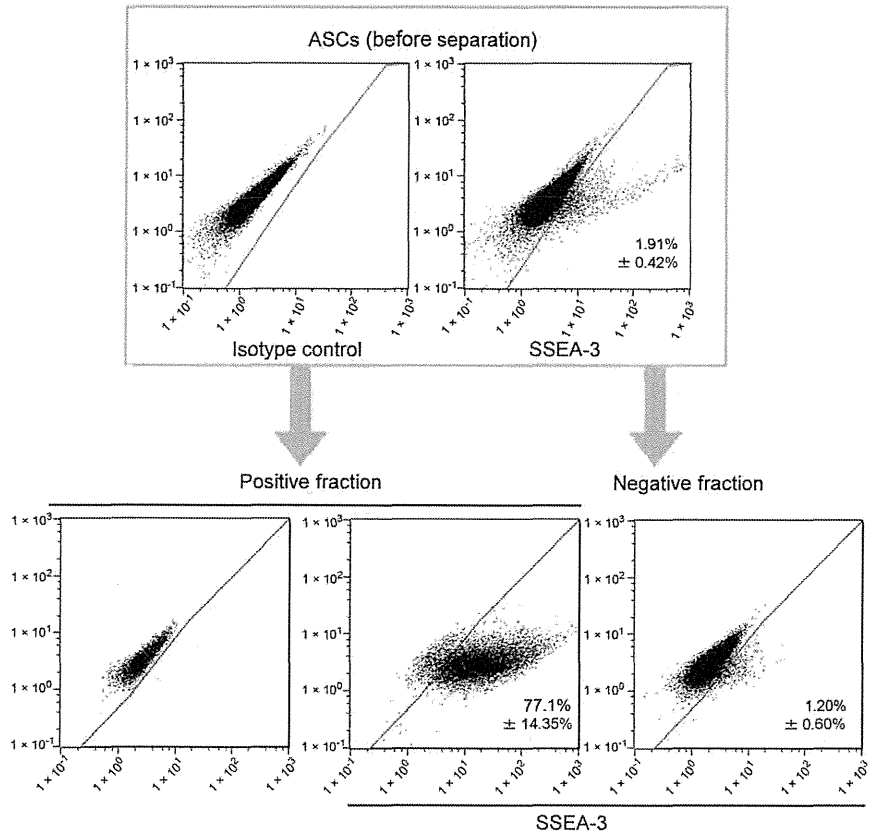

Figure 2. Flow cytometry analyses for SSEA-3 expression before and after enrichment of Muse cells using magnetic-activated cell sorting (MACS). An example of flow cytometry analysis performed to measure SSEA-3$^+$ cells before and after MACS cell enrichment and separation is shown. Cultured human ASCs were processed using MACS separation to obtain SSEA-3$^+$ cells. The positive and negative cell fractions after MACS separation were used as Muse-rich and Muse-poor cell populations, respectively, in subsequent experiments. Abbreviations: ASCs, adipose tissue-derived stem/stromal cells; SSEA-3, stage-specific embryonic antigen-3.

Cytokine Production Assays (Enzyme-Linked Immunosorbent Assays)

Muse-rich and Muse-poor cell populations were seeded at 4.0 × 10^5 cells in 60-mm dishes and cultured in DMEM without serum under hypoxic (1% O_2) or normoxic (6% O_2) conditions. After 48 hours, the culture media were collected and filtered using a 0.22-μm filter (Millex-GV filter; EMD Millipore, Billerica, MA, http://www.emdmillipore.com). Enzyme-linked immunosorbent assays (ELISA) kits for hepatocyte growth factor (HGF), stromal cell-derived factor 1 (SDF-1) (both from R&D Systems, Minneapolis, MN, http://www.rndsystems.com), and a cytokine array kit for vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF)-BB, nerve growth factor-β (NGF-β), stem cell factor (SCF), tumor necrosis factor-α (TNF-α), basic fibroblast growth factor (bFGF), and transforming growth factor-β (TGF-β) (catalog no. EA-1101; Signosis, Inc., Santa Clara, CA, http://www.signosisinc.com) were used to detect the cytokines. The absorbance was spectrophotometrically measured at 450 nm using an infinite microplate reader (M1000; Tecan Group, Männedorf, Switzerland, http://www.tecan.com).

Microarray Analyses

The samples were prepared as follows. Cultured hASCs were harvested with 0.25% trypsin and 2 mM EDTA for 5 minutes at 37°C. Portions of the cell suspensions were collected and dissolved with Isogen (Nippon Gene, Tokyo, Japan, http://www.nippongene.com) as a control sample, and other portions were used for sorting with autoMACS. After sorting, cell suspensions of Muse-rich populations and Muse-poor populations were dissolved with Isogen as a Muse-rich sample and a Muse-poor sample, respectively. Total RNA was purified using the RNeasy mini kit (Qiagen, Hilden, Germany, http://www.qiagen.com) according to the manufacturer's recommendations. Fluorescent-labeled cRNA was synthesized using a Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA, http://www.agilent.com). Labeled cRNAs were hybridized with the SurePrint G3 Human GE microarray 8 × 60K (G4851A; Agilent Technologies). Microarrays were scanned using a G2505C microarray scanner, and the results were analyzed using Feature Extraction Software (Agilent Technologies). Finally, we analyzed gene expression using GeneSpring GX software, version 12.5 (Agilent Technologies).

Preparation of Immunodeficient Diabetic Mice

Five-week old male severe combined immunodeficient (SCID) mice (C.B-17/Icr-*scid/scid*Jcl) were purchased from CLEA Japan, Inc. (Tokyo, Japan, http://clea-japan.com). All animal experiments were performed with approval from the Institutional Animal Care and Use Committee of the University of Tokyo. After the SCID mice were fasted for 24 hours, freshly prepared

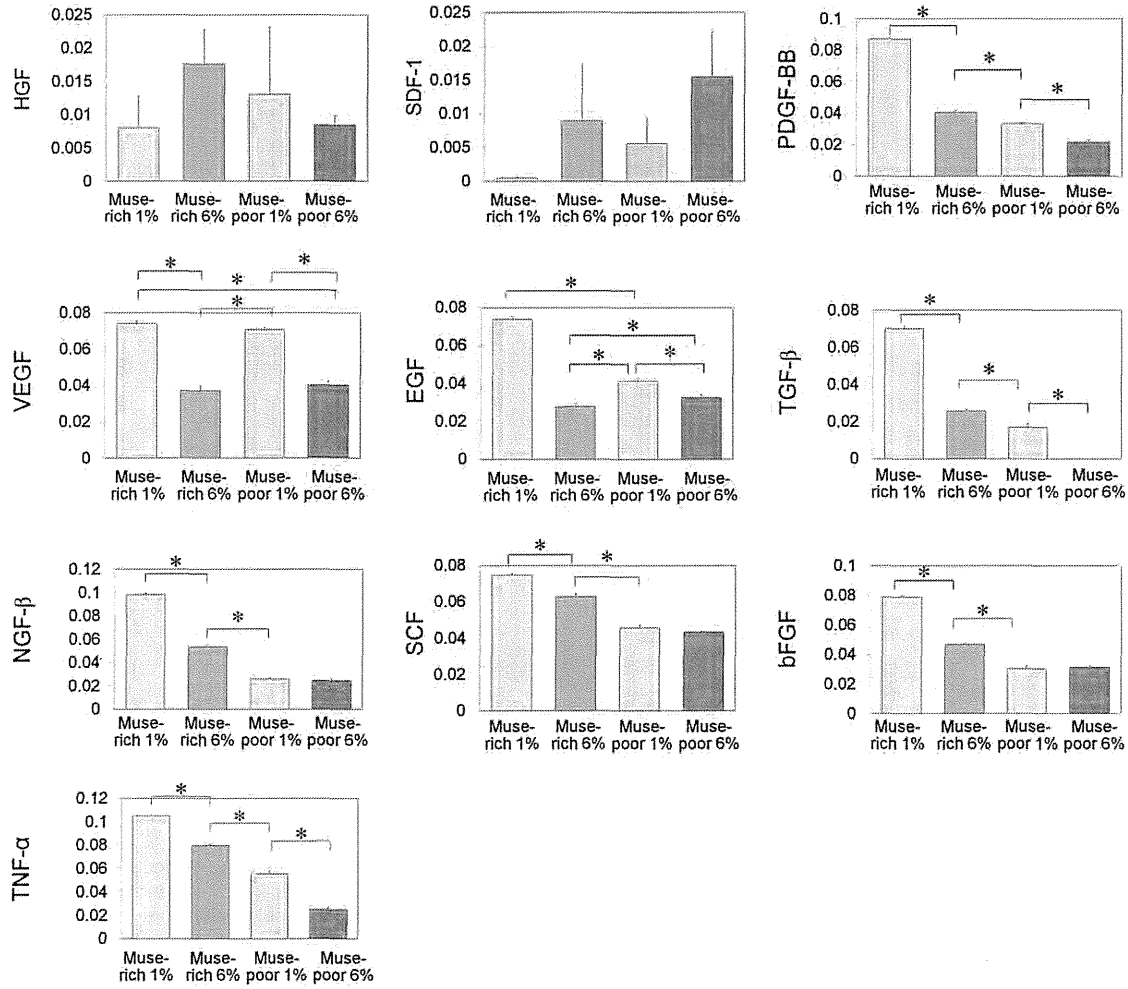

Figure 3. Enzyme-linked immunosorbent assay (ELISA) analyses for growth factor production under hypoxic and normoxic conditions. The relative growth factor production values were measured with ELISA in Muse-rich and Muse-poor cell fractions cultured under hypoxic (1% O_2) or normoxic (6% O_2) conditions for 48 hours. The measured growth factors included HGF, SDF-1, PDGF-BB, VEGF, EGF, TGF-β, NGF-β, SCF, bFGF, and TNF-α. The y-axis indicates absorbance at 450 nm. The samples were collected from three independent experiments, and three replicates were used in each measurement. Data are presented as the mean ± SD ($n = 3$). *, $p < .05$. Abbreviations: bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; HGF, hepatocyte growth factor; Muse, multilineage-differentiating stress-enduring; NGF-β, nerve growth factor-β; PDGF-BB, platelet-derived growth factor-BB; SCF, stem cell factor; SDF-1, stromal cell-derived factor 1; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

streptozotocin (STZ; 150 mg/kg; Sigma-Aldrich, St. Louis, MO, http://www.http://www.sigmaaldrich.com) in citrate-saline buffer (pH 4.5) was injected intraperitoneally. The blood glucose levels were measured using a glucometer and test strips (Glucose Pilot; Aventir Biotech LLC, Carlsbad, CA, http://www.aventirbio.com) 3 days after STZ injection. When the blood glucose levels were greater than 300 mg/dl, the mice were considered to have diabetes mellitus (DM). The mice that did not show hyperglycemia (>300 mg/dl) received a second injection of STZ (150 mg/kg), and the blood glucose levels were monitored 3 days later (Fig. 1A).

