqMan® Universal Master Mix II (Applied Biosystems), 0.5 μL primer, and 3.5 μL RNAse free water (Applied Biosystems) were added to yield a total volume of 10 μL. Primers for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), BMP-2, VEGFA, SDF-1, and IL-6 were purchased from Applied Biosystems. Reactions were performed using an ABI PRISM 7700 sequence detection system (Applied Biosystems) with 40 cycles of 50 °C for 2 minutes, 95 °C for 10 minutes, 95 °C for 15 seconds, and 60 °C for 1 minute and amplification. The expression ratios of BMP-2, VEGFA, SDF-1, and IL-6 to GAPDH were calculated for each sample.

Statistical Analysis

This study was performed as an observer-blinded study. Coefficient of variation was measured in all tests. There were no differences between groups. As determined by power analysis, our sample size was 60 rats (three groups of 20 each). This size was based on a desired alpha level of 0.05 with an 80% power of detecting a 5% difference in bone density. Experimental results were expressed as mean ± SD. A t-test was performed to compare two groups. Multiple comparisons among groups were made using the Kruskal-Wallis and Steel-Dwass tests. Differences were considered significant when $p < 0.05$.

Results

Radiographic Evaluation

Three weeks after completion of distraction, the distracted calluses of the ADRC group became fused and gradually consolidated (Fig. 1). The distracted calluses of the other groups also fused at 4 weeks after the end of elongation.

The bone density was greater in the ADRC group as compared with the control group at 3, 4, and 6 weeks (mean ± SD, Week 3, $p = 0.036$, Cohen's $d = 1.20$, 95% confidence interval [CI] ± 0.137; Week 4, $p = 0.033$, Cohen's $d = 1.10$, 95% CI ± 0.152; Week 6, $p = 0.003$, Cohen's $d = 10.2$, 95% CI ± 0.180) or the collagen-treated group at 4 and 6 weeks (mean ± SD, Week 4, $p = 0.033$, Cohen's $d = 1.00$, 95% CI ± 0.139; Week 6, $p = 0.018$, Cohen's $d = 0.91$, 95% CI ± 0.152) (Fig. 2). The control and collagen groups had similar bone densities throughout the testing period. Bone regeneration was greater in the ADRC group than in any other group.

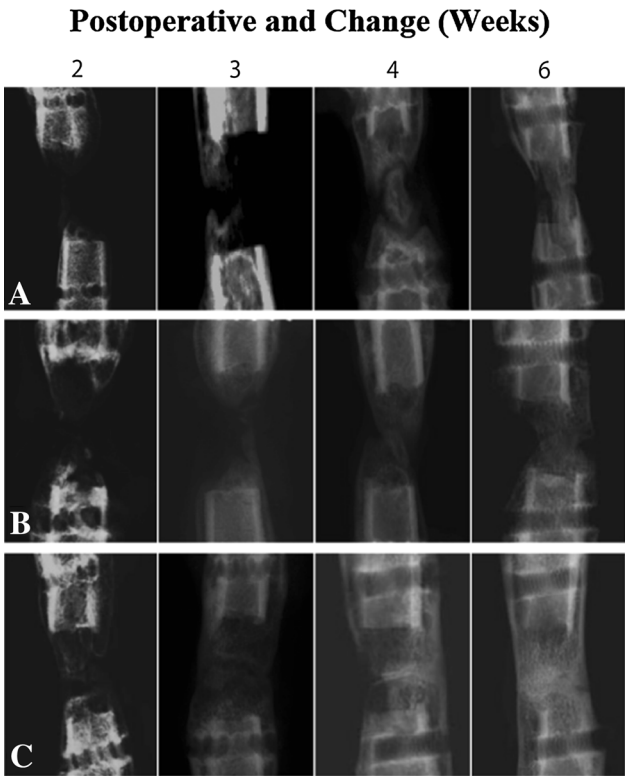

Fig. 1A–C Radiographs of femurs from (**A**) control, (**B**) collagen, and (**C**) ADRC groups showing distracted calluses at 2, 3, 4 and 6, weeks after termination of lengthening. Callus formation appeared to occur earlier in the ADRC group than in the other groups.

