Aesth Plast Surg (2019) 43:498–513
https://doi.org/10.1007/s00266-018-01304-2

ORIGINAL ARTICLE BASIC SCIENCE/EXPERIMENTAL

Effects of Stromal Vascular Fraction on Breast Cancer Growth and Fat Engraftment in NOD/SCID Mice

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Received: 6 October 2018 / Accepted: 23 December 2018 / Published online: 11 January 2019

Abstract

Background To overcome unpredictable fat graft resorption, cell-assisted lipotransfer using stromal vascular fraction (SVF) has been introduced. However, its effect on cancer growth stimulation and its oncological safety are debatable. We investigated the effect of SVF on adjacent breast cancer and transplanted fat in a mouse model.

Methods A breast cancer xenograft model was constructed by injecting 2×10^6 MDA-MB-231-luc breast cancer cells into the right lower back of 40 NOD/SCID mice. Two weeks later, cancer size was sorted according to signal density using an in vivo optical imaging system, and 36 mice were included. Human fat was extracted from the abdomen, and SVFs were isolated using a component isolator. The mice were divided into four groups: A, controls; B, injected with 30 µl SVF; C, injected with 0.5 ml fat and 30 µl saline; group D, injected with 0.5 ml fat and 30 µl SVF. Magnetic resonance imaging and three-dimensional micro-computed tomography volumetric analysis were performed at 4 and 8 weeks.

Results Tumor volume was 43.6, 42.3, 48.7, and 42.4 mm^3 at the initial time point and 6780, 5940, 6080, and 5570 mm^3 at 8 weeks in groups A, B, C, and D, respectively. Fat graft survival volume after 8 weeks was 49.32% and 62.03% in groups C and D, respectively. At 2-month follow-up after fat grafting in the xenograft model, SVF injection showed an increased fat survival rate and did not increase the adjacent tumor growth significantly.

Conclusion Fat grafting with SVF yields satisfactory outcome in patients who undergo breast reconstruction surgery.

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Keywords Stromal vascular fraction · Breast cancer · Fat graft · Fat injection

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Introduction

As an autologous tissue, fat has no antigenicity or allergic reactions, and enough supply of fat is available. Moreover, the technique is convenient, and fat can be harvested repeatedly. Consequently, fat is considered the most ideal soft tissue filler. Accordingly, fat grafting has been successfully used for facial volume augmentation, rejuvenation of the lips and hands, and physique correction [1–5].

Fat grafting on women's breasts has a long history since it was first performed by Czerny [6]. However, due to the lack of studies on its safety, fat grafting has remained a

controversial topic even in recent times. The breasts require a relatively large amount of graft; hence, adverse effects such as microcalcification and cysts may occur, which can be mistaken for breast cancer on mammography, thereby interfering with early breast cancer detection. However, the engraftment rate has improved due to advances in surgical techniques for fat grafting. Moreover, a differential diagnosis between calcification and early breast cancer has become possible due to advances in radiological diagnostic methods, which has led to the interest being focused again on fat grafting for breasts in the 2000s. The recent trend is to use fat grafting not only for simple breast augmentation but also for correction of asymmetric breasts and supplementing soft tissues of the breasts [7].

Although many authors believe that fat grafting for breasts can help improve the outcome of breast reconstruction surgery in patients with breast cancer, the final outcomes are difficult to predict due to varying levels of resorption of fat grafts [8–11]. There have been continued advances in fat harvesting and manipulation methods to overcome this problem. Recently, studies that used adipose-derived stem cells (ASCs) or stromal vascular fraction (SVF) to increase the engraftment rate of fat graft have reported good outcomes [12–14].

SVF is a population of heterogeneous cells isolated from adipose tissues using enzymes, such as collagenase. SVF contains preadipocytes, mesenchymal stem cells, pericytes, endothelial cells, and macrophages, which have progenitor activity [15]. Some studies have reported on the potential effects of SVF in facilitating angiogenesis and adipocyte progenitor proliferation and differentiation [16, 17]. However, the actual mechanism of SVF involved in the repair and regeneration process remains unknown [18].

The method of cellular adjuncts, such as pluripotent ASCs, for improving the engraftment rate of autologous fat grafts has produced good results. However, no clear evidence is available whether these cells cause proliferation, differentiation, or metastasis of adjacent tumor cells. Additionally, findings in preclinical and clinical trials have been inconsistent and unclear [19]. In particular, a consensus has not been reached to date whether SVF affects the proliferation or metastasis of adjacent breast cancer due to lack of data from animal studies or clinical trials.

There have been reports that fat grafts affect oncological safety. Thus, this study aimed to investigate the effects of SVF on adjacent breast cancer and transplanted fat in an animal model. A breast cancer model was established by inoculating NOD/SCID mice with breast cancer cells and performing grafts using SVF cells together with human-derived adipocytes. Given that the fat graft used human-derived adipocytes, this mouse model was used to minimize the effects of the immune response. After the graft, radiological examinations, including magnetic resonance imaging (MRI) and three-dimensional (3D) micro-computed tomography (CT), and histological examinations were performed to investigate the effects of SVF cells on the growth of adjacent breast cancer and engraftment of the fat graft. Therefore, in this experiment, we aimed to perform fat grafting in a cancer model and analyze its effects.

Materials and Methods

Materials

To minimize suffering, the mice were anesthetized with intraperitoneal injections of 10 mg xylazine hydrochloride (Rompun®; Bayer Korea, Seoul, Korea) per kg and 100 mg of ketamine hydrochloride (Ketamine HCl®; Huons, Seoul, Korea).

Breast Cancer Cell Culture

MDA-MB-231-luc, the human-derived breast cancer cell line used in our study, was procured from PerkinElmer (Waltham, Massachusetts, USA). MDA-MB-231 is a triple-negative breast cancer cell line isolated from human metastatic pleural effusions. The culture broth used consisted of high-glucose Dulbecco's modified Eagle's media (HyClone, Logan, UT, USA) with 10% (v/v) heat-inactivated fetal bovine serum and 100 U/ml penicillin/streptomycin (both from Gibco-BRL, Grand Island, NY, USA). Culturing was performed in an incubator (Heracell 150i; Thermo Fisher Scientific, Waltham, MA USA) that maintained 5% CO_2 and 37 °C. When the cells covered approximately 80–90% of the cell culture dish, phosphate-buffered saline (PBS, pH 7.4) was used to wash the cell surface, and 0.25% trypsin-2.65 mM EDTA (Gibco-BRL) was then applied to isolate the cells. The abovementioned process was performed in class II, type A2 biological safety cabinets (Labconco Corporation, Kansas City, MO, USA), and the isolated cells were harvested through centrifugation at 1200 rpm for 3 min. A Countess™ Automated Cell Counter (Invitrogen, Carlsbad, CA, USA) was used to count the number of cells, whereas IVIS was used to identify the fluorescence.

NOD/SCID Mice

A total of 40 four-week-old NOD/SCID mice (NOD.CB17-Prkdc scid/J, female, mean weight of 14 g) were procured from Charles River Laboratories (Yokohama, Japan) and reared in the re-entry animal room in the Laboratory Animal Center at DGMIF. The animals were divided into eight groups of five animals and were kept in individually

ventilated cages (IVC rack size, width × depth × height, 391 × 199 × 160 mm) for rearing in an area that maintained the following conditions: indoor temperature, 23 °C ± 3 °C; relative humidity, 55% ± 10%; ventilation rate, 10–20 times/h; 12-h lighting cycle (fluorescent lighting, 07:00 light-on, 19:00 light-off); illuminance, 150–300 lx. Food (solid food for experimental animals) and water (reverse osmotic water) were provided ad libitum, and the cages were changed twice per week. They were quarantined upon arrival and after a 1-week acclimation period (including quarantine) when the animals were monitored for general symptoms; all animals were used in the experiment. During the entire experimental period (8 weeks), the animals were individually identified using the tail tattoo method and placing labels specifying the experimental conditions. The condition of the animals was monitored by gross observation at least twice a day. In addition, an animal laboratory use record log was placed on the entryway of the animal laboratory, which contained information on the experiment number, experiment title, duration of laboratory use, principal investigator, and sub-investigators. The mice were killed after confirming that anesthesia was induced. Pain alleviation was not implemented, since this can influence the effects of cancer growth, conflicting with the overall purpose of the experiment. Euthanasia was performed using CO_2 gas. The criteria for euthanasia were as follows: sudden weight loss of over 20% of normal body weight; necrosis, infection, or ulcers severe enough to impair feeding or drinking; or inability to continue with normal activity (e.g., inability to walk). Half of the mice were killed after 4 weeks post-fat grafting and the other half after 8 weeks post-fat grafting.

