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Cell-Assisted Lipotransfer: Supportive Use of Human Adipose-Derived Cells for Soft Tissue Augmentation with Lipoinjection

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ABSTRACT

Injective transfer of autologous aspirated fat is a popular option for soft tissue augmentation, but several issues require attention, including unpredictability and a low survival rate due to partial necrosis. In this study, histologic features and yield of adipose-derived stromal (stem) cells (ASCs) were compared between human aspirated fat and excised whole fat. Aspirated fat contained fewer large vascular structures, and ASC yield was lower in aspirated fat. Aspirated fat was transplanted subcutaneously into severe combined immunodeficiency mice with (cell-assisted lipotransfer; CAL) or without (non-CAL) vascular stromal fractions containing ASCs isolated from adipose tissue. The CAL fat survived better (35% larger on average) than non-CAL fat, and microvasculature was detected more prominently in CAL fat, especially in the outer layers. DiI-labeled vascular stromal fraction cells were found between adipocytes and in the connective tissue in CAL fat, and some of these cells were immunopositive for von Willebrand factor, suggesting differentiation into vascular endothelial cells. Another experiment that used vascular stromal fractions taken from green fluorescent protein rats also suggested that ASCs differentiated into vascular endothelial cells and contributed to neoangiogenesis in the acute phase of transplantation. These findings may partly explain why transplanted aspirated fat does not survive well and suggest clinical potential of the CAL method for soft tissue augmentation.

INTRODUCTION

ADIPOSE-DERIVED STROMAL (STEM) CELLS (ASCs) can be obtained from liposuction aspirates[1,2] and differentiate into multiple lineages of mesodermal or ectodermal origins. Human ASCs were shown by several *in vitro* and *in vivo* studies to differentiate into adipogenic, osteogenic,[3–7] chondrogenic,[8–10] myogenic,[11,12] cardiomyogenic,[13,14] and neurogenic[15–17] lineages. Adipose tissue is known to be rich in microvasculature,[18] and ASCs were shown to have angiogenic characteristics and to differentiate into vascular endothelial cells experimentally.[19–21] Human ASCs are distinct from other mesenchymal progenitors in surface marker expression profile; notably, only ASCs express stem-cell–associated marker CD34 in higher percentages compared with bone marrow–derived mesenchymal stem cells and dermal fibroblasts.[22]

ASCs are being studied in clinical trials, including those investigating bone defect[23] (fresh ASCs) and rectovaginal fistula[24] (cultured ASCs) treatments and soft tissue augmentation by cell-assisted lipotransfer[25] (our unpublished data; fresh ASCs). If ASCs are harvested from a large volume (e.g., 1 L) of liposuction aspirates, ASCs can be used

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clinically without cell expansion because a sufficient number can be obtained. Furthermore, the use of minimally manipulated fresh cells may lead to greater safety and efficacy in actual treatments.

Aspirated fat is also used as injection material for soft tissue augmentation to reconstruct inborn or acquired tissue defects, or for such cosmetic treatments as breast enhancement or facial rejuvenation. Although lipoinjection has some problems to be resolved, such as unpredictability and a low survival rate due to partial necrosis,[26,27] it is almost the only method of soft tissue augmentation that can be performed without detectable scarring on a donor or a recipient site and without complications associated with foreign materials. Thus, if the clinical efficacy and safety of the procedure can be improved, it could be a strong cosmetic and reconstructive tool for soft tissue augmentation.

Here, we provide evidence to support a novel method of autologous tissue transfer, which we named cell-assisted lipotransfer (CAL). CAL is a concurrent transplantation of aspirated fat and ASCs (i.e., transplantation of ASC-rich aspirated fat). In CAL, ASCs were supportively used to boost the efficacy of autologous lipoinjection (resulting in a higher survival rate and persistency of transplanted fat) and to decrease known adverse effects of lipoinjection, such as formation of fibrosis, pseudocyst, and calcification. In this study, aspirated fat was compared with excised whole fat (nonaspirated fat) in microscopic and electromicroscopic histology and adherent cell yields. In addition, we evaluated the effectiveness of CAL in animal models compared with non–cell-assisted lipotransfer (non-CAL), and examined the fate of ASCs in fat transplanted with the CAL method.