Histological Examination

We performed the histological examination to evaluate callus formation qualitatively. Hematoxylin and eosin staining of tissue from the ADRC group showed evidence of greater amounts of longitudinal bony trabeculae (Fig. 3). In addition, remodeling of the distracted callus and rebuilding of the medullary canal were completed by 4 weeks after completion of bone lengthening. Meanwhile, in the control and collagen groups, maturation of the distracted callus was poor, and the central radiolucent area between the proximal and distal calcified trabecular area was composed mainly of masses of fibroblast-like cells, which is in contrast to the ADRC group that showed mature trabeculae.

Biomechanical Analysis

The fracture strength as measured by the three-point bending test was greater in the ADRC group as compared

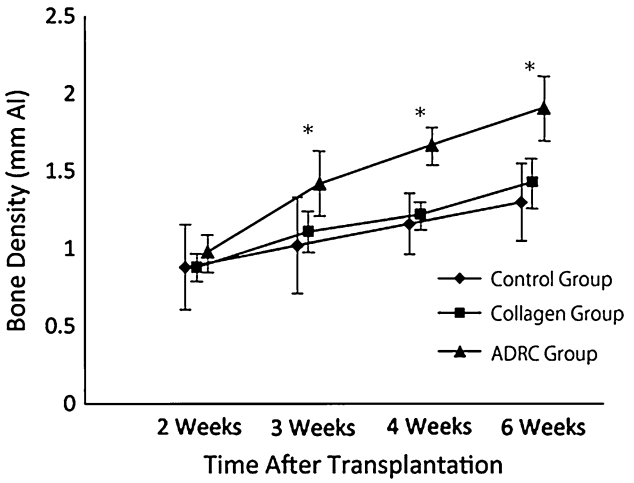

Fig. 2 Time course of bone density changes after lengthening termination. The ADRC group bone density was significantly greater at 3, 4, and 6 weeks than in the control group (*$p < 0.05$).

with the control group at 4 and 6 weeks (mean ± SD, Week 4, $p = 0.009$, Cohen's $d = 0.84$, 95% CI ± 0.152; Week 6, $p = 0.006$, Cohen's $d = 1.32$, 95% CI ± 0.180) or the collagen-treated group at 4 and 6 weeks (mean ± SD, Week 4, $p = 0.009$, Cohen's $d = 0.80$, 95% CI ± 0.139; Week 6, $p = 0.006$, Cohen's $d = 1.19$, 95% CI ± 0.152) (Fig. 4).

DiI Labeling

DiI labeling suggested that the transplanted cells had survived by 3 weeks posttransplantation. The distributions of DiI-positive (red) areas were detected during the 4 weeks after transplantation. The transplanted cells survived up to 2 weeks after transplantation with some cells being focally distributed in the trabecular bone (Fig. 5).

Real-time RT-PCR

The ADRC group had higher levels of BMP-2, VEGFA, and SDF-1 expression compared with the collagen group (Fig. 6). BMP-2, VEGFA, SDF-1, and IL-6 expression levels were detected both in the regenerated tissue in the collagen and ADRC groups. The expression ratio of BMP-2 was 1.01 ± 0.19 in the collagen group and 6.21 ± 0.81 in the ADRC group ($p = 0.047$). The expression ratio of VEGFA was 1.13 ± 0.29 in the collagen group and 6.81 ± 0.97 in the ADRC group ($p = 0.048$). The expression ratio of SDF-1 was 1.10 ± 0.18 in the collagen group and 4.3 ± 0.46 in the ADRC group ($p = 0.037$). The expression ratio of IL-6 was 4.25 ± 0.36 in the collagen group and 5.80 ± 0.31 in the ADRC group ($p = 0.055$).

Discussion

A variety of adjuvant treatments [8, 17] have been used in an attempt to shorten external fixation time during distraction osteogenesis, because the longer the external fixator is applied, the greater the number of complications and the more prolonged the patient's discomfort. Shortcomings with the adjuvants now in use include (1) that the isolation of BMSCs frequently yields low numbers of stem cells; (2) that the BMSC isolation procedure is invasive for donors and patients; and (3) that BMP is expensive. In this study, we found that ADRC transplantation promoted efficient ossification as determined by radiography and histology. These favorable effects of ADRCs promoted bone formation as evidenced by a biomechanical three-point bending test. DiI labeling indicated that transplanted ADRCs had survived in new bone for at least 4 weeks after transplantation. We thought that transplanted cells might differentiate into some kind of osseous tissue. Paracrine effects of ADRCs were also demonstrated by real-time RT-PCR to determine levels of osteogenic and angiogenic cytokines.