Human Fat Harvest

The tissue donor was a 52-year-old woman with no underlying diseases who was scheduled to undergo abdominal liposuction. After explaining about the objective of the study and obtaining the consent from the donor (Institutional Review Board at Kyungpook National University Medical Center; IRB approval number KNUMC 2016-12-015-006), fat tissues were harvested from the lower abdomen via liposuction in accordance with the principle of the Coleman technique, using a suction cannula connected to a 10-ml syringe [20]. Lipoaspirates were centrifuged at 1000 rpm for 3 min, and after removing the serum and oil fraction, clean fat samples were obtained for use in the in vivo animal study.

SVF Isolation Using SmartX kit®

The fat tissue harvested using the same method mentioned above was treated with type 1 collagenase at a ratio of 1:1 and was allowed to react for 30 min in a 37 °C incubator. Then, the syringe containing the enzyme-treated fat tissue was connected to the distribution tube of the component isolator (SmartX kit®; DongKoo Bio & Pharma Co Ltd., Seoul, Korea). After injecting the fat tissue into the component isolator, centrifugation was performed at 3000 rpm for 3 min. The pressure members were used to remove most of the fat layer and supernatant by moving the plunger to the upper part, and with only a part of the bottom layer remaining, physiological saline was added. Centrifugation was performed again at 3000 rpm for 3 min. After repeating this washing process three times, the SVF remaining at the bottom layer was collected.

For accurate count of nucleated cells within the collected SVF cells, cell pellets were resuspended in 1 ml of PBS, and 1 ml of red blood cell (RBC) lysis buffer (CEFO Co., Ltd., Korea), the solution used to remove RBCs, was added; the mixture was allowed to react for 3 min while being shaken slowly. After additional washing by adding more PBS and centrifuging at 3000 rpm for 3 min, the solution was diluted by a set ratio. Subsequently, 10 µl of the diluted sample was drawn and stained using trypan blue; then, the nucleated cells were microscopically counted using a hemocytometer.

Experimental Animals and Experimental Groups

A total of 40 five-week-old female NOD/SCID mice were selected to establish the breast cancer animal model (NOD.CB17-Prkdc scid/J; mean body weight, 15.64; range, 12.26–18.10 g, control group, 11 mice; SVF group, 9 mice; fat group, 11 mice; fat + SVF, 9 mice). The lower back area was selected as the site of breast cancer cell injection. This area has little subcutaneous fat and would allow relatively less restriction in the movement of the mice when the tumor volume would subsequently increase and fewer motion artifacts caused by breathing during imaging. Two weeks after subcutaneous injection of 2.0×10^6 MDA-MB-231-luc breast cancer cells into the right lower back of NOD/SCID mice, an IVIS spectrum imager was used to quantify the size by measuring the intensity of the signal emitted from the breast cancer cells. Among the animals, 36 were selected and assigned to the following four groups depending on the IVIS signal intensity (Fig. 1): group A: control ($n = 9$; mean IVIS signal = 1.661×10^8); group B: injected with 30 µl SVF ($n = 5$; mean IVIS signal = 1.438×10^8); group C: injected with 0.5 ml fat and 30 µl of physiological saline ($n = 11$; mean IVIS signal = 1.479×10^8); group D: injected with 0.5 ml fat and 30 µl SVF ($n = 11$; mean IVIS signal = 1.484×10^8). These are raw numerical values and do not have units.

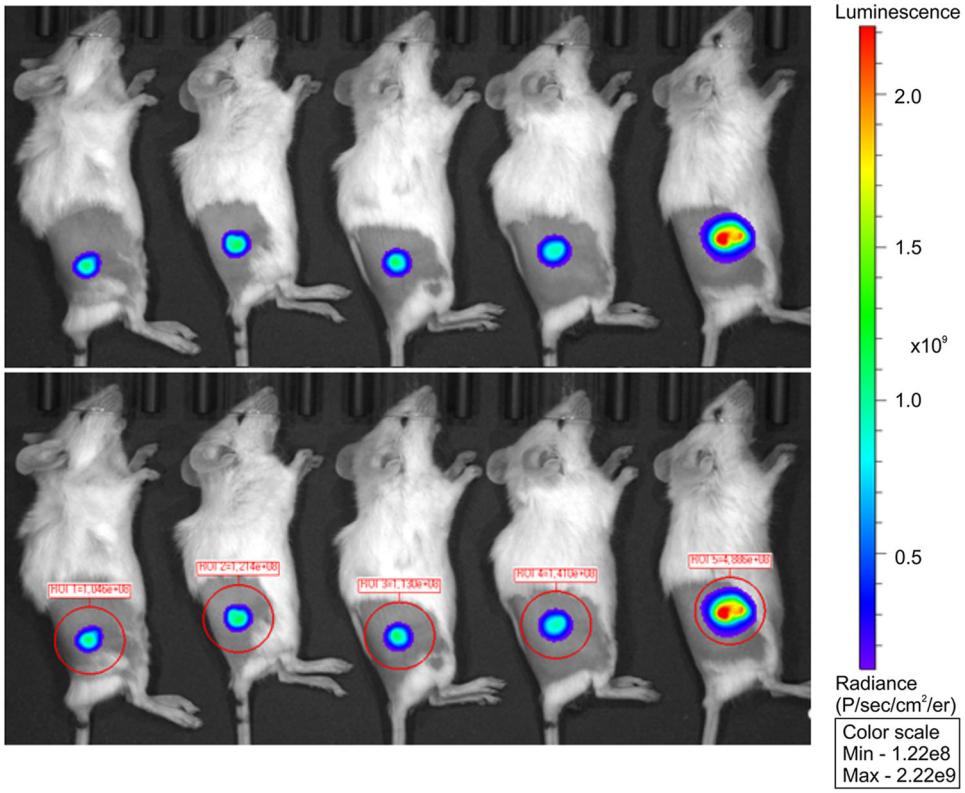

Fig. 1 Acquired bioluminescent images of NOD/SCID mice. Bioluminescent in vivo imaging using the IVIS spectrum imager at 2 weeks after injection of MDA-MB-231-luc into the right flank of the NOD/SCID mice. Four experimental groups were divided according to signal density

Methods

For the surgery, general anesthesia was performed using isoflurane (Isotroy; Troikaa Pharmaceuticals, Gujarat, India), an inhalation anesthetic, which was used to maintain a concentration of 0.5–3% in a mixture with oxygen. After the NOD/SCID mice were sufficiently anesthetized, the right lower back area was shaved and disinfected with povidone-iodine and alcohol; then, the area was adequately draped. After confirming that a palpable tumor had been created in the right lower back area from the engraftment of the breast cancer cells that were previously injected, the groups were treated as follows: group B, subcutaneous injection of 30 µl SVF near the tumor site using a 50-µl Hamilton syringe; group C, subcutaneous injection of 0.5 ml fat near the tumor site using a 21-G cannula, followed by injection of 30 µl of physiological saline; group D, subcutaneous injection of 0.5 ml fat and 30 µl SVF (Fig. 2). When the increased tumor size caused further problems, such as necrosis, infection, or ulcers that interfered with normal feeding/drinking or gait, the animals were killed according to the criteria for euthanasia.

Experimental Evaluation

On the day after the fat graft and SVF injection procedures, images were acquired using MRI, 3D micro-CT, and IVIS spectrum imager to measure the initial (baseline) volume of the grafted fat. Subsequently, follow-up imaging examinations were performed at weeks 4 and 8, and pathological examination was performed after autopsy. The body weight of each mouse was measured each week to check for growth.

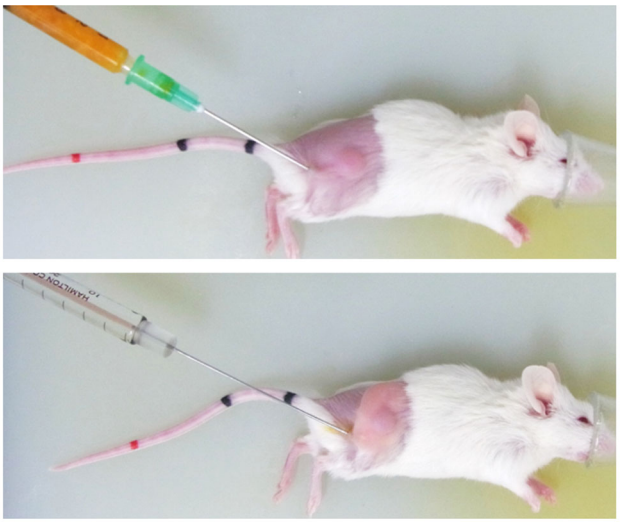

Fig. 2 Experimental animal model. Depending on the experimental group, 0.5 ml of purified human lipoaspirate was injected subcutaneously around the existing tumor using a 21-G cannula and 30 µl of stromal vascular fraction or saline was injected using a 50-µl Hamilton syringe

Radiological Evaluation

MRI volume analysis was performed using MRI data acquired from the RARE sequence of a 9.4-T MR scanner (Bruker, Germany). MRI was performed under general anesthesia using Isotroy, and the relevant parameters were as follows: repetition time, TR = 5000 ms, echo time, TE = 20 ms, matrix = 128 × 128 × 96, field of view (FOV) = 30 × 30 × 22.5 mm^3,
resolution = 0.234 × 0.234 × 0.234 mm^3.