MATERIALS AND METHODS

Human tissue sampling

We obtained liposuction aspirates from healthy female donors undergoing liposuction of the abdomen or thighs. Participants provided informed consent, and an institutional review board—approved protocol was used. From 3 patients who underwent both liposuction and tummy tuck, excised adipose tissue (20–30 g) was also taken. Aspirated fat was used as the cell source of vascular stromal fraction (SVFs) containing ASCs. The excised fat obtained from patients who underwent a tummy tuck was also used for isolation of SVFs. Both the aspirated fat and the excised fat were also used for histologic examination.

Cell isolation and culture

SVFs were isolated from the fatty portion of liposuction aspirates by using a procedure modified from Zuk *et al.*[1] Briefly, the aspirated fat was washed with phosphate-buffered saline (PBS) and digested on a shaker at 37°C in PBS containing 0.075% collagenase for 30 min. Mature adipocytes and connective tissues were separated from pellets by centrifugation (800 g, 10 min). The pellets were resuspended and filtered with a 100-μm mesh (Millipore, MA). Freshly isolated SVFs were plated (30,000 cells/cm^2) on gelatin-coated dishes and cultured at 37°C in an atmosphere of 5% carbon dioxide (CO_2) in humid air. The culture medium was M-199 containing 10% fetal bovine serum, 100 IU penicillin, 100 mg/mL streptomycin, 5 μg/mL heparin, and 2 ng/mL acidic fibroblast growth factor. After 7 days, attached cells were passaged by trypsinization and cultured in the same medium. Medium was replaced every third day. The excised fat from patients who underwent a tummy tuck was first minced with scissors into 3-mm pieces and then processed in the same manner as aspirated fat.

Mouse models for transplantation of human aspirated fat

Seven-week-old male severe combined immunodeficiency (SCID) mice housed with free access to water and standard chow diet were anesthetized by intraperitoneal injection of 5 mg/mL pentobarbital. For preparation of graft material, human aspirated fat was washed with saline and poured into 10-mL syringes, which were then placed upright at room temperature for 10 min. The infranatant fluid was then discarded. The human aspirated fat (1 mL = 900 mg) was subcutaneously injected into the back of the SCID mice with or without freshly isolated SVF cells. For CAL, SVFs taken from 4 mL of aspirated fat was mixed with 1 mL of aspirated fat. Four weeks later, transplanted fat was harvested, weighed, and fixed with 4% paraformaldehyde, and 4 μm sections were stained with hematoxylin-eosin.

Mouse models for tracing human SVF cells in CAL

For tracing the SVFs, SVF cells freshly isolated from human aspirated fat were labeled by incubated with 5 μg/mL CM-DiI (Molecular Probes, Eugene, Oregon) for 1 h before transplantation. DiI-labeled SVFs taken from 4 mL of aspirated fat were mixed with 1 mL of aspirated fat and then injected under the back skin of a SCID mouse. In the same manner, human aspirated fat was injected without DiI-labeled SVFs as control. Four weeks later, transplanted fat was harvested, fixed with 4% paraformaldehyde, and embedded in optimal cutting temperature (OCT) compound by freezing in isopentene cooled with liquid nitrogen. Then, 20-μm-thick frozen sections were examined with a confocal microscope system (Leica TCS SP2, Leica Microsystems GmbH, Wetzlar, Germany).

Rat models for tracing rat green fluorescent protein–labeled SVF in CAL by using rat minced fat

Inguinal adipose tissue excised from green fluorescent protein (GFP) rats [SD TgN(act-EGFP)OsbCZ-004; gener-