This study had a number of limitations. First, we did not confirm the differentiative capacity of transplanted ADRCs. DiI-labeled ADRCs existed in the newly formed bone. However, the survived cell number was too small to confirm their osteogenic differentiative potential. Second, we did not confirm the best timing and number of injections. Especially, there is a possibility that multiple injections induce better effect. We will need to examine the details in the future.

Several studies of tissue/organ regeneration with ADSCs showed direct evidence of the capacity of ADSCs to differentiate into multiple lineage cells in vivo and in vitro. Although ADSCs can survive a long period and undergo osteogenic differentiation in vivo [10], they may also contribute through paracrine pathways. The adipose-derived cells secrete angiogenic cytokines such as BMP-2, VEGF, SDF-1, and IL-6, which are postulated to contribute to the osteogenic and angiogenic properties of ASDCs [14, 16]. Our study supports these existing data both in terms of the survival potential and paracrine effects of ADRCs.

There are many opinions about the survival potential of ADSCs. For example, after intravenous injection, ADSCs were found to persist in mice even after 8 months [24]. In contrast, in other models, in which simple subcutaneous injection was used, ADSCs dissipated after as few as 5 days [26]. In our study, we showed the continued presence of ADRCs in the distracted callus up to 3 weeks after transplantation. This result suggests that ADRCs may survive for long periods after transplantation into the confined distracted callus. The ADRCs survived locally, and the number of surviving ADRCs was fairly small. As