The volume in the MR images was measured using the Python program. Both the tumor and fat regions were drawn as the 3D region of interest (ROI), and all regions with a signal below a certain range were delineated from the 3D ROI. The volume was calculated by the number of voxels in the ROI drawn and the product of voxels (Fig. 3).

For imaging of the grafted fat volume, 3D micro-CT reconstruction (Quantum FX; PerkinElmer) was performed under general anesthesia using Isotroy. The imaging parameters were as follows: voltage, 90 kVp; current, 180 μA; FOV, 120 mm; scan time, 34 s. The Analyze 12.0 program (Overland Park, KS, US) was used for the 3D reconstruction of the acquired images.

For optical imaging using the IVIS spectrum imager, D-luciferin was dissolved in Dulbecco's PBS at a concentration of 30 mg/ml and sterilized using a 0.2-μm filter. The images were acquired 10–15 min after intraperitoneal injection of 100 μl of D-luciferin. Optical imaging was performed under general anesthesia using Isotroy. The imaging parameters were as follows: excitation, block; emission, open; binning, 4; exposure time, auto.

Histological Evaluation

For confirmation of invasion of breast cancer into the adjacent tissue and successful fat engraftment, the tumor and fat graft were excised together and fixed in 10% neutral buffered formalin. To prepare the paraffin blocks, the

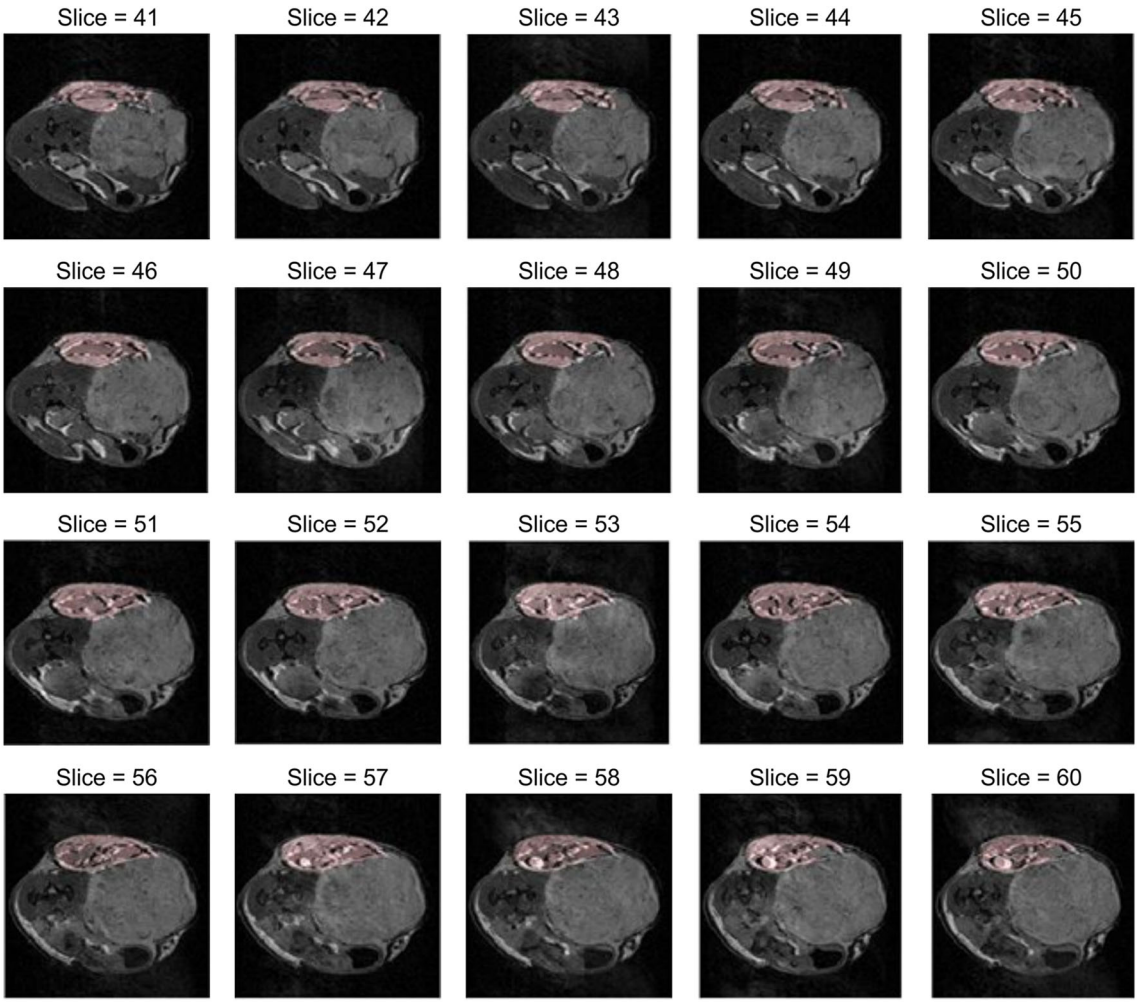

Fig. 3 Volumetric analysis based on magnetic resonance images using the Python program. Manual region-of-interest (ROI) delineation of tumor and fat was drawn on every slice, and the volume was calculated by summation of ROIs

sample in the cassette was transplanted on the mold removed from the tissue storage tank and placed on the hot spot. Then, the paraffin was lightly dispensed. After positioning the sample, it was transplanted on the mold and transferred to the cold spot where its position was fixed. After dispensing the paraffin with the cassette placed on top of the mold, the paraffin was hardened by transferring it to the cold plate. As the paraffin began to harden, it was moved to a 4 °C refrigerator for further hardening. Once the paraffin hardened completely, the mold and paraffin block were separated. The paraffin blocks were stored until trimming, and the mold that was used was placed back into the tissue storage tank. Tissue slices with 4-μm thickness were prepared from all samples, which were then stained with hematoxylin and eosin (H&E). The samples were analyzed using CaseViewer version 2.1 (3DHISTECH Ltd., Hungary) after digital imaging of the tissue samples using a slide scanner.

Immunohistological Evaluation

We compared the density of the newly generated blood vessels in the fat tissue grafted using CD31 antigen, an endothelial marker, and CD45 antigen, a hematopoietic marker.

After rehydration following the deparaffinization of the paraffin block samples, antigen retrieval was performed using citric acid to expose the hidden antigens by removing the cross-links created by the fixing solution. After washing with PBS, hydrogen peroxide treatment was applied for 10 min, which was followed by another round of washing with PBS and intrinsic enzyme blocking for 6 min using 5% normal goat serum. Anti-CD31 antibody (PA5-14372, Invitrogen) and anti-CD45 antibody (ab10559, Abcam) were added as primary antibodies, and the samples were incubated overnight and then washed with PBS. To visualize the primary antibody with optical microscopy, a 3,3′-diaminobenzidine (DAB) detection system (DAKO EnVisionTM plus System; Dako Cytometrics, Carpinteria, CA, USA) was used to analyze the samples after binding the secondary antigens for 60 min, staining for 5 min with DAB chromogen kit, counterstaining for 1 min with Mayer's hematoxylin, dehydrating, and mounting. The samples were observed using an optical microscope (Leica DM750; Leica Microsystems (Switzerland) Limited), and Leica Application Suite version 3.4.0 (Leica Microsystems (Switzerland) Limited) software was used to acquire the digital images.

Statistical Analysis

Statistical analysis of the measured values was performed using SigmaPlot (Systat Software, Inc., San Jose, CA, USA). Mean values were compared using paired t tests. The statistical significance of surviving fat volume (%) and changes in tumor size (%) between the experimental groups was analyzed using one-way analysis of variance. The statistical significance level was set at $< 5\%$ ($p < 0.05$).