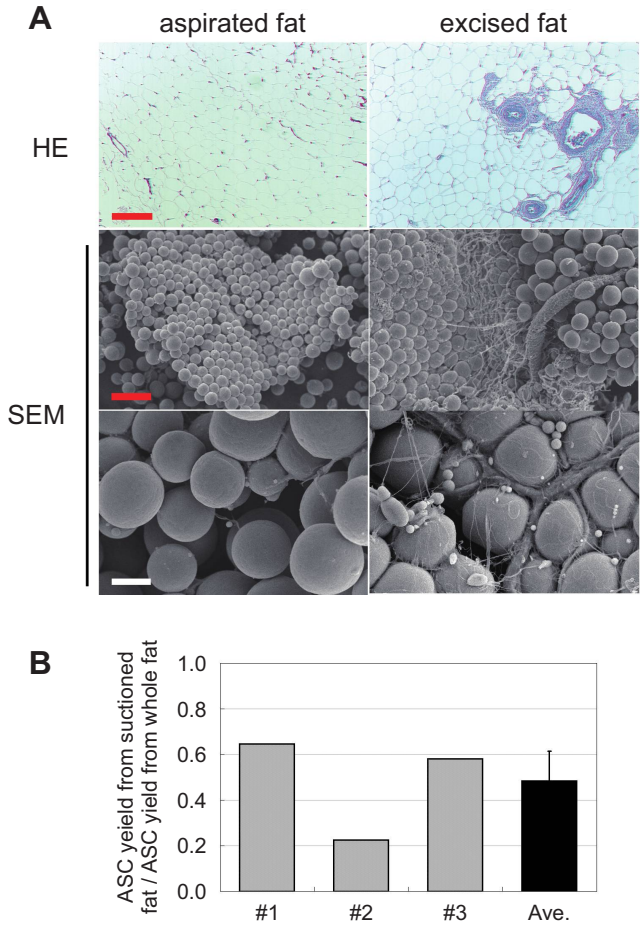

FIG. 1. Comparison of human aspirated fat and excised whole fat obtained from a single site of a single patient. (**A**) Histologic features of aspirated fat and excised fat. (Hematoxylin-eosin [HE]–stained microphotographs and scanning electron micrograph [SEM] photos; red scale bar = 200 μm, white scale bar = 40 μm). The basic structure of adipose tissue was preserved in the aspirated fat, while vascular vessels, especially large one, were significantly less detected in aspirated fat than in excised fat. (**B**) Adipose-derived stromal (stem) cell (ASC) yield from aspirated fat and excised fat. Both tissues were processed for isolation of) vascular stromal fractions, which were then cultured for 1 week. Ratios of ASC yield from aspirated fat to that from excised fat of the same volume were calculated; data from 3 patients (#1–#3) and their average value were demonstrated. ASC yield from aspirated fat was significantly less (48% ± 13%) than that from excised fat.