Wound Healing Mice Models

To evaluate skin wound healing, skin defects were created on the back of the mice, as previously described [13, 14]. In brief, the mice were individually anesthetized via an intraperitoneal injection of pentobarbital (65 mg/kg). After removing the hair using an electric trimmer and depilatory cream, two full-thickness skin wounds (6 mm in diameter), extending through the panniculus carnosus layer, were made on the dorsum of mice using a sterile circular biopsy punch (Kai Industries Co., Tokyo, Japan, http://www.kai-group.com/global/en/). To avoid wound contraction, a donut-shaped silicon splint (internal and external diameter of 9 and 15 mm, respectively; 1.0-mm-thick silicone rubber sheet; Kyowa Industries, Saitama, Japan, http://www.kyowakg.com) was applied and fixed using a 6-0 nylon suture to avoid wound contraction (Fig. 1A). Occlusive dressing (Perme-roll; Nitto Medical, Osaka, Japan, http://www.nitto.com/jp/en/) was used to prevent wound drying and scab formation.

We prepared five experimental groups: wild-type mice (C.B-17/Icr), non-DM SCID mice (C.B-17/Icr-*scid*/*scid*Jcl), DM-induced SCID mice, DM-induced SCID mice treated with a Muse-rich cell population, and DM-induced SCID mice treated with a Muse-poor cell population. Six mice were used in each group. After mixing with 0.1 ml of cross-linked hyaluronic acid (Restylane; Galderma, Watford, U.K., http://www.galderma.com), Muse-rich

or Muse-poor cells (1.0×10^5 cells per mouse) were injected into the subcutis at four points (0.025 ml at each point) around the wound. Macroscopically, we examined the interval to wound closure (the number of days until the skin defect had closed completely). The wound was sequentially photographed on days 0, 3, 7, 10, and 14 using a digital camera (IXY Digital 90; Canon, Tokyo, Japan; http://www.canon.com). The photographs were evaluated to measure the wound area using image analysis software (Photoshop CS6; Adobe Systems, San Jose, CA, http://www.adobe.com).

Histological Analyses

The mice skin samples were embedded in optimal cutting temperature compound (Sakura Finetek, Tokyo, Japan, http://www.sakura-finetek.com), frozen in liquid nitrogen, and stored at −80°C until sectioning. The frozen sections (8 μm) were placed on slides, air dried at room temperature for 20 minutes, fixed in 4% paraformaldehyde (in PBS) for 1 minute, and washed for 5 minutes in PBS. The slides were stained with H&E and processed for immunohistochemistry.

Immunohistochemistry was performed as described previously [6]. To detect injected human cells in the mouse skin, the sections were treated with 0.3% hydrogen peroxide in methanol for 30 minutes to inactivate intrinsic peroxidase activity and incubated sequentially with rabbit anti-human 58K Golgi protein (dilution 1:100; Abcam, Cambridge, U.K., http://www.abcam.com) and donkey anti-rabbit IgG-horseradish peroxidase (dilution 1:500; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, http://www.jacksonimmuno.com). Expression of human 58K Golgi protein was visualized by the peroxidase reaction with 3,3′-diaminobenzidine tetrahydrochloride. For laser confocal microscopy, cryosections were incubated with the following primary antibodies: mouse anti-human mitochondria (dilution 1:100, Abcam), rabbit anti-human 58K Golgi protein, or goat anti-human platelet endothelial cell adhesion molecule-1 (PECAM-1; dilution 1:50, Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com). Next, the sections were incubated with the following secondary antibodies: donkey anti-mouse IgG-Alexa 488 or rabbit anti-goat IgG-Alexa 488 (all dilutions, 1:500; Invitrogen, Carlsbad, CA, http://www.invitrogen.com). Vessels (vascular endothelial cells) were stained with Alexa Fluor 594-conjugated isolectin GS-IB_4 (Molecular Probes, Eugene, OR, http://probes.invitrogen.com) and nuclei with Hoechst 33342 (Dojindo Molecular Technologies Inc., Kumamoto, Japan, http://www.dojindo.com) and inspected using confocal microscopy (C1si Nikon; Nikon, Tokyo, Japan, http://www.nikon.com).

Statistical Analysis

The results are expressed as the mean ± SD. Tukey's tests and Steel-Dwass tests were applied to evaluate the differences between each group; $p < .05$ was considered statistically significant.

Results

Detection and Separation of Muse Cells From Cultured hASCs

hASCs were obtained by culturing SVF obtained from lipoaspirates. Flow cytometry analyses revealed that cultured hASCs at passage 2 contained a low percentage of SSEA-3^+ Muse cells (1.91% ± 0.42%) (Fig. 2). Using MACS sorting, we collected Muse-rich and Muse-poor cell populations, both of which were used in animal wound healing experiments. In the Muse-rich population, 77.1% ± 14.35% of cells were SSEA-3^+. In contrast, in the Muse-poor population, 1.20% ± 0.6% of the cells were SSEA-3^+, suggesting that SSEA-3^+ ratio in Muse-poor population is very close to that in the original ASCs (Fig. 2).

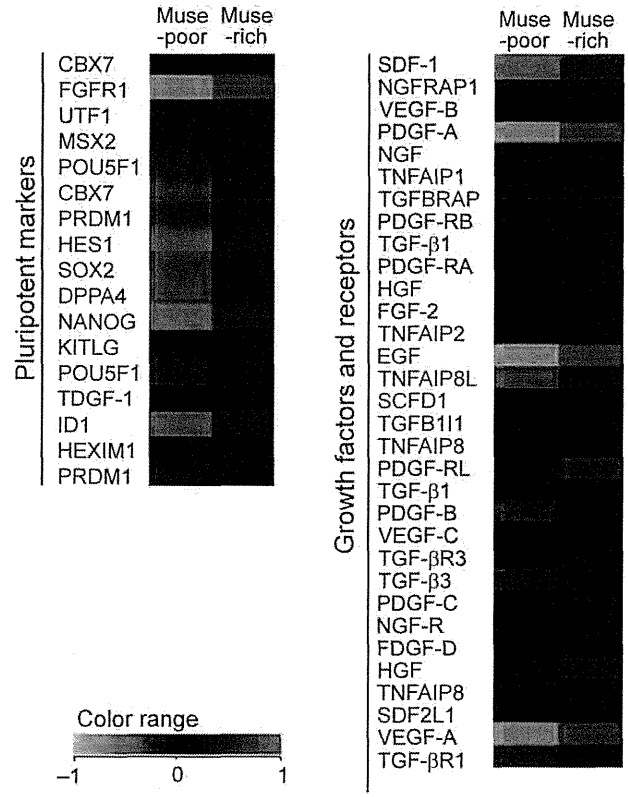

Figure 4. Microarray analyses of Muse-rich and Muse-poor cell populations. Heat maps for pluripotent markers, growth factors, and receptors indicate that pluripotent markers, including NANOG and FGFR1, were upregulated in the Muse-rich population compared with the Muse-poor population. The Muse-rich population also showed higher gene expression of growth factors such as PDGF-A, EGF, and VEGF-A (supplemental online Figure 1). Abbreviations: EGF, epidermal growth factor; FGFR1, fibroblast growth factor receptor 1; HGF, hepatocyte growth factor; Muse, multilineage-differentiating stress-enduring; NGF, nerve growth factor; PDGF-A, platelet-derived growth factor-A; SDF-1, stromal cell-derived factor 1; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

Cytokine Secretion by Muse Cells Under Normoxic and Hypoxic Conditions

We compared the cytokine concentrations in culture media after 48 hours of adherent culture of Muse-rich and Muse-poor populations under normoxic (6% O_2) or hypoxic (1% O_2) conditions (Fig. 3). The Muse-rich population released greater amounts of EGF, PDGF-BB, NGF-β, SCF, TNF-α, bFGF, and TGF-β compared with the Muse-poor population cultured under the same oxygen tension (Fig. 3). In addition, the concentration of VEGF, EGF, PDGF-BB, NGF-β, SCF, TNF-α, bFGF, and TGF-β increased under hypoxic conditions compared with normoxic conditions, particularly in the Muse-rich population.

Comparative Gene Expression Profiles of Muse-Rich and Muse-Poor Cell Populations

Microarray analyses were performed to analyze differences in gene expression between the Muse-rich and Muse-poor populations (n = 1). Gene ontology analyses of the genes differentially expressed between the Muse-rich and Muse-poor populations indicated several characteristic ontologies. For example, "blood vessel morphogenesis" genes were upregulated in Muse-rich cells and "mitotic cell cycle" genes were upregulated in Muse-poor cells (supplemental online Fig. 1). We found that Muse-rich cells had upregulated expression of pluripotent markers, including NANOG and Sox2 (Fig. 4), as described previously [6]. In addition, the Muse-rich population highly expressed growth factors/cytokines such as SDF-1, PDGF-A, EGF, and VEGF-A. All microarray data obtained from our gene expression analyses were deposited with the National Center for Biotechnology Information Gene Expression Omnibus database (accession no. GSE55526).