Results

A breast cancer animal model was established by xenograft of human-derived breast cancer cells in 40 NOD/SCID mice. Among those animals, 36 were selected and divided into groups A, B, C, and D according to SVF and/or fat injection. SVF was obtained by isolating 3 ml (2.2×10^6 cells/ml, viability 89%) from 50 ml of lipoaspirate. Radiological and pathological evaluations were performed at 4 and 8 weeks after fat grafting. On day 38 of the experiment, one animal in group C died of unknown cause, whereas all other animals survived until the end of all experiments.

No symptoms requiring the experiment to be stopped, such as seizure or paralysis, were observed. In addition, the tumor burden size did not reach 20 mm or higher in any dimension, i.e., the conditions for the termination of the animal experiment were not met.

Radiological Findings of Breast Cancer

MRI

The mean tumor volumes immediately after SVF and/or fat graft in groups A, B, C, and D were 43.6 ± 4.8, 42.3 ± 9.9, 48.7 ± 3.5, and 42.4 ± 10.6 mm^3, respectively. None of the groups showed statistically significant differences as compared with their baseline values ($p > 0.05$).

In the follow-up observations, the mean tumor volumes in groups A, B, C, and D were 1530 ± 280, 1170 ± 230, 1010 ± 170, and 790 ± 160 mm^3 at week 4, respectively, and 6780 ± 360, 5940 ± 1900, 6080 ± 380, and 5570 ± 570 mm^3 at week 8, respectively. The results did not show statistically significant differences in tumor volume between the groups ($p > 0.05$; Fig. 4).

Relative to their baseline values immediately after the graft, groups A, B, C, and D showed a mean percentage increase of 2630%, 2600%, 2100%, and 1990% at week 4, respectively, and 12,270%, 12,610%, 12,530%, and 14,060% at week 8, respectively (Fig. 5).

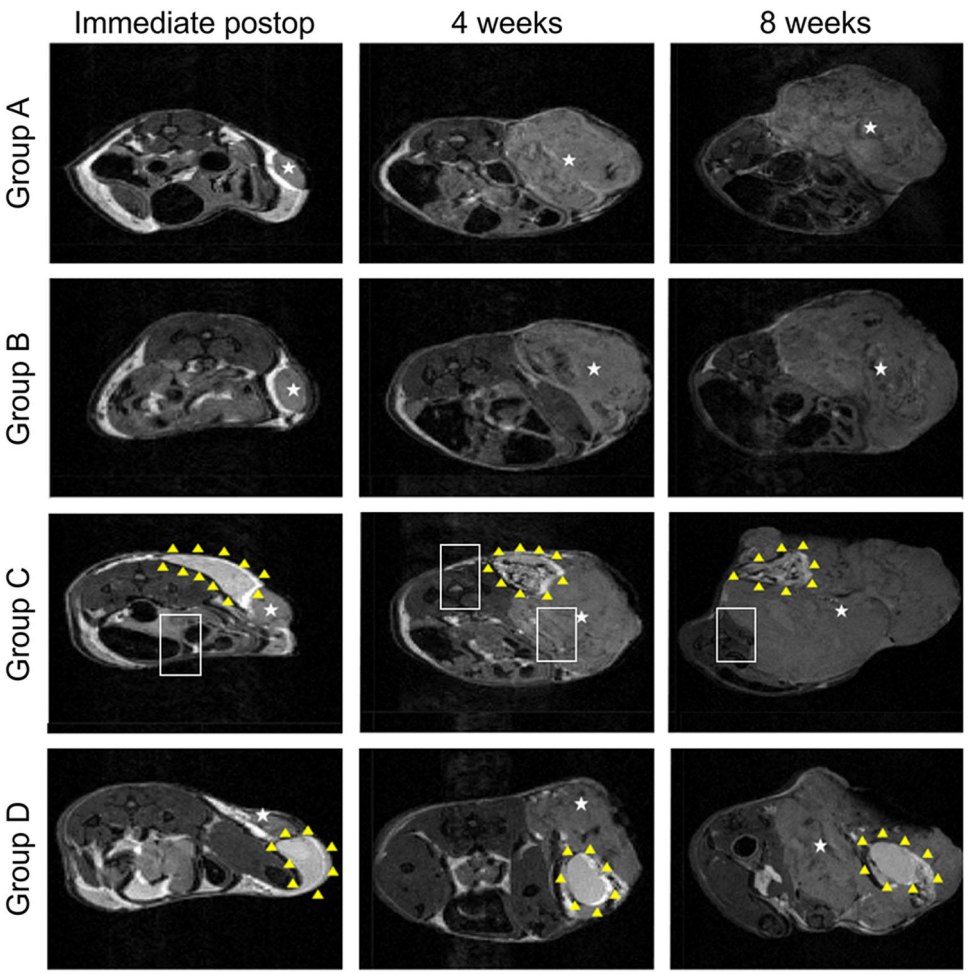

Fig. 4 Magnetic resonance (MR) images of tumor and grafted fat in the NOD/SCID mice. The MR images demonstrate a significant increase in tumor volume (groups A, B, C, and D) and gradual reduction of grafted fat volume (groups C and D). White stars indicate tumor and yellow triangles indicate engrafted fat tissue

IVIS Spectrum Imager

In the follow-up imaging at 4 weeks after the graft, accurate quantification of the optical image signals was impossible due to the presence of necrosis at the center of the tumor in all groups. Increased necrotic tissue is accompanied by increased discharge, and alongside tumor growth, the tissue itself started to fall out, rendering signal detection impossible. Up to a follow-up at 8 weeks after the graft, no findings of distant metastasis were found in any group (Fig. 6).

Radiological Findings of Grafted Fat Tissues

MRI

The mean grafted fat volumes immediately after SVF and/or fat graft were 370.3 ± 61.5 and 307.9 ± 51.6 mm^3 in groups C and D, respectively, showing no statistically significant difference in the initial volume between the two groups ($p > 0.05$).

In the follow-up observations, groups C and D showed mean grafted fat volumes of 318.7 ± 87.2 and

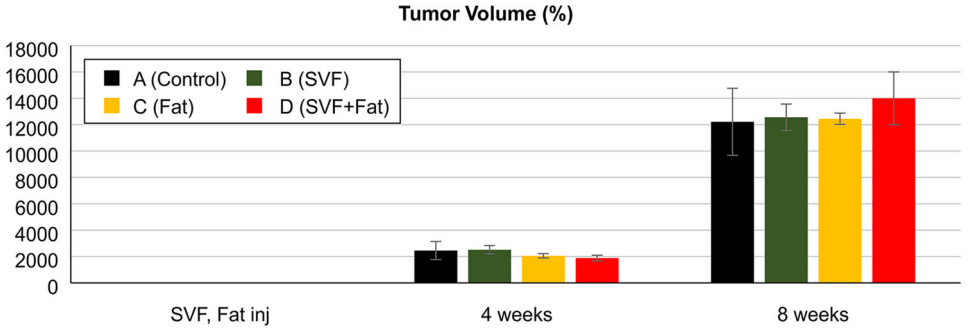

Fig. 5 Proportion of tumor volume at 4 and 8 weeks compared with the initial volume. No statistical differences in tumor growth rate were found among groups A to D

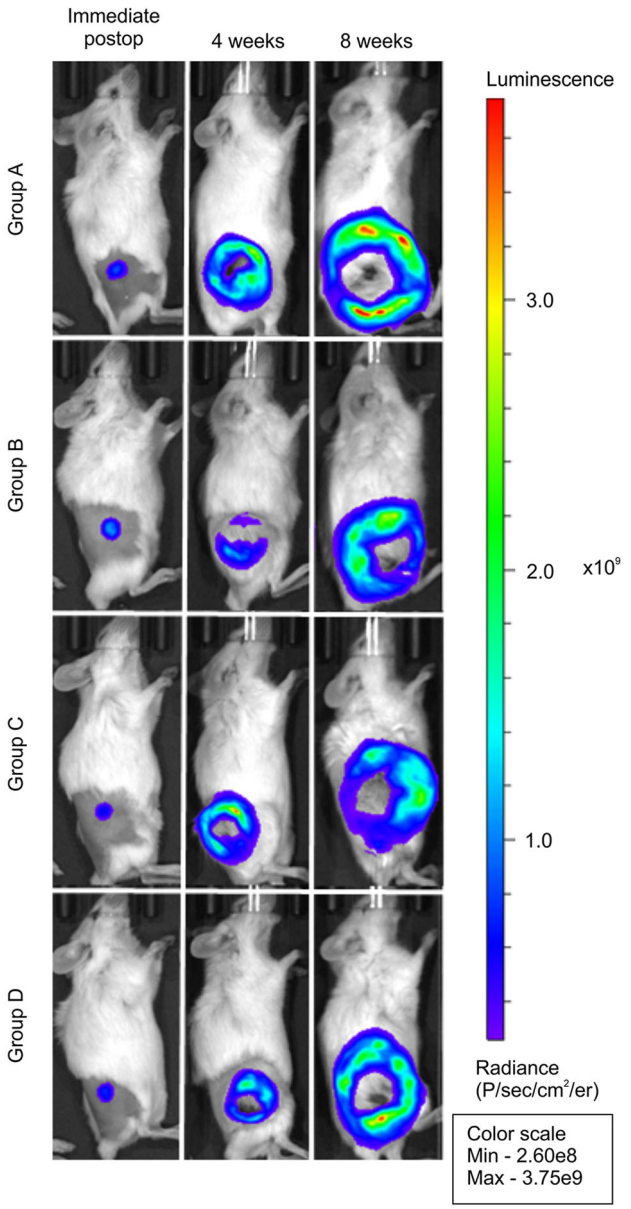

Fig. 6 IVIS images of tumor in the NOD/SCID mice. Central necrosis of the tumor was found at 4 weeks, and no distant metastases occurred in any group at 8 weeks