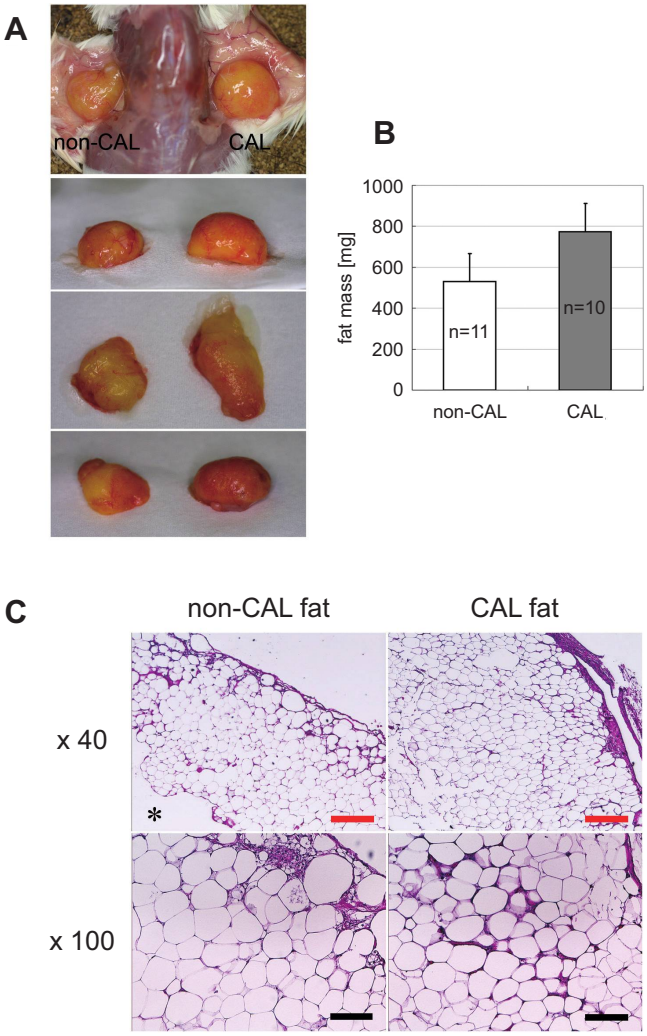

FIG. 2. Cell-assisted lipotransfer (CAL) and non-CAL using human aspirated fat and vascular stromal fractions. (**A**) Photographs of CAL fat (right) and non-CAL fat (left) in severe combined immunodeficiency mice. Human aspirated fat was transplanted with (CAL fat) or without (non-CAL fat) human vascular stromal fractions freshly isolated from the same aspirated fat. Transplanted fat was harvested at 4 weeks. (**B**) Weight of harvested CAL fat and non-CAL fat. CAL fat weighed significantly more than non-CAL fat ($p < .05$). (**C**) Histologic features of CAL fat and non-CAL fat samples (red scale bar = 250 μm, black scale bar = 100 μm). Central necrosis (*) was almost always seen in non-CAL fat, and the surviving layer of CAL fat was thicker than that of non-CAL fat. At higher magnification, microvasculature was more frequently seen in CAL fat, especially in the outer layers, than in non-CAL fat.

ated from Sprague-Dawley (SD) rats] was processed in the same way as human excised fat. GFP-SVF taken from 1 mL of fat of the GFP rat was mixed with minced inguinal adipose tissue (1 mL) harvested from SD rats and then injected under the back skin of an SD rat. Four weeks later, transplanted fat was harvested and forwarded to immunohistology as well.

Immunostaining

Frozen sections were prepared from transplanted fat embedded in OCT compound. After fixation in 100% acetone for 10 min at 4°C, sections were rehydrated and blocked with 0.5% goat serum in PBS. Paraffin-embedded sections

were dewaxed, washed in PBS, and treated with proteinase K (DakoCytomation, Carpinteria, CA) for 6 min and blocked with 0.5% goat serum in PBS. Primary and secondary antibody stainings were performed for 60 min each at room temperature. 4′,6-diamidino-2-phenylindole (DAPI) staining was performed with DAPI containing mounting medium (Vector Laboratories, Burlingame, CA). The following antibodies and dilutions were used: anti-green fluorescent protein (mouse, 1:500; Molecular Probes), von Willebrand factor (rabbit, 1:500; DakoCytomation), and Alexa Fluor 488 or Alexa Fluor 546 conjugated antimouse or antirabbit antibodies (goat, 1:200; Molecular Probes). Sections were imaged on a confocal microscope system (Leica TCS SP2).

Scanning electron microscopic study

Aspirated and excised fat were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.2 M cacodylate buffer for 1 week at room temperature, and then fixed in 1% osmium tetroxide. After dehydration, they were dried with a super-critical-point CO_2 dryer (HCP-2, Hitachi, Tokyo, Japan), sputter-coated with platinum-palladium, and examined with a scanning electron microscope (S3500N, Hitachi).

Statistical analyses

Results were expressed as the mean ± standard error. The data were statistically analyzed using an unpaired Student *t*-test. A p value less than .05 was considered to represent a statistically significant difference.

RESULTS

Comparison of histologic features and ASC yield between aspirated fat and excised fat

Both aspirated fat and excised fat were harvested from the 3 patients who underwent the tummy-tuck operation. Histologic examinations with a light microscope and scanning electron microscope showed that aspirated fat preserves basic structures of adipose tissue and that mature adipocytes keep normal adhesions with each others. The only difference detected was that aspirated fat contained fewer vascular structures, especially large ones, compared with excised fat.

The same weights of aspirated fat and excised fat were processed for isolation of SVFs. The cell isolation process was performed within 2 h after harvest. The isolated SVFs were cultured for 1 week, and the numbers of adherent ASCs from the aspirated fat and the excised fat were counted and compared. In all 3 patients, the number of adherent ASCs at 1 week was greater in the excised fat than in the aspirated fat. The ratio of normalized ASC number from aspirated fat to that from excised fat was 0.48 ± 0.13 ($n = 3$) (Fig. 1B).

Human aspirated fat transplantation with or without SVF

Human aspirated fat was transplanted with or without freshly isolated SVFs containing ASCs taken from the same patient. The experiments were done 3 times using aspirated fat from 3 patients.

Transplanted adipose tissue with or without ASCs (CAL fat and non-CAL fat) was 712.3 ± 45.3 mg ($n = 10$) or 520.6 ± 40.8 mg ($n = 11$), respectively, while fresh adipose tissue (1 mL) before transplantation was approximately 900 mg (Fig. 2A and B). Histology of transplanted fat samples showed that the central region was necrotic in non-CAL fat samples and that the survived layer was thicker in CAL fat than in non-CAL fat (Fig. 2C). Microvasculature microscopically detected appeared to be prominent in CAL fat, especially in the outer layers, but not in non-CAL fat (Fig. 2C).