Induction of DM in SCID Mice by STZ Injection

STZ damages the pancreatic β cells and induces type 1 DM; however, the dose and method of STZ administration have differed among previous reports [15–17]. When we administered 200 mg/kg STZ, the SCID mice frequently died of severe weight loss and metabolic abnormalities within 1 week of administration. However, injection of 150 mg/kg STZ into SCID mice after 24 hours of fasting successfully induced hyperglycemia with relative consistency, and the DM status (>300 mg/dl blood glucose) was maintained for longer than 30 days (Fig. 1B). DM-induced SCID mice (DM-SCID), which were successfully prepared using one (9 of 29 mice; 31.0%) or two (13 of 29 mice; 44.8%) STZ injections, were used in wound healing experiments 30 days after the final STZ injection.

Would Healing in DM-SCID Mice With or Without Cell Treatment

Compared with wild-type (WT) mice (n = 6) or non-DM-SCID mice (n = 6), wound healing was significantly delayed in the DM-SCID mice (Fig. 5). WT and non-DM-SCID mice showed wound sizes of 56.9% ± 12.0% and 67.5% ± 6.5% on day 7, respectively. In contrast, DM-SCID mice (n = 6) showed wound sizes of 95.4% ± 3.1% (WT vs. DM-SCID; $p < .0001$). The DM-SCID mice also presented with larger wound sizes on day 14 compared with the WT or non-DM-SCID mice. Thus, the DM-SCID mice were confirmed to be an immunodeficient animal model with impaired wound healing.

The skin wounds in the DM-SCID mice were treated with a subcutaneous injection of either Muse-rich or Muse-poor cells. Muse-rich cell-treated DM-SCID mice (n = 6) showed significantly better wound healing with a statistically significant difference compared with DM-SCID mice treated with Muse-poor cells (n = 6), although the treatment using Muse-poor cells significantly accelerated wound healing compared with that of the DM-SCID mice with no treatment (Fig. 5). The wound size on day 7 was 51.05% ± 7.2% and 74.0% ± 6.6% in the DM-SCID mice treated with Muse-rich and Muse-poor cells, respectively ($p < .0001$). The wounds had healed completely in the DM-SCID mice with Muse-rich treatment by day 14, although the wound size in the DM-SCID mice with Muse-poor treatment was 30.3% ± 6.7% (p = .0235). The wound healing in the DM-SCID mice with Muse-rich treatment on day 14 was even significantly better than that in the WT group. The effect of the vehicle (hyaluronic acid) used in the cell treatments was also evaluated and showed no significant promoting effects of the vehicle (supplemental online Fig. 2).

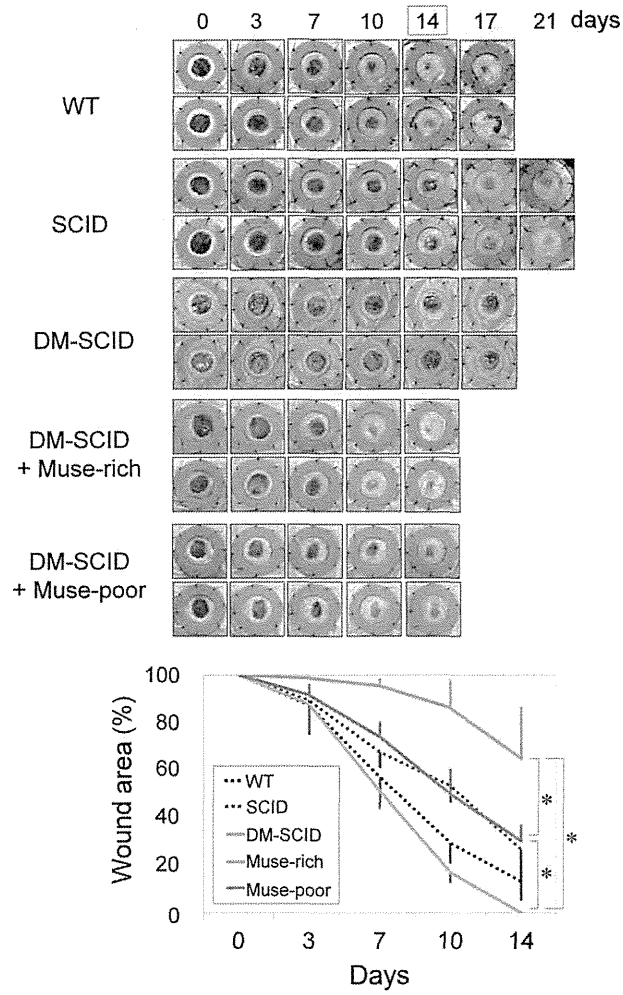

Figure 5. Wound healing of skin ulcers treated with Muse-rich and Muse-poor cell populations. Wound healing of skin defects (6 mm in diameter) was sequentially evaluated for up to 14 days. The wound area was photographed, and the percentage of the wound area to the original wound size was calculated using digital imaging software. Although SCID mice originally showed slightly delayed wound healing compared with their WT counterparts, the wound healing in the STZ-induced DM-SCID mice was more impaired than that in the SCID mice. Wound closure was significantly faster in the DM-SCID mice treated with the Muse-rich cell population than in Muse-poor-treated DM-SCID mice, which showed significantly better wound healing than the nontreated DM-SCID mice. Six mice were used in each group. *, $p < .05$. Abbreviations: DM-SCID, diabetes mellitus-induced SCID mice; Muse, multilineage-differentiating stress-enduring; SCID, severe combined immunodeficiency; STZ, streptozotocin; WT, wild-type.

Immunohistological Tracing of Transplanted Human Cells

The immunohistologic findings for the human Golgi complex suggested that transplanted human cells were detected in the dermis of the wounded area on day 14 in both Muse-rich and Muse-poor samples. However, the human Golgi complex-positive cells were not observed in the surrounding intact area (Fig. 6). Human Golgi

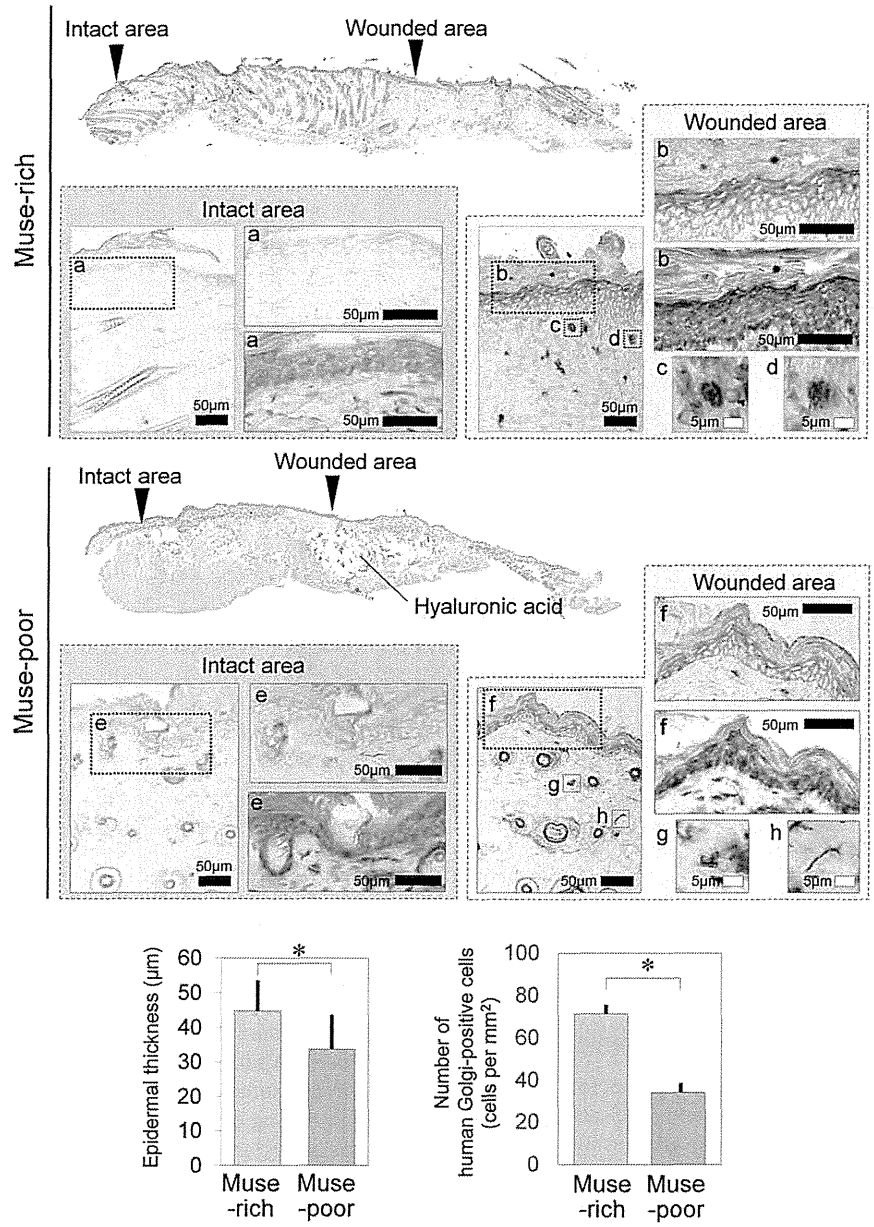

Figure 6. Immunohistologic findings for human Golgi complex of diabetes mellitus-induced severe combined immunodeficiency wounds treated with Muse-rich or Muse-poor cell populations. Shown are high-magnification views of the intact epidermis (a), repaired epidermis (b), and wounded area (c, d) in Muse-rich sample. Also shown are high-magnification views of the intact epidermis (e), repaired epidermis area (f), and wounded area (g, h) in Muse-poor sample. Human Golgi complex-positive cells, which are equivalent to transplanted human cells, were observed in the epidermis and dermis of the wounded area in both Muse-rich and Muse-poor samples after 14 days. However, human Golgi complex-positive cells were not detected in the surrounding intact area in either group. Transplanted human cells were significantly more frequently detected in Muse-rich samples compared with Muse-poor samples ($p = .0006$). A significantly thicker epidermis was also noted in Muse-rich samples ($p = .0053$). *, $p < .05$. Abbreviation: Muse, multilineage-differentiating stress-enduring.