278.8 ± 75.3 mm^3, respectively, at week 4, and 175.9 ± 43.0 and 194.5 ± 69.1 mm^3, respectively, at week 8, showing no statistically significant differences between the two groups ($p > 0.05$; Fig. 4).

Relative to their baseline values from immediately after the graft, groups C and D showed a mean percentage increase of 85.6% and 89.0% at week 4, respectively, and 49.3 and 62.0% at week 8, respectively (Fig. 7).

Micro-CT

Changes in the grafted fat volume were examined by obtaining the density of fat through micro-CT and performing 3D reconstruction. The results showed that group D showed relatively less change in the morphology of the grafted fat than group C while also well retaining the volume (Fig. 8).

Macroscopic Findings

The body weight of the mice was measured each week to check for normal growth. When the breast cancer model was established, the mean body weights in groups A, B, C, and D were 15.7 ± 1.1, 14.9 ± 1.5, 14.9 ± 0.8, and 16.7 ± 1.2 g, respectively. Subsequently, the mean body weights in groups A, B, C, and D were 17.4 ± 1.5, 16.5 ± 2.0, 16.6 ± 1.3, and 18.2 ± 1.1 g, respectively, immediately after the SVF and/or fat graft; 19.9 ± 1.8, 20.2 ± 1.5, 20.3 ± 1.1, and 21.5 ± 1.0 g, respectively, at week 4, and 25.9 ± 0.7, 25.4 ± 1.9, 26.7 ± 2.5, and 25.0 ± 1.1 g, respectively, at week 8. The results confirmed that the animals in each group showed normal growth with no statistical differences ($p > 0.05$; Fig. 9).

We observed the breast cancer and grafted fat tissues excised together from the euthanized mice. At 4 weeks after the graft, necrosis was found in the breast cancer tissues in all groups, and by 8 weeks after the graft, the tumor had grown to infiltrate the right hind leg of the mice. The fat tissues were successfully engrafted in both groups C and D, as indicated by fresh yellow fat tissues (Fig. 10). Overall, no macroscopically distinguishable findings were found between the groups.

Histological Findings

H&E Stain

With respect to the tumor tissues, the cancer cells were confirmed to infiltrate the skin, causing destruction of normal skin structure and some necrosed tissues at week 4, and at week 8, the cancer cells had infiltrated the external oblique muscle below the tumor in all animals (Fig. 11).

With respect to the engrafted fat cells, several intact fat cells were found in both groups C and D at week 4, whereas no clear signs of fibrosis or infiltration by inflammatory cells were observed. However, at week 8, irregularly shaped fat cells and some damaged cells were found in both groups, but this finding was relatively more severe in group C (Fig. 12).

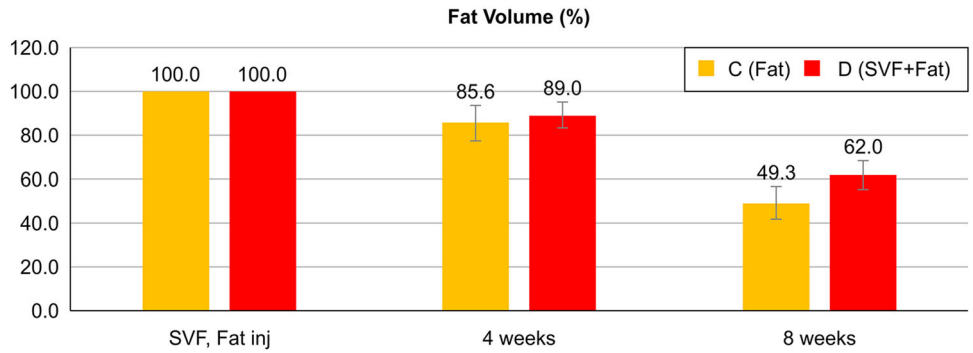

Fig. 7 Proportion of residual fat volume at 4 and 8 weeks compared with the initial volume. At 8 weeks, group D showed a 25.8% more superior engraftment rate compared with group C

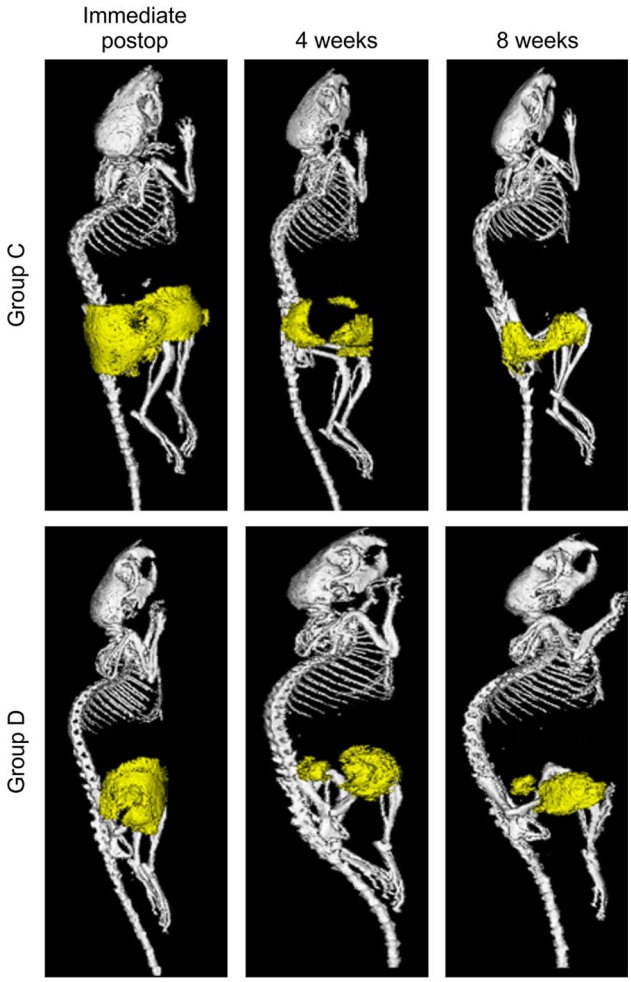

Fig. 8 Micro-computed tomography images of grafted fat in the NOD/SCID mice. Three-dimensional volume reconstructions of the injected fat (yellow) are shown. At 8 weeks, the structure of the grafted fat was better maintained in group D

Immunohistological Findings

In group D, both CD31 and CD45 antigens were stained relatively denser between the engrafted fat cells at week 4, and while the density decreased slightly at week 8, such findings were still noticeable. In group C, both CD31 and CD34 antigens were hardly stained at weeks 4 and 8 (Figs. 13, 14).

Discussion

In 1895, the German surgeon Czerny performed the first transplantation on breast defect area using lipoma excised from the lumbar spine region [6]. Over a century later, fat grafts have been used to achieve larger and esthetically enhanced breast shapes. In 2007, Coleman and Saboeiro [7] published a report on 17 cases of breast augmentation and reconstruction using autologous fat grafting, and Delay et al. [21] also reported good outcomes from using fat graft in breast reconstruction surgeries starting from the early 2000s.

Autologous fat grafting in the breast area has been widely used because the use of a natural filler offers several advantages over artificial implants, in addition to having therapeutic potential [22].

Although thousands of cases of fat grafting for breasts are being performed worldwide as an alternative to using breast implants, such as silicone, its oncological safety remains controversial. Consequently, many surgeons have been reluctant in using fat grafting for breasts, whereas even the surgeons who use fat grafting for breasts have been concerned about whether using fat grafting may increase the recurrence of breast cancer. These results were due to concerns on whether ASCs transplanted during fat grafting may reactivate dormant tumor cells in the breast.