Fate of human ASCs transplanted with human aspirated fat

To trace human ASCs, SVFs freshly isolated from aspirated fat were labeled with DiI and then transplanted with human aspirated fat. In CAL fat, DiI-labeled ASCs were occasionally detected between mature adipocytes and in the connective tissue of the transplanted fat (Fig. 3). ASCs positive for both DiI and von Willebrand factor were detected in CAL fat, suggesting that some transplanted ASCs were differentiated into vascular endothelial cells (Fig. 4).

Fate of ASCs derived from GFP rats in rat CAL models

To trace ASCs transplanted with fat, minced fat of SD rats was transplanted with (CAL) or without (non-CAL) SVFs isolated from the inguinal adipose of GFP rats (GFP-SVF) (Fig. 5A). In CAL fat, GFP-positive cells, which are supposed to be transplanted GFP-ASCs, were detected within connective tissue and in some vessels (Fig. 5B). Most of the vessels were derived from host (von Willebrand factor–positive, GFP-negative), but vessels positive for both von Willebrand factor and GFP were occasionally detected. The GFP-positive cells partly or entirely covered the inner surface of vessels (Fig. 5B). It was suggested that some ASCs differentiated into endothelial cells and contributed to angiogenesis during the surviving process of the adipose transplantation.

DISCUSSION

Adipose tissue is predominantly composed of extracellular matrix; mature adipocytes; ASCs; vascular cells, such as endothelial cells; and mural cells, such as pericytes and vascular smooth muscle cells. We recently reported the cell

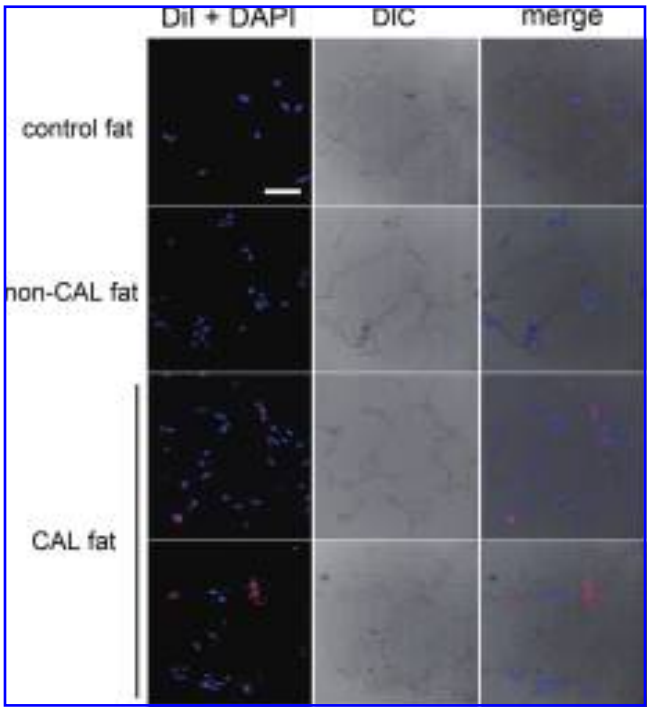

FIG. 3. Histologic features of cell-assisted lipotransfer (CAL) fat using human aspirated fat and DiI-labeled vascular stromal fractions. Normal excised fat and non-CAL fat were also used for comparison. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). In CAL fat, DiI-labeled cells, which are supposed to be co-transplanted adipose-derived stromal (stem) cells, are located between mature adipocytes and in interstitial connective tissue. The frequency of DiI-positive cells was 10%–30%, although it varied among samples and at locations in a sample. Scale bar = 50 μm. The right column shows merges of DiI and DAPI, and differential interference contrast (DIC) images.