complex-positive cells were detected in the Muse-rich samples at significantly larger numbers compared with the Muse-poor samples (Muse-rich, 71.4 ± 4.6 cells per mm^2; Muse-poor, 34.2 ± 4.6 cells per mm^2; $p = .0006$). A significantly thicker epidermis was also noted in the Muse-rich samples ($p = .0053$). Injected hyaluronic acid deposits were generally recognized in the subcutaneous layer. The cells positive for human Golgi complex were also positive for PECAM-1 (Muse-rich, 186.1 ± 9.8 cells per mm^2; Muse-poor, 156.7 ± 13.9 cells per mm^2; $p = .144$). In contrast, many Golgi complex-positive cells in the middle to lower dermis were not positive for human PECAM-1 or isolectin in the Muse-rich samples at day 14 (Fig. 7). The number of PECAM-1-positive vascular endothelial cells was comparable between the Muse-rich and Muse-poor samples (Muse-rich, 186.1 ± 9.8 cells per mm^2; Muse-poor, 156.7 ± 13.9 cells per mm^2; $p = .144$), although the ratio of human-derived cells in the PECAM-1-positive cells was higher in the Muse-rich samples (Muse-rich, 22.8% ± 3.2%; Muse-poor, 12.5% ± 1.1%; $p = .02$). These data suggest that transplanted Muse cells survived in the dermis and have differentiated into vascular endothelial cells or other cell types.

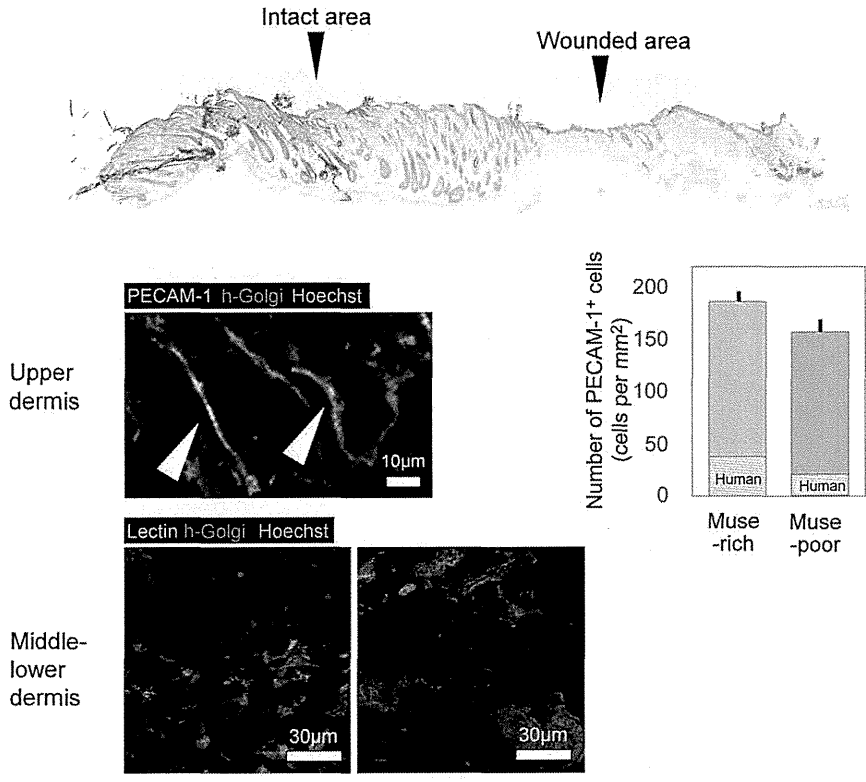

Figure 7. Immunohistologic findings of differentiation markers expressed by transplanted Muse-rich cells. Double immunohistochemistry was performed for human-specific proteins (human Golgi complex) and differentiation markers (PECAM-1 or isolectin) to characterize transplanted Muse-rich cells at day 14. Some cells expressing human Golgi complex were positive for PECAM-1 or isolectin, suggesting differentiation into vascular endothelial cells in the upper dermis; however, human Golgi complex-positive cells in the middle and lower dermis were negative for PECAM-1 and isolectin. The number of PECAM-1^+ cells per microscopic field was counted, with no significant difference between the two groups ($p = .144$), although the ratio of human-derived cells was higher in the Muse-rich samples ($p = .02$). Abbreviations: h-Golgi, human Golgi complex; Muse, multilineage-differentiating stress-enduring; PECAM-1, platelet endothelial cell adhesion molecule-1.

Discussion

In the present study, we used a single pluripotent stem cell marker, SSEA-3, for Muse cell isolation and purification. Previous reports have investigated Muse cells derived from human adipose tissue (previously termed "adipose Muse" [10], "Muse-AT" [9], or, in our study, Muse ASCs), which were shown to also be positive for CD105 and to differentiate into cells representative of the three germ layers from a single cell [10]. In addition, Muse cells could be efficiently isolated as cells single positive for SSEA-3 in human dermal fibroblasts, because nearly all dermal fibroblasts were positive for CD105 [6, 12]. Although fluorescence activated cell sorting (FACS) or stress conditions were used in previous studies [6, 9, 12, 18, 19], we used autoMACS, with double collection of the positive fraction after preliminary optimization experiments. MACS purification of Muse cells was not perfect (less than 90%). However, MACS appears to be the most practical method to purify Muse cells, because it is a clinically approved cell separation method and enabled higher Muse purification than other isolations using FACS or stress application. Although Muse cells were initially identified as stress-tolerant cells [6], selection using SSEA-3 collected the same Muse population [10, 12]. Because it remains challenging to efficiently expand the number of Muse cells, we used regular adherent cell culture with hundreds of culture dishes to achieve sufficient numbers of Muse cells for the animal experiments.

We confirmed the superiority of Muse cells compared with non-Muse MSCs (i.e., nearly equal to general ASCs in the present study) in promoting wound healing in diabetic mice, which have impaired healing of skin defects. Ulcers treated with Muse-rich cells healed faster with a thicker epidermis than those treated with Muse-poor cells, with the duration of wound closure even shorter than that in WT mice. In order to test the therapeutic value of human Muse cells for diabetic ulcers, we prepared a diabetic immunodeficient mice model by inducing DM in SCID mice using STZ injections. This mouse model showed impaired wound healing. Our protocol of STZ injection induced DM in SCID mice with a consistent success rate of approximately 75%. Hyperglycemia was well maintained, even 1 year after induction (data not shown). Although systemic administration procedures (e.g., intravenous injection) of Muse cells were reported previously [6, 8], we used local injection of Muse cells with hyaluronic acid as the carrier to treat localized skin wounds to avoid unfavorable cell migration and achieve better concentrated treatment effects. The injections were histologically confirmed and localized in the subcutis around the wound. Hyaluronic acid has been used as a preferable carrier or scaffold, although the appropriate state (cross-linked or non-cross-linked) and concentration are controversial and should be optimized in future studies [20–22].

The mechanism of the wound healing-promoting effects of Muse cells remains to be elucidated. Although Muse-poor ASCs also promoted wound healing in DM-SCID mice, the Muse cells

in the ASC populations showed a higher power to accelerate tissue repair, suggesting that SSEA-3$^+$ Muse ASCs are a selected ASC population with distinct therapeutic potential. Our histological assays showed that Muse-rich ASCs were integrated into the repaired dermis. Some of the injected cells were detected as vascular endothelial cells and as other cells in the upper and lower dermis, respectively. Although the proliferative capacity of Muse cells is not that great, they were previously mentioned as pluripotent stem cells but are nontumorigenic and might be a potent tool for clinical use [6, 9, 10, 18], which was partly supported our findings.

In addition, ASCs have been reported to home to damaged regions and secrete the growth factors required for the inflammatory and proliferating phases of wound healing, such as PDGF, bFGF, TGF-β, and EGF [23]. Microarray assays in our study showed that the expression of growth factors, including PDGF-A, EGF, and SDF-1, was upregulated in Muse-rich cells cultured under hyperoxic conditions (20% O_2), although a limitation existed in that the array used only one sample from each group. In addition, upregulation of pluripotent markers, such as NANOG, suggest a high differentiation potential for Muse cells. Our ELISA results also indicated that Muse cells secrete several growth factors, including PDGF-BB, TGF-β, bFGF, and TNF-α, in larger amounts than in non-Muse cells, particularly under hypoxic (1% O_2) conditions compared with normoxic (6% O_2) conditions. PDGF-BB, TGF-β, and bFGF are known to be involved in the initial coagulating phase of wound healing to promote a series of subsequent events [24]. In contrast, TNF-α is released during the acute inflammatory phase and triggers the inflammatory cascade [25, 26]. TNF-α null mice showed delayed epithelialization in dorsal full-thickness skin defects, suggesting that TNF-α is essential for wound healing [27]. These data collectively suggest that Muse cells might function better under stressful conditions (e.g., hypoxia) and coordinate cellular events in the wound healing process by releasing soluble factors. The diabetic skin ulcers used in the present study are generally refractory to healing, with ischemia and chronic inflammation present, and appear to be a reasonable therapeutic target of Muse cells.