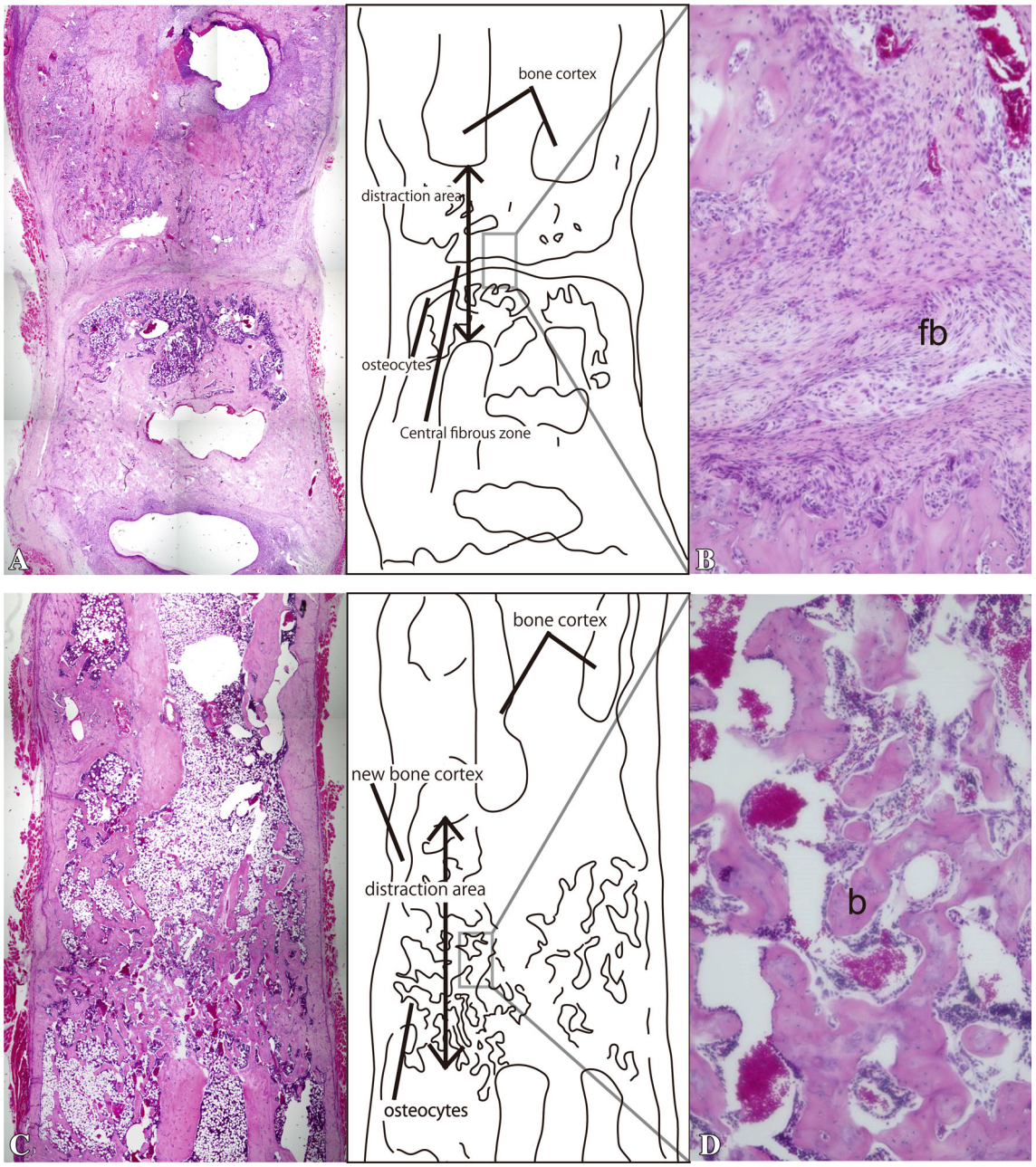

Fig. 3A–D Photomicrographs and schemes of (**A**–**B**) collagen and (**C**–**D**) ADRC groups prepared 4 weeks after termination of lengthening. The ADRC group showed mature trabeculae (Stain, hematoxylin and eosin; original magnification, X20, X200). b = trabecular bone; fb = fibroblast-like cells.

such, the osteogenic differentiative potential of ADSCs among ADRCs is not the only mechanism by which distracted callus maturation is promoted. Thus, secretion of some type of humoral factor by ADRCs may also promote osteogenic differentiation and angiogenesis. The ability of adipose tissue to promote tissue regeneration through secretion of various cytokines is well known. Previous reports revealed expression of angiogenic cytokines (eg, hepatocyte growth factor, VEGF) [12, 19] and enhanced neovascularization that in turn promoted bone formation. In addition, the chemokine SDF-1 was reported to play a role in ADRC-mediated angiogenesis [9]. ADSCs also secrete larger amounts of inflammatory cytokines such as IL-6, which is reported to inhibit osteoclast differentiation [29] such that IL-6 might therefore promote distraction osteogenesis. Various cytokines and other factors such as BMP-2, insulin like growth factor-1, and histone deacetylase inhibitors (eg, valproic acid) are known to stimulate

osteogenic differentiation among ADSCs [11, 25, 27]. In our study, the paracrine effects of ADRCs were shown by quantitative real-time RT-PCR using rat-specific primers for osteogenic and angiogenic cytokines.

Cellular factors have not seen widespread clinical applications for ethical and technical reasons [19]. Pluripotent stem cells have a high ability to differentiate. However, they have an associated risk of neoplastic transformation. Cell culture also has a higher risk of infection and immune reactions. In this study, we showed bone formation after distraction osteogenesis using ADRCs that can be easily isolated in large amounts from

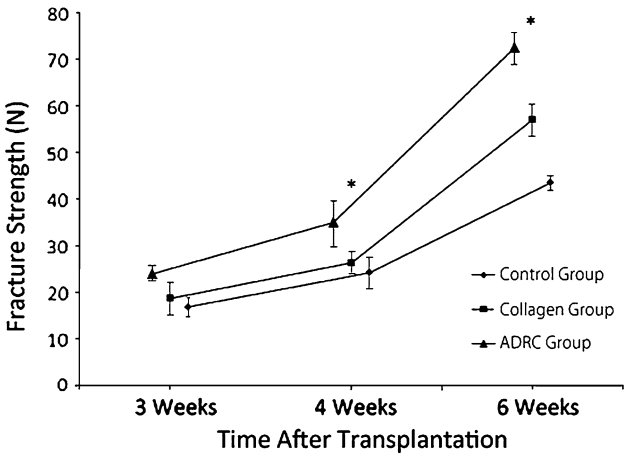

Fig. 4 Time course of changes in fracture strength as measured by a three-point bending test. At 4 and 6 weeks, the fracture strength in the ADRC group was significantly higher than that in the control and collagen groups.