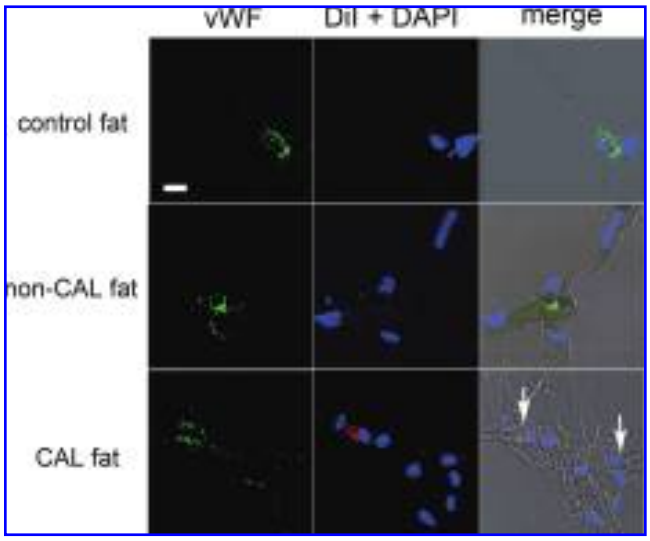

FIG. 4. Immunohistology of cell-assisted lipotransfer (CAL) fat using human aspirated fat and DiI-labeled vascular stromal fractions. Normal excised fat (control fat) and non-CAL fat were also assessed for comparison. Endothelial cells were immunostained with von Willebrand factor (vWF). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Cells double-positive for vWF and DiI (arrows), which were suggested to be vascular endothelial cells differentiated from co-transplanted DiI-labeled adipose-derived stromal (stem) cells, were observed in connective tissue or between adipocytes. Scale bar = 10 μm. The right column shows merges of immunostaining of vWF, DiI, DAPI, and differential interference contrast images.

composition of SVFs freshly isolated from liposuction aspirates, which is important information for the clinical use of SVFs.[22] The SVFs are composed of heterogeneous populations, including blood-derived cells, which are composed of 50%–70% SVF cells. Adipose-derived cells ($CD45^-$cells) in SVFs isolated from aspirated fat were of the following types: ASCs (70%–90%; $CD31^-$ $CD34^+$ $CD45^-$ $CD90^+$ $CD105^-$ $CD146^-$), vascular endothelial (progenitor) cells (3%–9%; $CD31^+$ $CD34^+$ $CD45^-$ $CD90^+$ $CD105^{low}$ $CD146^+$), pericytes (2%–5%; $CD31^-$ $CD34^-$ $CD45^-$ $CD90^+$ $CD105^-$ $CD146^+$), and other cells. The cell composition of the SVFs was measured after the cell isolation process with collagenase digestion for 30 min, but not all adipose-derived cells can be isolated from the SVFs in this process; thus, the cell composition may not accurately correspond to the actual cell composition of the adipose tissue. The location of ASCs in the adipose tissue is not clearly understood. Some ASCs are supposed to be located in the connective tissues in adipose, and others are located between adipocytes or around microvasculature or macrovasculature.

Liposuction aspirates consist of 2 parts: a floating fatty portion and an infranatant fluid portion. We have investigated cells derived from these portions of liposuction aspirates and found that a substantial number of adipose-derived cells, including ASCs and endothelial cells, can be isolated from the fluid portion, although the number from the fluid portion is smaller than that from the fatty portion.[22] This finding supports a result of this study showing that aspirated fat is relatively stem cell–deficient compared with excised whole fat. The reason was not elucidated here, but mechanical injury during the liposuction procedure and digestion by endogenous proteases during the surgery or subsequent storage periods probably induces the release of ASCs from harvested aspirated fat into the fluid portion. In addition, as shown in this study, the basic structure of adipose tissue was preserved in the aspirated fat, but vascular vessels, especially larger ones, are significantly less detected in aspirated fat than in excised fat. It is well known that the honeycomb structures of vascular and neural perforator networks are left intact in aspirated sites after liposuction operation. (Click here for supplementary material.) Thus, it is reasonable that fewer ASCs, which also reside around capillaries and vessels, were isolated from aspirated fat, which does not contain larger vessels, than excised fat. Unlike our results, a recent