Conclusion

In the present study, a selected population of ASCs, namely Muse cells, was shown to have greater therapeutic effects in accelerating the impaired wound healing associated with type 1 DM. On the basis of the suspected mechanisms, our data further suggest their clinical potential in a variety of stem cell-depleted or ischemic conditions of any organ or tissue. Adipose tissue has been gaining more attention as a practical source of adult stem cells. The therapeutic potentials of ASCs were suggested for treating various DM conditions, such as hyperglycemia [28] and autoimmune DM [29]. ASCs were shown to share most biological characteristics, and to have comparable functions, with bone marrow-derived MSCs. Recent studies have suggested that ASCs (and also other tissue-resident MSCs) can be supplied from the bone marrow as tissue-localized stem/progenitor cells [30, 31]. Bone marrow is the central factory and bank of stem/progenitor cells, which are mobilized and delivered on demand by peripheral organs. These data suggest that invasive damage to the bone marrow while harvesting bone marrow-derived cells might have been underestimated. ASCs can be obtained in much larger quantities using minimally invasive approaches that will not damage the bone marrow, suggesting a clinical potential for Muse ASCs in the future.

Acknowledgments

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Author Contributions

K. Kinoshita: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; S.K., H.I., and N.A.: collection and/or assembly of data, data analysis and interpretation; K.M., H.K., K.D., K. Kanayama, J.F., and T.M.: collection and/or assembly of data; A.K.: data analysis and interpretation; K.Y.: conception and design, data analysis and interpretation, financial support, administrative support, final approval of manuscript, manuscript writing.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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Therapeutic Potential of Adipose-Derived SSEA-3-Positive Muse Cells for Treating Diabetic Skin Ulcers

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Complications of Fat Grafting

How They Occur and How to Find, Avoid, and Treat Them

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KEYWORDS

• Fat grafting • Adipose-derived stem/stromal cell • Tissue necrosis • Oil cyst • Calcification
• Blindness • Infection

KEY POINTS

- Blindness and stroke have occurred as a result of arterial injection of fat tissue in almost every part of the face. The injection of large boluses and the use of sharp needles/cannulas should be avoided in the face.
- Most of the common complications such as no/minimal graft retention, infection, oil cysts, and calcifications are related to necrosis of grafted fat. To minimize fat necrosis, surgeons should optimize each step from liposuction to lipoinjection. Injection as small aliquots/noodles of fat (preferably 2 mm in diameter) is particularly important.
- Although fat grafting is a minimally invasive surgical procedure, surgeons have to be cautious to avoid any unexpected damage to the donor and recipient sites to minimize the perioperative risk and complications.

INTRODUCTION

Recent technical and scientific advances in fat grafting procedures and concepts have improved predictability of fat grafting. Large-volume fat injection is gaining much attention as an attracting procedure for body contouring and reconstruction, but an increasing number of complications also has been recognized over the world.[1] In this article, typical complications after fat grafting are described, as well as an explanation of how and why they occur, and how surgeons can avoid and treat complications.

COMPLICATIONS AFTER FAT GRAFTING PROCEDURES

Most of the common complications are related to necrosis of grafted fat, which can be minimized by technical improvements, but there are rare but catastrophic complications such as blindness and stroke.[2] Typical complications and possible complaints by patients will be discussed.

Embolization: Blindness, Strokes, and Skin/Tissue Necrosis

Probably the most devastating potential complication of fat grafting is embolization after intravascular injection. Blindness from a fat injection was first reported in 1988.[3] Few details were given, but the basic presentation was identical to the later reports. The patient experienced excruciating pain accompanied by immediate and permanent loss of vision in 1 eye. There have been reports of permanent unilateral blindness from central retinal artery occlusion by fat grafting, frequently

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accompanying stroke and skin necrosis. Although most instances of central retinal artery occlusion and blindness resulted from fat injection in the nose or periorbital region,[3–5] some were reported with fat injection into the nasolabial folds[6] or even the lower lip.[7]

Obviously, artertial embolization can also affect the mucosa, conjunctiva, or skin and result in necrosis. There has been a report of blindness, stroke, and skin necrosis from the injection of only 0.5 mL of filler into the left side of the nasal bridge.[8] Even a small amount injected into the lower face has been reported as having devastating complications; unilateral blindness and brain infarction occurred after the injection of only 0.5 mL into a nasolabial fold.[6]

How it occurs?

The retinal artery and posterior ciliary arteries are proximal branches of the ophthalmic internal carotid arteries. If the opening to the needle is in the lumen of an artery, the filler will be injected into the lumen of the cannulated artery. As more pressure is applied to the plunger, the filler displaces the arterial blood and travels as a column proximally past the origin of the retinal artery. A tiny amount of the filler slipping into the retinal artery can precipitate a central retinal artery blockage, usually resulting in permanent blindness. It is also possible to force the column back into the internal carotid artery and embolize into any area supplied by the internal carotid area, and this may result in a stroke.

How to avoid?

To avoid such complications, do not use sharp needles. Additionally, one should limit bolus size, limit syringe size (only 1 mL syringe to the face), and avoid using ratcheting guns. Small and sharp needles/cannulas are much more likely to perforate the wall of an artery and cannulate the artery lumen than are the larger, blunter instruments. Therefore, extreme caution should be taken when sharp needles of any type are used to inject particulate matter into the face.

The volume placed with each pass of the cannula should also be limited. Infiltration of less than 0.1 mL with each pass of the cannula is recommended in the face. To be especially safe, aliquots of less than 0.033 or 0.02 mL are preferable in the periorbital region. Additionally, a vasoconstricted artery is harder to cannulate than a vasodilated one, so epinephrine should be considered for use at the injection site for the placement of fillers. When using a larger syringe (10 or 20 mL) for infiltration of the fat, the surgeon's control over the volume injected is less than with the smaller syringe, so it is easier to mistakenly inject a larger amount or to inject with a high pressure. Thus, it is strongly recommended to use only 1 mL Luer-Lok syringes for subcutaneous infiltration into the face. Blindness has also occurred following soft tissue injections with assistive mechanical devices that may create strong pressures during the injection of soft tissues.

Fat Necrosis: Calcifications and Oil Cysts

Necrosis of grafted fat tissue induces cicatrization, calcifications, and oil cysts if the necrosis size is substantial. Although a single dead adipocyte (50–150 μ) can be completely absorbed, significant numbers of oil drops are replaced with collagen matrix (cicatrization).[9] If the cicatrization has a central tiny oil drop (<1 mm), chronic inflammation persists and a sand-like macrocalcification (0.3–2 mm) can develop over the first 5 years. In the event that the fat necrosis is large in size (>10 mm), the necrotic tissue becomes an oil cyst within 6 to 12 months after grafting, which presents never-ending inflammation. Oil cysts are permanently problematic; they neither become silent, nor reduced in size.[1]

Oil cysts can occur after roughly performed fat injection and are more likely to be seen after large-volume fat grafting such as the breast and buttock. It should be noted that the well-projected breast and buttock with a tight skin envelop are uncommon outcomes after fat grafting (common after synthetic implant placement) and can result from oil cyst formation (**Fig. 1**).

How it occurs?

Dead adipocytes become oil droplets and are first surrounded by infiltrated M1-type (inflammatory) macrophages for phagocytosis.[9] The absorption of oil is a very slow process; it takes weeks for a 1 mm oil droplet to be completely absorbed. At a later stage, stratified layers of M2-type (anti-inflammatory) macrophages surround the M1 macrophages and form a fibrous cyst wall. The formation of cyst wall stops the oil absorption process, and the size of oil cysts will not change any later than 1 year after surgery, although the calcification process of the cyst wall continues to progress over several years due to the persisting inflammation.[1]

How to avoid?

Recent findings on the mechanism of fat graft survival and regeneration suggested that fat particles with a more than 2 to 3 mm diameter cannot be engrafted at 100%. Fat necrosis after grafting largely depends on the injection technique/volume and microenvironments of the recipient site. If one