Laboratory studies have shown that adipocytes are capable of stimulating breast cancer cells. White adipose tissue is an endocrine organ with the potential to secrete hormones, growth factors, and cytokines, such as insulin, leptin, adiponectin, hepatocyte growth factor, and insulin-like growth factor I. These adipokines are not only essential components of metabolism, but they may also facilitate the progression of nearby cancer [23, 24].

Petit et al. [25] conducted a retrospective study on 646 cases of fat grafting for the breasts in a 2011 European multi-institutional study and reported that the risk of

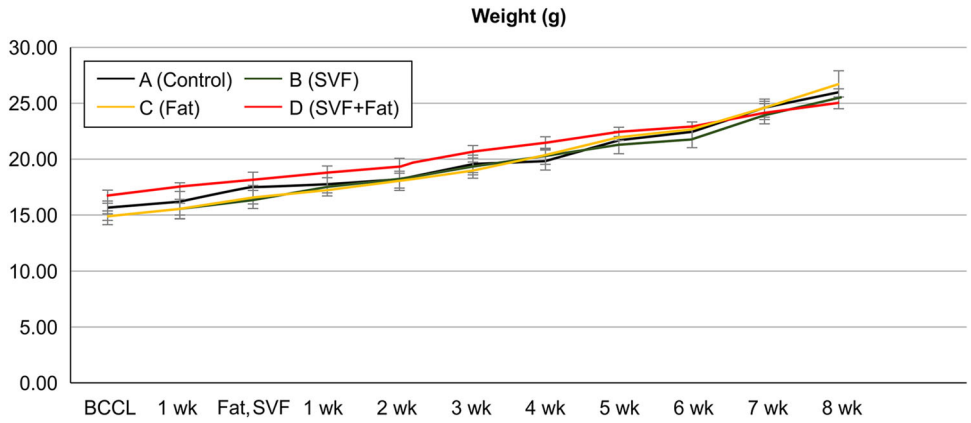

Fig. 9 Body weight graph of the NOD/SCID mice. All groups showed normal growth, with no statistical difference among the groups

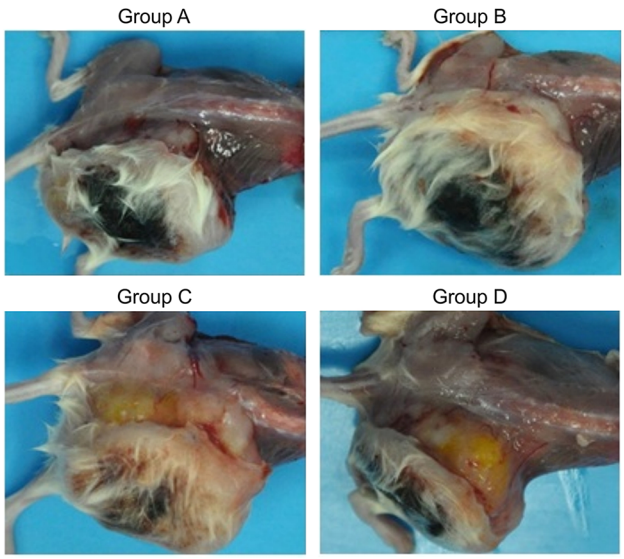

Fig. 10 Necropsy findings in the NOD/SCID mice at 8 weeks. Central necrosis of the tumor was found in all groups, and fresh yellow fat tissues were observed in groups C and D

recurrence of breast cancer was higher in patients with in situ carcinoma than in patients with invasive breast cancer. Subsequently, a follow-up observation study on 59 patients with in situ carcinoma reported that the locoregional recurrence rate was 18% when fat grafting for the breasts was used, which was higher than 3% for when it is not used [26].

In contrast to the study by Petit et al., Kronowitz et al. [27] conducted a large-scale comparative study on 719 cases of patients with breast cancer and 305 cases of patients with and without breast cancer with fat grafting versus 670 cases of breast cancer control group with no fat grafting between 1981 and 2014. The results showed that the fat graft and control groups showed locoregional recurrence rate of 1.3% and 2.4%, respectively, and systemic recurrence rates of 1.3% and 2.4%, respectively. Moreover, not even a single case of breast cancer occurred in the patients without breast cancer who received fat grafting for the breasts. Hence, Kronowitz et al. reported that no statistical differences were observed in locoregional and systemic recurrence rates and occurrence of secondary breast cancer between the two groups.

Gale et al. [28] conducted a comparative study on 211 cases of fat grafting after breast cancer surgery. The cases involved in situ carcinoma (13%) and infiltrating cancer (87%), and in these cases, mastectomy and breast preservation surgery were performed in 83.4% and 16.6% of the cases, respectively. Follow-up observations at 88 months after breast cancer surgery and 32 months after fat grafting showed that the group that received fat grafting and the control group showed no statistically significant differences, with local recurrence rates of 0.95% and 1.90%, regional recurrence rates of 0.95% and 0%, and distant metastasis rates of 3.32% and 2.61%, respectively.

Petit et al. [29] conducted another study in 2017, which compared 322 patients who received fat grafting after breast preservation surgery. No statistically significant differences were observed between the two groups (control group: not undergone fat injection, experimental group: undergone fat injection), with ipsilateral recurrence in 14 and 16 cases, axillary or regional lymph node metastasis in 3 and 6 cases, distant metastasis in 14 and 15 cases, and contralateral breast cancer in 4 and 5 cases, respectively. Additionally, statistical differences were observed between the two groups with respect to previous in situ breast cancer [26]. However, statistical differences could not be found in long-term observations on the same patient group [29].

Based on such results from recent large-scale comparative clinical trials, the oncological safety of fat grafting for breasts has been proven to a certain degree. However, fat grafting for the breasts still faces problems such as the need for large amount of graft and low engraftment rate. Given such unpredictability in the engraftment of fat graft, several rounds of surgery may be required to obtain the desired volume, whereas fat that did not engraft successfully can remain as necrotic tissue. To overcome such disadvantages,

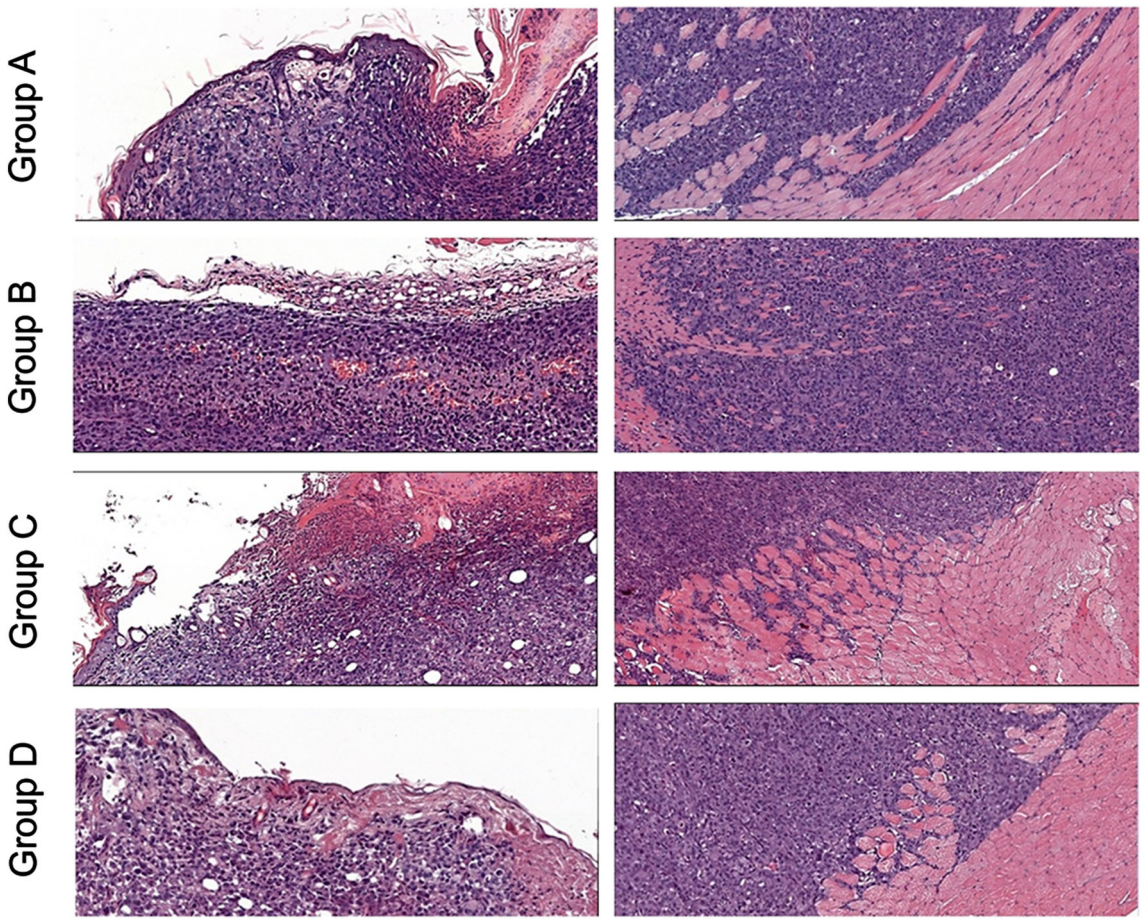

Fig. 11 Typical sample photographs of hematoxylin and eosin–stained tumor tissue specimens. The specimens were harvested at 8 weeks from each group. Cancer cells invaded the skin, and destruction of normal skin structure was confirmed in all groups (left). Moreover, the cancer cells invaded the underlying external oblique muscle layer in all groups (right) (original magnification × 100)

numerous studies have examined the use of chemical cell-stimulating factors, such as insulin, vitamin, growth factor, platelet-rich plasma, and SVF [30–32].