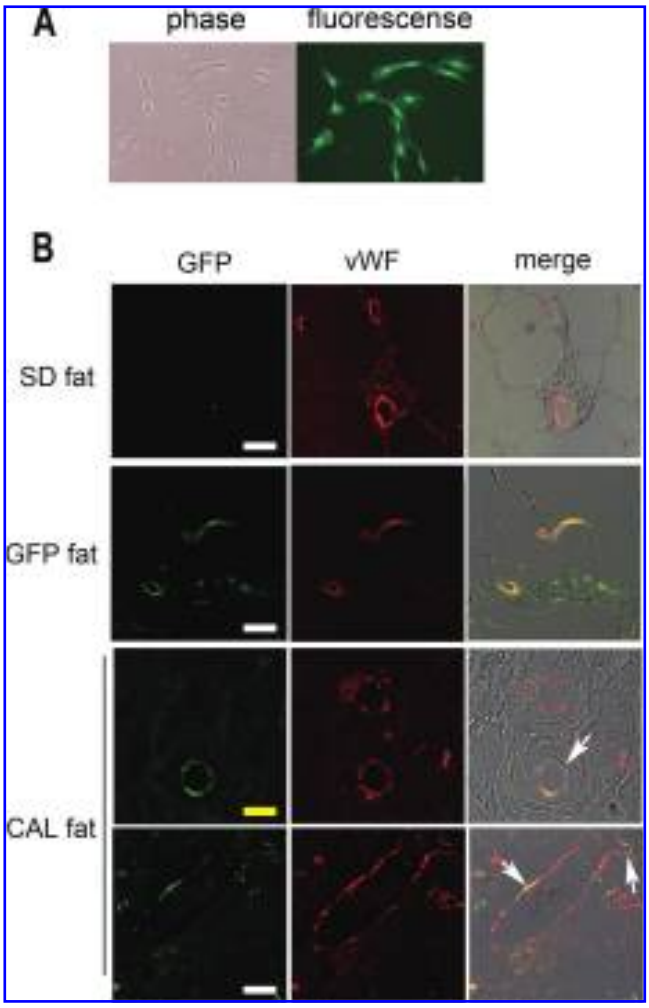

FIG. 5. Cell-assisted lipotransfer (CAL) fat in rat models (fragmented fat of Sprague-Dawley [SD] rat and green fluorescent protein – vascular stromal fractions [GFP-SVF rat]). (**A**) Cultured GFP–adipose-derived stromal (stem) cells (ASCs) (phase-contrast and fluorescence images). When GFP-SVF cells were cultured, all adherent cells were GFP-positive. (**B**) Immunohistology of cell-assisted lipoprotein fat in rat models. GFP was detected by immunostaining with anti-GFP antibody. Adipose tissue of SD rats and GFP rats were also demonstrated for comparison. Vascular endothelial cells are only von Willebrand factor (vWF) positive in SD fat but were all double positive for vWF and GFP in GFP fat. In CAL fat, capillaries with endothelial cells partly or entirely double positive for GFP and vWF (arrows) were detected, suggesting that they were differentiated from co-transplanted GFP-ASCs. The other endothelial cells were only positive for vWF, suggesting that they were derived from the host SD rat. White scale bar = 30 μm, yellow scale bar = 10 μm. The right column shows merges of immunostaining of vWF, DiI, 4′,6-diamidino-2-phenylindole, and differential interference contrast images.

study[28] that compared viable cell yield from fresh aspirated versus fresh excised fat did not detect a significant difference. However, this finding was probably occurred because the investigators centrifuged the aspirated fat and removed fibrous structures and visible vessels from excised fat during the cell isolation process.

The results of the present study suggest that addition of ASCs to aspirated fat improves the efficacy of adipose transfer, although the exact mechanisms remain to be elucidated. By addition of ASCs to relatively ASC-poor fat (aspirated fat), the aspirated fat is theoretically converted to relatively ASC-rich fat. A recent study using fragmented omentum tissue suggested the effects of co-transplantation with preadipocytes, but the fragmented adipose tissue in that study was not ASC-poor.[29]

Partly on the basis of the present data, we can speculate on the fate and roles of ASCs as follows. First, as shown in this study as well as in previous studies,[19–21] ASCs can differentiate into vascular endothelial cells that may contribute to neoangiogenesis during the healing process after transplantation. This effect may contribute to the decreased amount of central necrosis and marked microvasculature in the outer layers of CAL fat seen in this study.

Second, as suggested in this study, some ASCs were located between mature adipocytes and in the connective tissue of CAL fat, as they had been in normal fat before liposuction. They may play a role as adipose progenitor cells for future turnover of adipocytes. The relative deficiency of ASCs in aspirated fat compared with excised fat may contribute to the low long-term survival rate in non-CAL fat, which is a well-known clinical phenomenon.[26,27] This hypothesis is also supported by a recent study[29] showing that fragmented omentum (mainly composed of adipose) with or without preadipocytes (ASCs) was transplanted and the postoperative atrophy of transplanted tissue was suppressed when transplanted with preadipocytes. As it was reported that adipocytes are replaced with the next generation every 1–2 years in normal adipose,[30] the relative deficiency of tissue-specific progenitor cells in non-CAL fat may affect the coming turnover of the tissue and lead to its long-term atrophy. Turnover of adipocytes may occur in the early stage after transplantation in transplanted adipose tissue because its vascularity was temporarily damaged; this outcome may be the reason that atrophy of transplanted adipose is clinically seen during the first 3 months.