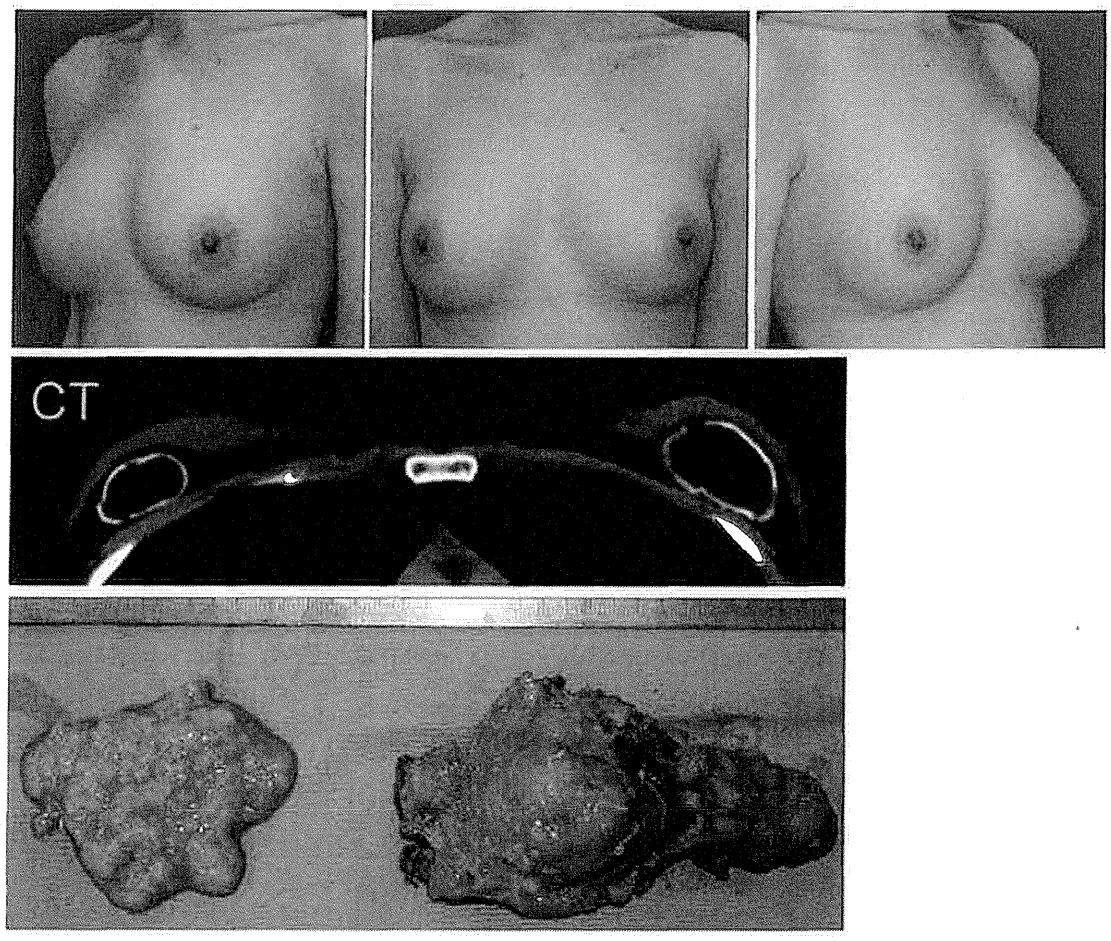


Fig. 1. A case of oil cysts. A 24-year-old woman underwent fat grafting for cosmetic breast augmentation 2 years earlier. She recognized hardness of the entire breast at 6 months and gradually recognized tenderness and abnormal sensations. (*Top*) She had well-projected breasts with tight skin. The contour of the upper pole looked similar to a breast with implant contracture. (*Middle*) Preoperative computed tomography (CT) scan showed that there was a single large calcified oil cyst under each mammary gland. It was suspected that 100 to 200 mL of fat tissue had been introduced in a bolus injection before. (*Bottom*) Removed oil cysts were filled with muddy content caused by fat necrosis. The oil cyst wall had innermost and outermost fibrous layers. (*Adapted from* Mineda K, Kuno S, Kato H, et al. Chronic inflammation and progressive calcification as a result of fat necrosis: the worst outcome in fat grafting. Plast Reconstr Surg 2014;133:1064–72; with permission.)

wants to make a fat column (noodle) with 2 or 3 mm diameter, 1 mL fat has theoretically to be a noodle of 32 or 14 cm long, respectively. Such a careful and meticulous injection technique is critical to avoid a large fat necrosis forming an oil cyst. One needs to use smaller injection syringes such as 1 mL syringe for the face and 3 mL syringe for the body. Special injection syringes/devices may be helpful if they facilitate a controllable precise infiltration of fat tissue.

How to treat?

Once an oil cyst is established, never-ending inflammation and progressive calcification of the cystic wall can induce symptoms such as hard lumps, tenderness/pain, abnormal/heating sensation, contracture, projected breast with tight skin envelop, and/or general fatigue. It is not easy to treat the oil cyst and its associated symptoms without surgical resection. Another option is to partially cut the cystic wall with an 14–18G needle and squeeze it, leading to leakage and phagocytosis of oil or necrotic tissue.

Calcifications in mammogram

Small fat necrosis induces a fibrous deposit, which later develops into a sand-like micro/macro-calcification over years (**Fig. 2**), whereas large fat necrosis induces an oil cyst, of which fibrous wall will be calcified over time and present an

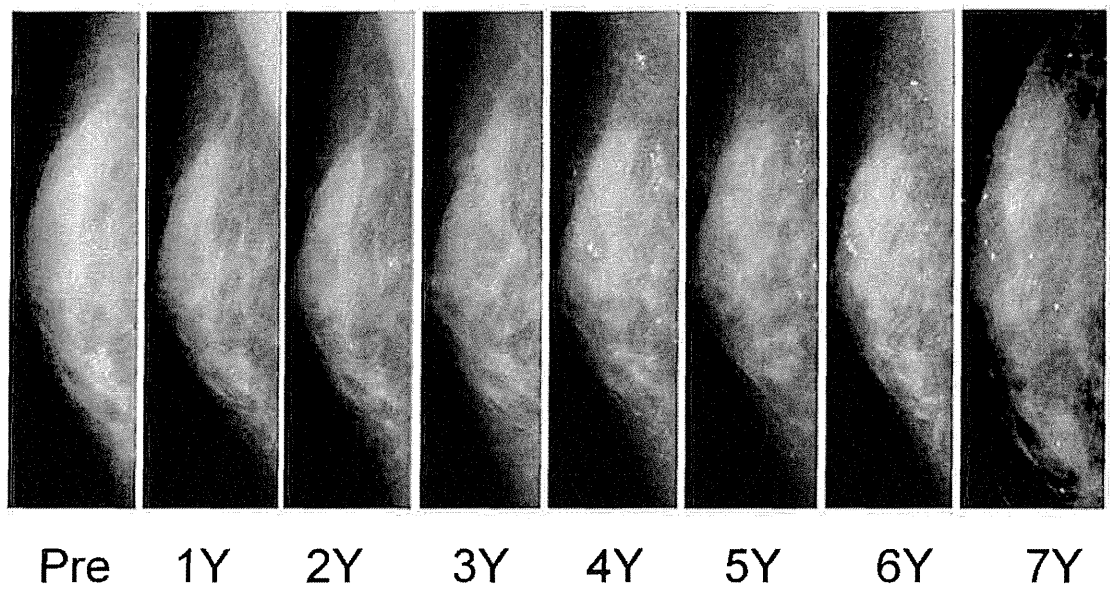


Fig. 2. Sequential mammography images of a case with fat grafting to the breast. Mammograms were taken sequentially from a patient (30-year-old woman) who underwent fat grafting to the breast with no postoperative lumps. Calcification was not apparent at 1 year, but sand-like macrocalcifications were clearly detected at 2 years and progressed over time, indicating that calcifications grow progressively at least up to several years even with no noticeable lump. It is suspected that small necrotic areas become sand-like calcifications, while larger necrotic areas becomes oil cysts with eggshell-like calcification. (*Adapted from* Mineda K, Kuno S, Kato H, et al. Chronic inflammation and progressive calcification as a result of fat necrosis: the worst outcome in fat grafting. Plast Reconstr Surg 2014;133:1064–72; with permission.)

egg-shell-like calcification (**Fig. 3**). The former-type calcification usually shows no clinical symptoms, though the latter can be troublesome. When there are numerous number of sand-like and/or egg shell-like calcifications detected in the mammogram, they interfere with detailed evaluation of mammographic images and precise diagnosis of breast cancer. Visual features of each calcification induced by fat necrosis are generally distinct and can be differentiated by professional diagnostic radiologists from those of microcalcifications by breast cancer, and therefore, it is not a problem that there are several typical sand-like macrocalcifications seen in the mammogram. Plastic surgeons should be careful not to inject fat into the mammary gland and to avoid any bolus injections. Ultrasound assessment at 1 month after lipoinjection is particularly valuable for a surgeon to detect even tiny oil drops and learn how well the injection procedure was performed.

Other Complications

Infection

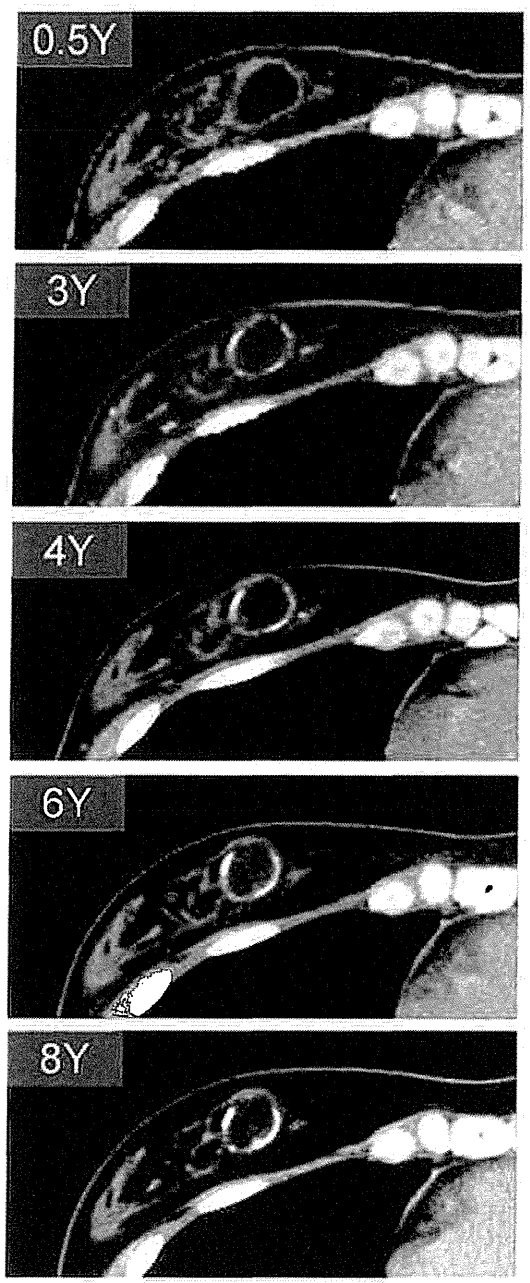
Although infection is uncommon with fat grafting, infection following fat necrosis or hematoma can occur. As the grafted fat is not vascularized, it can be a focus of infection once severely contaminated by bacteria. Sterile technique should be observed at all times. Intraoperative antibiotics are recommended to use, but perioperative use of antibiotics is not recommended unless there is a specific indication. In cases of delayed infection, a high index of suspicion should be maintained for mycobacterial or other unusual infections.

Damage to underlying structures

Even a blunt cannula, when inserted for removal and placement of fat, can damage underlying structures such as nerves, muscles, glands, and blood vessels using this technique; however, permanent injuries are extremely rare.