ASCs were first characterized by Zuk et al. [33], and since then, extensive studies on their multipotency, paracrine effect, and effects in regenerative medicine have been conducted. SVF is a population of heterogeneous cells with adipose cell, connective tissue, and blood removed from lipoaspirates by enzymatic decomposition and centrifugation, and because it does not require culturing, it can be used more readily in clinical practice due to its relatively fast and simple isolation process as compared with that of ASCs [34]. According to the International Federation for Adipose Therapeutics and Science and the International Society for Cellular Therapy, SVF contains 25–45% of hematopoietic cells, 15–30% of stromal/stem cells, 10–20% of endothelial cells and progenitors, and $< 5\%$ of pericytes [35].

The SVF cell recovery rates in adipose tissues vary significantly, depending on the method or equipment used, and in the past, the recovery rate was reported to be lower when using mechanical methods than with the manual method [36]. However, with recent advances in SVF extraction equipment, automated or semiautomated mechanical systems are showing cell recovery rates that are reliable and comparable to the manual method [37]. The conventional enzymatic manual method has the possibility of obtaining not enough SVF layer or cell loss or damage due to aspiration of unwanted contaminants or some suspended cells, depending on the skill level of the operator. However, mechanical methods can be advantageous since the extraction process is a closed system, which minimizes the number of cells escaping from the pellet, whereas the fluctuations in cell loss can be minimized since the methods are less affected by the skill level of the operator. However, such mechanical systems are costly, with some automated systems having prices that exceed $50,000 [38].

Matsumoto et al. [39] introduced the concept of cell-assisted lipotransfer (CAL) through an animal model for increasing the engraftment rate of fat grafts. Half of the lipoaspirate was used to extract SVF by collagenase and centrifugation, whereas the other half was used as the

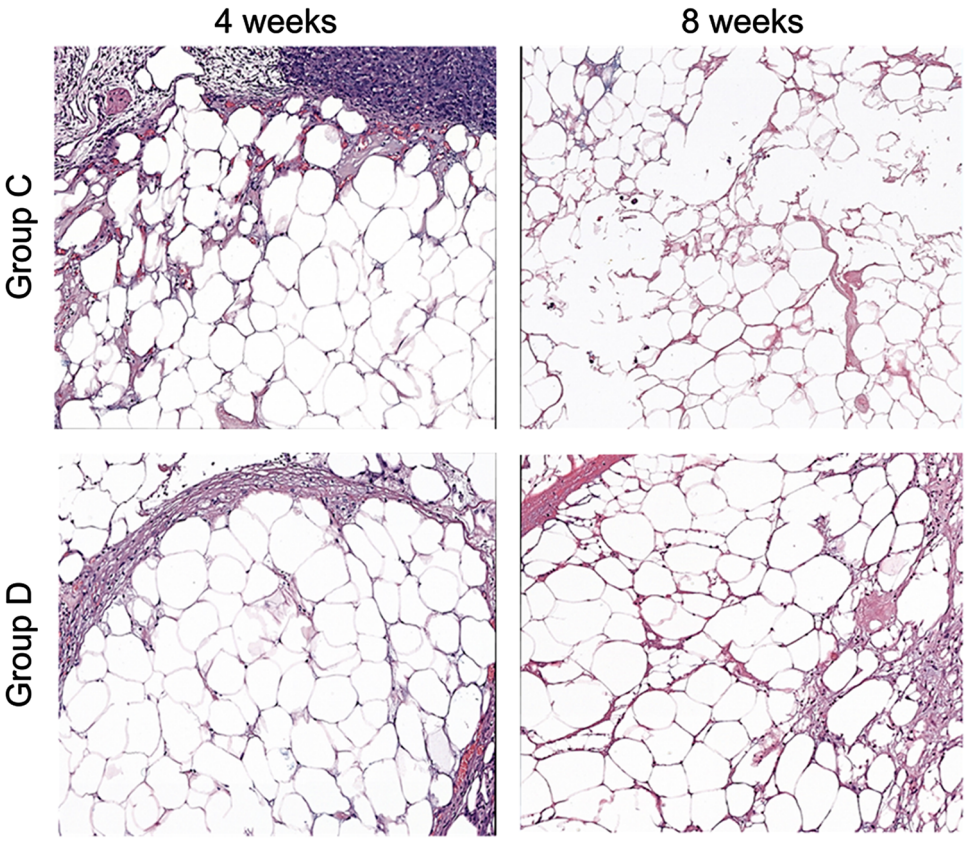

Fig. 12 Typical sample photographs of hematoxylin and eosin–stained grafted adipose tissue specimens. The specimens were harvested at 4 and 8 weeks from groups C and D. At 4 weeks, the integral architecture was well maintained in both groups. At 8 weeks, adipocytes were partially destroyed in group C, whereas the structure of the mature adipocytes was relatively well maintained in group D (original magnification × 100)

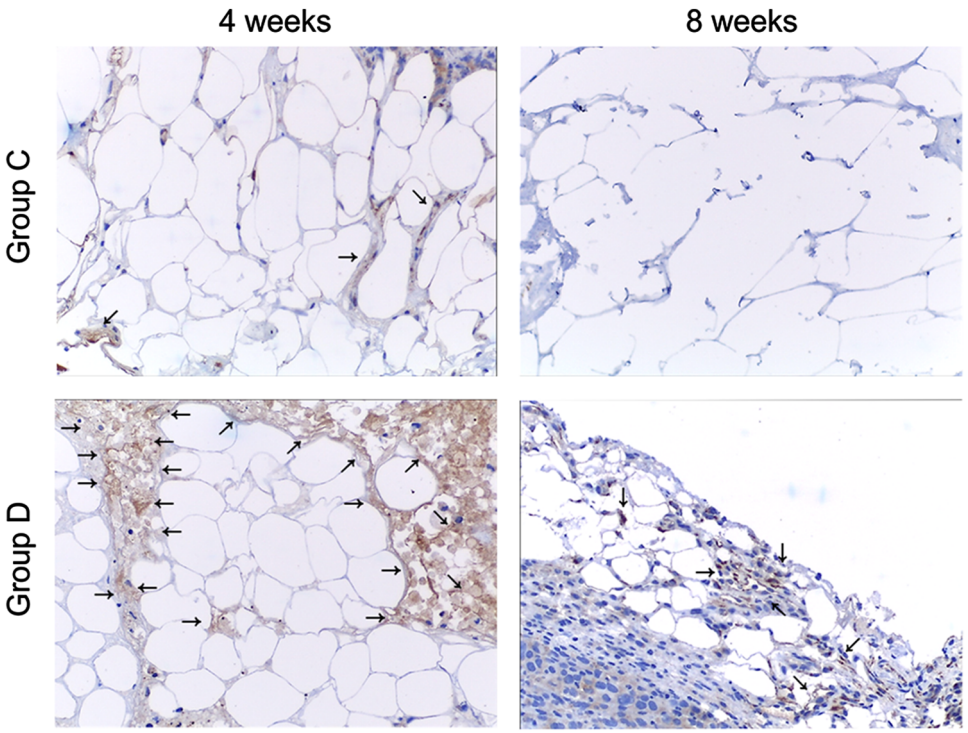

Fig. 13 Immunohistochemistry evaluation of human fat grafting in the NOD/SCID mice. Staining for endothelial marker CD31 in grafted fat and counterstaining with Mayer's hematoxylin were performed. Positive CD31 staining (black arrow) was more predominant in group D than in group C (original magnification × 200)

scaffold of SVF, which were combined in preparation for fat graft. Fat grafts containing SVF showed 35% higher graftment volume and more distinct microvasculatures than non-SVF fat grafts. Subsequently, the use of CAL expanded from animal model experiments to clinical trials, with Yoshimura et al. [32] using CAL to prove a volume