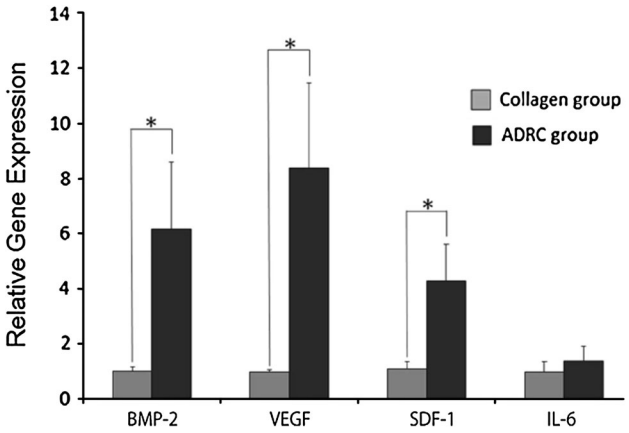

Fig. 6 Real-time RT-PCR. The ADRC group had significantly higher levels of BMP-2, VEGFA, and SDF-1 expression compared with the collagen group (*$p < 0.05$). There was a trend for higher expression of IL-6 in the ADRC group compared with the collagen group ($p = 0.055$).

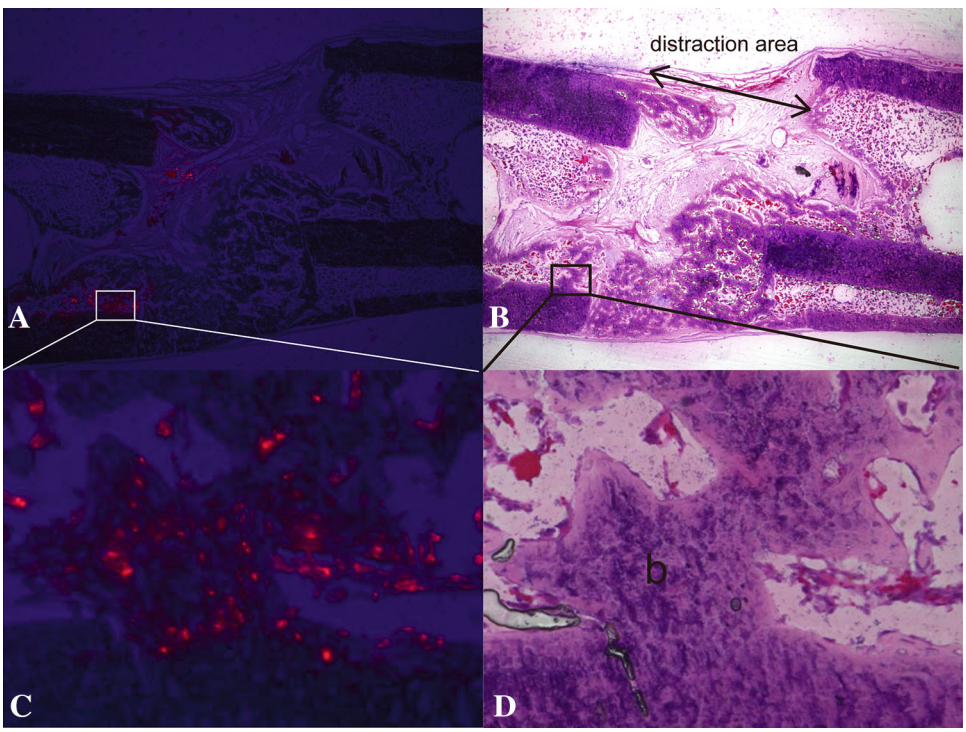

Fig. 5A–D DiI labeling (original magnification X20, X200). Two weeks after transplantation of ADRC labeled with DiI dye, frozen sections were prepared (**A**, **C**) and then stained with hematoxylin and eosin (**B**, **D**). Some cells survived and were focally distributed in the trabecular bone. b = new trabecular bone.

autologous adipose tissue. ADRCs were largely used without culturing or induction of differentiation, which would facilitate the future clinical applications of these cells. Collagen gel could be a suitable scaffold because we can easily apply it in a clinical application. To our knowledge, this study is the first to show the therapeutic potential of ADRCs that could be used with bioabsorbable scaffolds in animal models of distraction osteogenesis of the long bone. Our ultimate goal is to promote bone formation after distraction osteogenesis in human bones.

Our study showed that autogenous ADRCs with a collagen gel promoted bone formation in the distracted callus and shortened the consolidation period in vivo. The ADRCs show a survival capacity and potential capability of differentiating into osteogenic lineages during the early stages of distraction osteogenesis as well as paracrine effects for osteogenesis and angiogenesis in the distracted callus.

We hope to continue to develop this new cell-based therapy. Our next study will be to examine the best timing and frequency of injection in a large animal model. We also have to evaluate the risk of infection and immune reaction. Our ultimate goal is to develop a technique that will shorten treatment time with a low-invasive and useful method.

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