Pneumonia

In the learning curve of fat injection to the breast, it is rare but possible to induce pneumonia. Pneumonia is induced by damaging the pleura with an injection cannula/needle. Therefore, great care should be taken to avoid when introducing fat into the bottom layer close to the rib. Pneumonia is usually first recognized as a complaint of chest pain in the next morning and can be diagnosed by monitor of oxygen saturation, chest X-ray, and/or CT scan. If pneumonia is a minor one, it can be treated by a conservative treatment such as waiting with careful monitor of X-ray and symptoms.



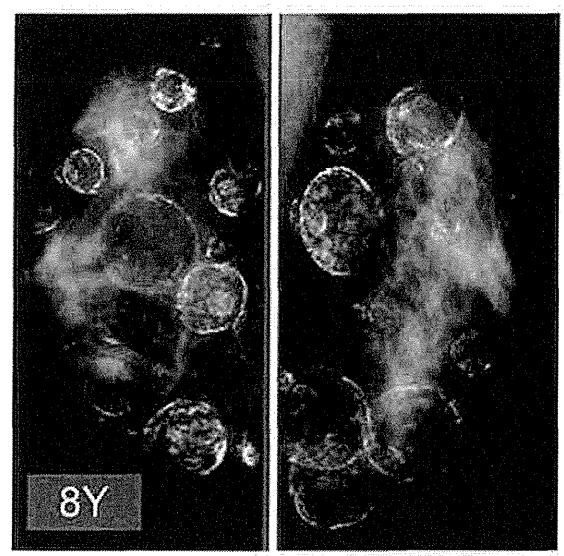


Fig. 3. Sequential CT images and mammography of oil cysts. (*Left*) CT was sequentially taken from a patient (34-year-old woman) with multiple oil cysts in both breasts who did not undergo any removal of the cysts. The size of oil cyst did not change between 3 and 8 years, but calcifications progressed during the period. (*Right*) Mammography of the same patient at 8 years. Many eggshell-like calcifications are shown. (*Modified from* Mineda K, Kuno S, Kato H, et al. Chronic inflammation and progressive calcification as a result of fat necrosis: the worst outcome in fat grafting. Plast Reconstr Surg 2014;133:1064–72.)

Aesthetic problems and complications

One of the most common complications after fat grafting is related to aesthetics, such as the placement of too much or too little fat in a specified area. The presence of irregularities, which can be caused by the intrinsic nature of the patient's body, from the technique used for placement, and from migration after placement, is also noted. Irregularities after fat grafting diminish significantly as the surgeon gains experience with the technique. In addition, the overgrowth of fat grafts can occur with weight gain. More perplexing, fat grafts can grow in people with stable weight. Such overgrowth, not related to weight gain, is most often seen in reaction to medications but can occur without apparent cause.

Swelling and downtime

One of the most difficult tasks for the surgeon is preparing patients to expect the bruising and swelling created by this technique. The placement of fatty tissue may create remarkable swelling in

the recipient tissues. This depends on a number of factors, including the amount of fat placed, the anatomic location to which it is grafted, the specific technique and instruments used, medications the patient takes, and the age and genetic makeup of the patient. Patient care after fat transplantation is directed at minimizing swelling and avoiding migration. Elevation, cold therapy, and external pressure with elastic tape or Tegaderm (3M, Maplewood, MN, USA) help prevent swelling. Certain medications (*Arnica montana* and bromelain) may also speed recovery. The patient is asked to avoid heavy pressure on the grafted areas for 7 to 10 days to avoid migration of the grafted fat.

Swelling can be especially bothersome and prolonged in the periorbital region, particularly the lower eyelid. Any pigmentation in the lower lid will appear darker with bruising, which can last even in a minimal form for many weeks. A slight staining of the skin, possibly hemosiderin deposits or other pigment changes, can remain for months in some patients after minimal fat grafting to the lower eyelid.

Donor site problems

Finally, many patients find the removal of fat and the body contouring performed at the same time to be advantageous, yet even a surgeon who is facile at liposuction may produce liposuction deformities. Furthermore, some patients simply do not have adequate donor sites, especially if they have previously undergone liposuction. Complications of the donor sites are rare, but irregularity of the surface could occur, particularly when an excessive volume liposuction is performed in very thin patients.

SUMMARY

Accidental injection of soft tissue fillers into the arterial system can result in catastrophic complications. Blindness and stroke have occurred as a result of the injection of soft tissue fillers in almost every part of the face. During injection of any soft tissue filler in the face, consideration should be given to the possibility of cannulation of arteries and to the volume of filler injected at any instant. The injection of large boluses of soft tissue fillers in the face and the use of sharp needles or cannulas that can easily perforate an arterial wall should be avoided. Another major complication after fat grafting is oil cyst formation and calcifications. Oil cysts show never-ending inflammation and progressive calcification in the cyst wall, which can induce many types of clinical complaints including unmanageable infection and pain. Oil cysts are a typical result of roughly done fat injection, and surgeons should avoid any bolus injection and introduce noodle-like columns of fat, which should be as small as 2 to 3 mm in diameter. Although fat grafting is a minimally invasive surgical procedure, surgeon have to be cautious to avoid any unexpected damage to the donor and recipient sites to minimize the perioperative risk and complications.

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Future Perspectives of Fat Grafting

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KEYWORDS

• Fat grafting • Soft-tissue augmentation • Soft-tissue reconstruction • Regenerative surgery
• Fat grafting research

KEY POINTS

- Future perspectives of fat grafting from 3 editors are summarized in this review. Fat grafting will continue to play an important role in cosmetic and reconstructive surgery.
- Fat grafting can be a good option to replace some of the "traditional" procedures in cosmetic and reconstructive surgery.
- Fat grafting may become a regenerative procedure that can be used to treat varieties of difficult clinical problems that have not been solved at the present time. More definitive studies are still needed in order to answer any specific questions related to fat grafting including the best technique and the role of ADSCs.

INTRODUCTION

Autologous fat grafting has become a popular procedure in both cosmetic and reconstructive plastic surgery. It has been a "hot topic" in almost all major plastic surgery meetings lately and many advances in fat grafting have been made in this exciting field of plastic surgery. Modern fat grafting started as a means for facial rejuvenation and correction for soft tissue counter deformity in the mid 1990s and championed by Coleman. It had a "bad reputation" for years, especially in the United States, as the procedure with unachievable or unpredictable outcome and uncertain safety.[1] However, as we learn more and more about fat grafting and its potential,[2] many reputable plastic surgeons are able to achieve good to excellent results with the procedure and to expand its role in many other areas of plastic surgery, including cosmetic and reconstructive surgery of the breast.[3] In this last article, 3 editors put together their perspective views on future autologous fat grafting.

FACIAL REJUVENATION

Autologous fat grafting will continue to play an important role in facial rejuvenation. As a matter of fact, it will, as a relatively less invasive surgical procedure, gradually replace many open approaches to early or even moderately facial aging.[4] It will also expand its role in combination with a traditional face lift surgery especially for correction of facial aging in the central portion of the face such as in the lid/cheek junction, tear trough, nasolabial fold, or perioral region because these areas are typically not corrected by a traditional face lift; fat grafting will likely achieve permanent improvement when comparing with a synthetic filler such as hyaluronic acid for the same purpose. Because of the unique regenerative potential of fat, presumably because of the potential effect of adipose derived stem cells, it has a unique feature not only to correct soft tissue deficiency but also to rejuvenate the skin of the face.[5] It is quite likely that fat grafting will play a more important role in facial rejuvenation and only stromal vascular fraction (SVF) other than fat will be injected for facial

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rejuvenation because of the regenerative potential of adipose derived stem cells within SVF. As surgeons gain more clinical experience and follow their patients for longer term, better results can be achieved by fat grafting in combination with a traditional open procedure for facial rejuvenation than that by an open face lift surgery alone.[6] It can also be true that fat grafting will replace some of traditional rhinoplasty procedures because it could improve the contour of the nose.[7]

CRANIOFACIAL DEFORMITY

Fat grafting is also going to play an increased role for correction of craniofacial deformities secondary to congenital deformity or traumatic injury. Fat grafting would correct soft tissue deformity and its preliminary results have been amazing.[8] It can also be an useful alternative to microvascular free tissue transfer to the face with a significant soft tissue deformity.[9] Fat grafting in combination with a traditional bony craniofacial reconstruction will provide the patient with much better clinical outcome than a traditional craniofacial approach with primary emphasis on bony reconstruction. In addition, the regenerative potential of adipose tissue cannot be replaced by any traditional craniofacial surgical approach in the head and neck region.[10] The regenerative nature of fat has been applied innovatively by our ENT colleagues to treat various vocal cord pathologies with good success and minimal or no serious complications.[11] Fat grafting as primary or adjunct procedure will be widely performed for management of many craniofacial pathologies.

COSMETIC AND RECONSTRUCTIVE BREAST SURGERY

The role of fat grafting in cosmetic and reconstructive breast surgery will continue to evolve. It will continue to become a widely used adjunctive procedure to improve the clinical outcome of both cosmetic and reconstructive breast surgery. Fat grafting for primary or secondary breast augmentation will be a common procedure of choice for both patients and plastic surgeons. Fat grafting will become a valid option for correction of lumpectomy defect for treatment of early breast cancer. In addition, fat grafting for total breast reconstruction after mastectomy may become a clinical reality and achieve the clinical outcome and patient satisfaction that cannot be achieved with an implant or flap surgery. The future of fat grafting in cosmetic and reconstructive breast surgery can be further classified into 4 major areas: breast augmentation, breast enhancement, correction of breast asymmetry and congenital deformity, and breast reconstruction.