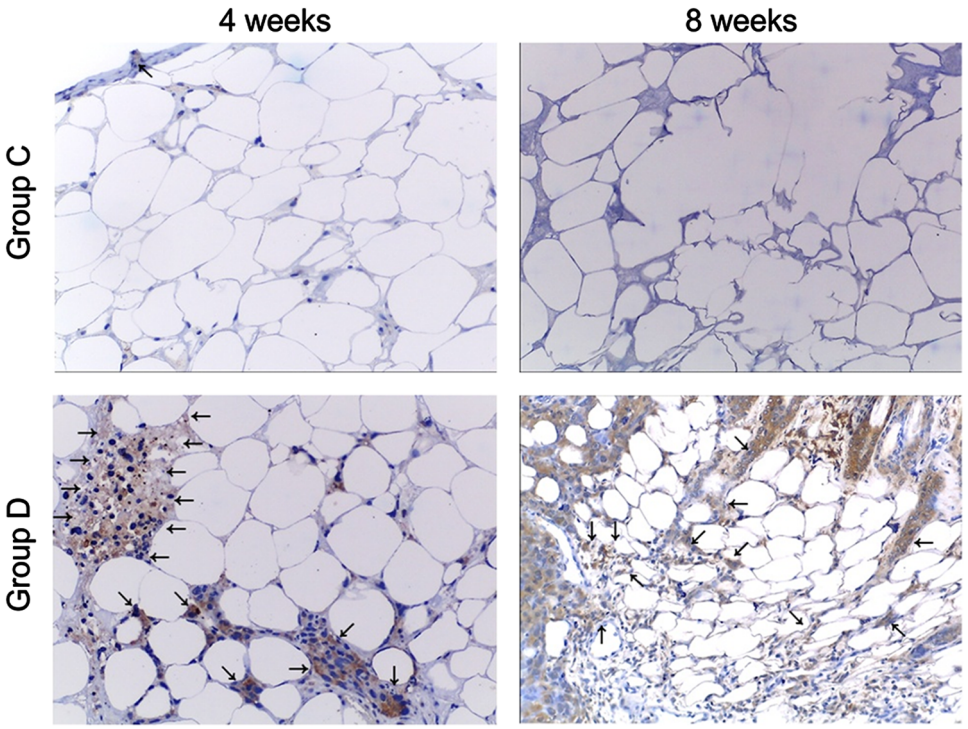

Fig. 14 Immunohistochemistry evaluation of human fat grafting in the NOD/SCID mice. Staining for hematopoietic marker CD34 in grafted fat and counterstaining with Mayer's hematoxylin were performed. Positive CD34 staining (black arrow) was more predominant in group D than in group C (original magnification × 200)

increase of 100–200 ml when applied in esthetic breast augmentation. Our study also performed fat graft combined with SVF on NOD/SCID mice, using the same ratio used by Matsumoto et al. [39], and as a result, an engraftment rate of 62% was found 2 months after the procedure, which represented a 25.8% superior engraftment rate than that of the control group.

Recent clinical trials are proving the therapeutic potential of CAL. A small-scale clinical trial on six patients by Charles-de- Sá et al. [40] demonstrated the anti-aging effect of ASCs and SVF on facial skin. In that study, no statistically significant difference was observed between SVF and ASCs, but both groups showed reversal of histological findings of aging, including decreased elastosis, new elastic fiber formation, vascular proliferation, and collagen remodeling. Given that SVF is easier and simpler to harvest than ASCs, such results are even more meaningful.

However, using CAL in breast reconstruction following mastectomy in patients with breast cancer remains controversial. Chatterjee et al. [41] demonstrated in an ex vivo experiment that SVF is a potent additive that can expand epithelial progenitors in breast tissues. Since previous studies have mentioned that ASCs found in the SVF area's potential source of proliferation, migration, and metastasis of breast cancer cells [42–44], caution should be taken when using SVF as an additive in breast reconstruction surgery following mastectomy. In our experiment, the group that received fat and SVF grafts together showed an increase of approximately 14.6% in breast cancer volume after 2 months than the control group. However, the difference between the two groups was not statistically significant. Unlike existing laboratory studies using high ratio of ASCs or SVF, our study grafted SVF using a ratio similar to CAL used by Matsumoto et al. [39], which is mostly used in clinical practice. Therefore, the SVF cells may not have had a significant effect on the growth of adjacent tumor.

Our experiment was conducted to investigate the possible effects of SVF, which was grafted together with fat, on the growth of adjacent breast cancer and engraftment of fat graft. MDA-MB-231, the cell line used in the experiment, takes on a volume of about 1.0 cm^3 after about 40 days after being injected [45]. Thus, the size could be measured using calipers to minimize errors. Prior to that time point, accurate measurement was difficult to obtain, and error rates may be high due to the small size. However, after about 40 days, the tumor size grows rapidly [45]; thus, there is a high probability that the fat and SVF injected during the experiment had a very weak effect on tumor growth. To overcome this issue, a cell line with luciferase was used, where millet-sized tumors at 2 weeks after the injection were used for group assignment based on IVIS signal intensity, and the outcomes from fat and SVF injection at a stage prior to rapid tumor growth were observed. Three evaluators used calipers to take the measurements at 2 weeks after the injection. However, because the tumor size was too small, large errors in the values

measured by each evaluator were detected, which made it difficult to use the values in the actual experiment.

Most of the experiments that investigated fat and tumor growth measured the volume directly after an autopsy. However, in doing so, neighboring tissues may be excised together, which may introduce errors, and especially, in cases involving fat graft, accurate measurement of volume is even more difficult since they are scattered, not lumped together. To overcome such errors, our experiment used MRI for volume measurement, which increased the reliability of data.

Moreover, the strengths of our study include the fact that we successfully grafted human-derived breast cancer cells, fat, and SVF and obtained meaningful data using NOD/SCID mice to exclude other factors, such as immune responses of mice.

This study has several limitations. NOD/SCID mice are expensive, and the equipment had some constraints, since it requires approximately 30 min to perform MRI on a single mouse. Given these factors, the number of mice used in the experiment was low, which presented limitations in obtaining statistically significant results. The group that was injected with SVF injected showed a relatively lower fat engraftment rate than that of other studies, which may be attributed to environmental peculiarity from having the fat injected close to the tumor. The quality of the fat being grafted, the ratio of ASCs included in the fat being grafted, and the nutrient supply to the graft site are well-known factors affecting the fat engraftment rate [46], and because regeneration occurs at the edges of the fat graft, ASCs can promote replacement and survival of adipocytes [47]. However, because tumor tissues were located adjacent to fat in our experiment, it might have a negative impact due to the volume effect from the continuously growing tumor and insufficient nutrient supply.

Moreover, the experimental model used in our study did not completely match the actual clinical setting for breast reconstruction following mastectomy. However, based on the data on the effects of SVF on adjacent tumor growth and fat graft survival obtained from the experiment, future studies with models using fat graft after excision of engrafted breast cancer tissue from injection of breast cancer cells should be conducted. Examining the changes in results is necessary based on the SVF cell count according to different concentrations of SVF in mixed injections.

Conclusions

Breast cancer is the second most common cancer among women in Korea, and instead of only a simple mastectomy, the demand for breast reconstruction surgery and oncoplastic surgery in consideration of postoperative quality of life continues to increase. For breast reconstruction using autologous tissue, fat graft has been used extensively in recent time for correction of asymmetry, shape enhancement, and supplementation of soft tissues in breast reconstruction using implants. The use of SVF may be considered as an option for overcoming the low engraftment rate of fat grafting for breasts. However, a consensus on its oncological safety has not been reached. As demonstrated in our animal experiment, using a proper ratio of SVF can increase the engraftment rate of the grafted fat, without any significant effect on the neighboring tumor tissues. Based on the findings from our study, satisfactory outcomes from fat grafting with SVF in patients who undergo breast reconstruction surgery can be expected.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have not conflicts of interest related to the manuscript.

Human and Animal Rights Statement This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal experiment was conducted after review and obtaining approval from the Institutional Animal Care and Use Committee of Daegu-Gyeongbuk Medical Innovation Foundation (DGMIF) (approval number: DGMIF-17021401-00).

Informed Consent For this type of study, informed consent is not required.

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