Third, some ASCs may differentiate into mature adipocytes and partly constitute transplanted fat. Although adipogenic differentiation of labeled ASCs was not detected in this study, the failure of detection may result from difficulty in detecting GFP-labeled cytoplasm of mature adipocytes, which are filled with lipid material; indeed, to our knowledge, no previous report has clearly demonstrated labeled cytoplasm of mature adipocytes.

Fourth, transplanted ASCs were kept in a hypoxic condition in the acute phase after transplantation and may release angiogenic soluble factors such as vascular endothelial growth factor and hepatocyte growth factor, accelerating neoangiogenesis from the surrounding host tissue in a paracrine manner. It was reported that cultured human ASCs produce and release these growth factors in a hypoxic con-

dition.[31] This effect may also contribute to the decreased volume of central necrosis and more prominent microvasculature in the outer layers of the CAL fat seen in this study.

Surgical injury accompanying the transplantation and the subsequent hypoxic condition and wound healing process, including inflammatory reactions, appear to trigger ASC differentiation into specific lineages, such as adipocytes, vascular endothelial cells, and mural cells. Because ASCs are known to undergo adipogenic differentiation when cocultured with mature adipocytes,[32] aspirated adipose tissue transplanted together with ASCs may contribute to acute adipogenic differentiation of ASCs.

In conclusion, aspirated fat contains less vasculature and fewer ASCs than excised fat. Transplanted aspirated fat survived better when transplanted with ASCs than without ASCs. These findings may partly explain why transplanted aspirated fat does not survive very well and suggest the clinical potential of the CAL method. Soft tissue augmentation with autologous fat, which leaves no incisional scar and lacks the complications associated with foreign materials, can be a cosmetically ideal tool when its effectiveness is improved.

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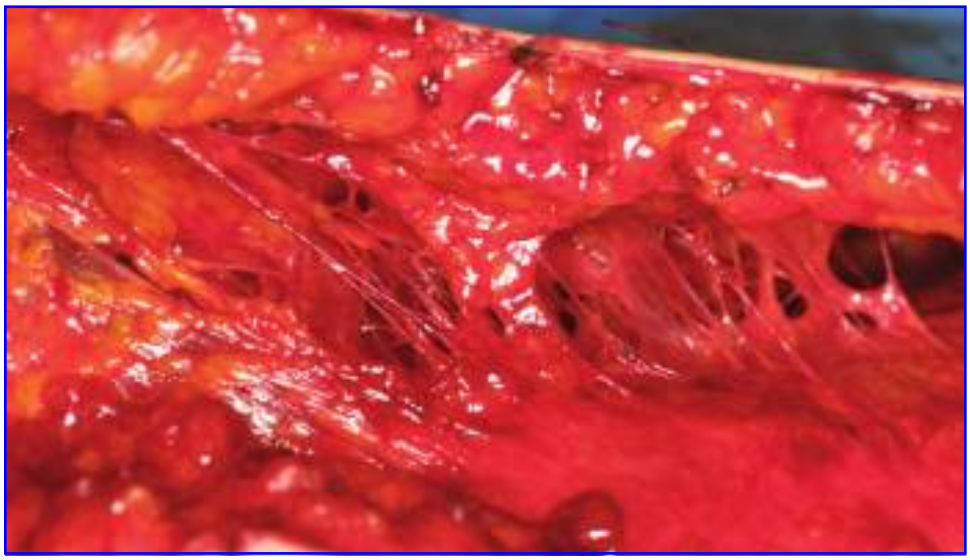

FIG. S1. Honeycomb structures in subcutaneous layers after liposuction. Vascular and neural perforators arising from the fascia or muscle are left intact after suctioning. Liposuction is usually performed with a metal cannula after infiltration of saline solution containing lidocaine and adrenaline. This fact clearly supports that aspirated fat contains fewer vascular structures compared to excised fat.