Breast Augmentation

Although fat grafting for primary breast augmentation had a "bad reputation" in the past, the procedure itself has gained more popularity recently and is being performed more and more by plastic surgeons worldwide for primary breast augmentation.[1,3] There are adequate studies in the literature to support the efficacy and safety of fat grafting for primary breast augmentation.[12–14] Although there is lack of standardized technique for fat grafting to the breast, plastic surgeons have improved their surgical technique of fat grafting for primary breast augmentation so that satisfactory results can be achieved in selective patients and there is no need for an implant-based breast augmentation.[15,16] More study needs to be conducted to further confirm the efficacy and safety of fat grafting as a primary means for breast augmentation. In addition, surgical techniques also need to be standardized to avoid complications and shorten the learning curve in fat grafting for primary breast augmentation.

Breast Enhancement

Fat grafting, as a valid option, will be used widely in conjunction with traditional mastopexy or implant exchange to achieve a better outcome in aesthetic breast surgery.[17,18] It provides another option to manage "soft tissue deficiency" for selected patients. In addition, the concept of composite breast augmentation with implant and fat grafting has been introduced, which may provide clinical outcome in breast augmentation for selected patients that cannot be achieved by either option alone.[19]

Correction of Breast Asymmetry and Congenital Deformity

Fat grafting as a popular procedure will probably replace most of the current techniques for correction of breast asymmetry.[20] This might be true for correction of some less significant breast asymmetries. Breast augmentation with implant, mastopexy, or breast reduction will continue to play a role for the correction of significant breast asymmetry. Fat grafting itself will become a primary option for correction of several breast congenital deformities, such as the Poland syndrome or tuberous breast. It will likely correct those congenital deformities without the need for a traditional breast implant or flap reconstruction.[21]

Breast Reconstruction

Fat grafting as a valid procedure will continue to be widely used in reconstructive breast surgery for a final touch up procedure after implant or flap reconstruction.[22] It has proven its role in correction

of soft tissue contour deformity related to implant-based breast reconstruction.[23] Multiple fat grafts followed by implant reconstruction has its unique value after total mastectomy with irradiation therapy because the quality of irradiated skin over the implant can be improved with fat grafting.[24,25] Fat grafting will become an effective and valid option for correction of lumpectomy or even partial mastectomy defect with radiation after conservative breast surgery for treatment of early breast cancer if such an approach can be proved to be safe with no issues on future breast cancer recurrence and detection. It can potentially revolutionize the surgical approach to what used to be an "uncorrectable" clinical condition in breast reconstructive surgery. With efficacy and safety of Brava continue to be firmed, mega volume autologous fat grafting may become a clinical reality for total breast reconstruction after mastectomy without radiation and replace the traditional approach to breast reconstruction with an implant or a surgical flap for breast reconstruction, although multiple sessions are needed.[26–28] However, a standardized technique for mega volume fat grafting needs to be established, just like an implant-based reconstruction or autologous flap-based breast reconstruction, so that the technique of mega volume fat grafting can be learned by most plastic surgeons to produce a consistent, if not better, clinical outcomes in total breast reconstruction for patients after mastectomy.

GLUTEAL AUGMENTATION

An implant-based gluteal augmentation has been adapted primarily by Brazilian plastic surgeons and an optimal outcome can be achieved with intramuscular placement of a gluteal implant.[29] Autologous fat grafting for gluteal augmentation has continued to evolve and recent experience from worldwide plastic surgeons has been good. Because fat grafting involves the harvest of fat from many unwanted areas in patients, fat grafting for gluteal augmentation will continue to be a popular and desirable approach to body contour surgery, requested by patients. With an improvement in standardized technique of fat grafting, fat grafting will play a more important role in gluteal augmentation and may replace implant-based gluteal augmentation if the patient has a great enough amount of fat as donor materials.[30,31]

HAND REJUVENATION

As hand rejuvenation becomes a demanding procedure for certain kind of patients, autologous fat grafting will continue to be safe and effective procedure for hand rejuvenation.[32] Because fat may not only serve as filler, but also has the regenerative potential to improve the quality of soft tissue and skin on the dorsal side of the hands, fat grafting can be an attractive procedure for hand rejuvenation and achieve better outcomes in the hand after fat grafting that cannot be accomplished by any other means, because synthetic fillers such as hyaluronic acid work poorly for hand rejuvenation and fat is definitively better filler to be used for such a clinical application.[4]

REGENERATIVE SURGERY

Autologous fat grafting has been used by some investigators to treat difficult wounds that have failed the conventional wound therapy.[33,34] Although the exact mechanism still remains unknown, most investigators believe the ability of fat grafting to heal the difficult wound would at least partially owing to the regenerative potential of adipose derived stem cells within the fat grafts.[33] If this concept can be confirmed in future studies, fat grafting will have an expanded role in reconstructive surgery to improve healing of difficult wounds and may replace some of the traditional surgical procedures, such as a flap reconstruction or skin grafting to facilitate wound closure. In addition, its regenerative effects can improve much functionality of the involved tissues such as elasticity, vascularity, pain release, reduced inflammation, and a lesser degree of immunoreaction. These effects, in general, cannot be achieved by any of "traditional" procedures in plastic surgery. This can be true when percutaneous aponeurotomy and lipofilling are performed to treat scar contracture as a regenerative alternative to a flap reconstruction.[35] This kind of new surgical approach is minimally invasive and "incisionless"; only needles, cannulas, and syringes are used for the procedure. It may revolutionize what we do now as reconstructive plastic surgeons because of the regenerative potential of fat grafting.

One of the great examples is that fat grafting can be performed after extensive percutaneous aponeurotomy for treatment of Dupuytren's contracture and fat grafts as lipofiller are placed between the skin and underlying structures of the finger and hand to lead to scarless supple skin and better functional outcome of the finger or hand.[36] It is possible that most patients with less severe Dupuytren's contracture can be treated with this new minimally invasive procedure once plastic surgeons have mastered their technique of percutaneous aponeurotomy and fat grafting.

Fat grafting may also be performed to the hand of patients with Raynaud phenomenon to reduce pain, cold attacks, and ulcerations, and to improve skin, soft tissue texture, and hand function. Several beneficial effects after fat grafting in this group of patients may be the contribution of adipose-derived stem cells, but the true mechanism of stem cells on ischemic tissue remains hypothetical.[37] However, fat grafting as a safe and well-recognized procedure provides a new alternative and potentially regenerative approach to this difficult clinical problem and may be used more often in the future.

Fat grafting will continually be performed to improve the appearance of scars, especially in the head and neck.[38,39] Once again, the observed effects of fat on scars are contributed mainly by adipose derived stem cells. Thus, fat grafting can be a new alternative for treatment of scars in selected patients with promising results and future potential.

Fat grafting, as an established procedure, has been performed for treatment of scar contracture induced pain in patients with "postmastectomy pain syndrome." The measurable improvements are partially attributed to scar remodeling through new collagen deposition, local hypervascularity, and dermal hyperplasia.[40] The regenerative role of fat in scarred areas is thought to be the result of releases of multiple nerve entrapments so that neuropathic pain is improved. In addition, the improvement in neurogenic pain may be maintained by placing fat grafts around the nerve to avoid the recurrence of scar contracture. Importantly, a well-conducted experimental study demonstrated that fat grafting can alleviate burn-induced neuropathic pain in rats.[41] If the efficacy and safety of fat grafting for treatment of neuropathic pain syndrome can be confirmed in future studies, this relatively simple approach will provide a new solution for this clinical condition, a pain syndrome that relates to various scar contracture or scar fibrosis in head and neck, breasts, or hands.

As the value of the SVF is recognized, SVF in combination with conventional fat grafting has been used clinically for improvement of the results in facial rejuvenation and primary breast augmentation with an approved success.[8,42] A level I clinical trial published in *Lancet* has clearly demonstrated the value of SVF to improve the survival of autologous fat grafting.[43] In addition, either noncultured or cultured SVF has been used by investigators to treat difficult wounds, arthritis, Crohn's disease with some amazing observed results. However, concerns about the use of "collagenase" during the isolation of SVF from lipoaspirates has been raised in the United States, which precludes the immediate clinical application of SVF-enriched fat grafting in patients. Nanofat grafting, as a possible alternative, has recently been described to obtain SVF-like cellular components without collagenase. It employs intense mechanical shearing to rupture adipocytes, leaving a nanofat sample that is rich in stem cell content. It has proven clinical efficacy in facial rejuvenation; however, the exact constituents and regenerative potential of this mix has yet to be defined.[44] In addition, SVF may be processed without the need of collagenase.[45] If the efficacy of this technique can be confirmed by future study, SVF-enriched fat grafting or SVF alone can be performed clinically for various conditions with potential better outcome.

RESEARCH IN FAT GRAFTING

It remains true that many questions need to be answered in fat grafting, for example, fat harvest, processing, and placement, preparation of the recipient site, and the role of stem cells.[46] We shall see more and more studies specifically designed to answer these questions related to fat grafting in the future because there are an increased number of publications in this exciting flied. We shall have better understanding of the mechanism how fat grafts survive; for example, the graft survival theory versus the host replacement theory or both.[47,48] Although we are just starting to realize the role of adipose-derived stem cells in autologous fat grafting, more definitive studies are needed to better elucidate the role of adipose derived stem cells in fat grafting. Expanding knowledge of adipose-derived stem cells would allow a more effective way to use SVFs for treatment of difficult clinical problems that have not been solved at the present time. In addition, cryopreservation of fat or adipose-derived stem cells may also explore possible future application of fat grafting because no additional harvest of fat grafts are needed and potentially cryopreserved SVF may provide younger adipose-derived stem cells from the same patient.[49]

STANDARDIZED TECHNIQUE IN FAT GRAFTING

Plastic surgeons will continue to refine their technique in fat grafting, but more important to standardize the technique for fat grafting probably based on the volume needed for their patients.[13] For example, small volume (<100 mL) fat grafting for facial rejuvenation or other regenerative approach, large volume (100–200 mL) for correction of significant contour deformities after breast