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Original Article

Novel breast reconstruction technique using ex vivo mononuclear (RE-01) cells and adipose-derived mesenchymal stem cells

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ABSTRACT

Introduction: Breast reconstruction using fat grafts presents challenges; for example, fat necrosis owing to inadequate blood flow results in reduced engraftment rates. Supplementation of adipose tissue with adipose-derived mesenchymal stem cells (ADSCs) to promote the rapid vascularization of transplanted tissue has been investigated. However, the vascularization of fat-grafted tissues using only ADSC transplantation is limited. *Ex vivo* cultured mononuclear cells (RE-01) are a cell population with highly vascular and tissue-regenerative properties. This study aimed to evaluate the effect of combining RE-01 cells and ADSCs on the engraftment rate of fat grafts and explore the potential of this approach as a new option for breast reconstruction surgery. We hypothesized that combining RE-01 with ADSCs might promote angiogenesis and improve the fat grafting rate, consequently reducing the number of ADSCs required.

Methods: ADSCs cultured from human adipose tissue discarded during liposuction were co-cultured with RE-01 cells produced from the peripheral blood of healthy volunteers. *In vitro* vascular regeneration and adipogenic differentiation potential were analyzed. In addition, fat grafting experiments were conducted using nude mice to verify the fat grafting efficacy of ADSCs after co-cultivation with RE-01.

Results: ADSCs co-cultured with RE-01 cells promoted angiogenesis and adipogenesis *in vitro*. This was evidenced by a significant increase in the expression of adipogenic markers *FABP4* and *PPARγ*, as well as enhanced lipid droplet formation observed through Oil Red O staining. The *in vivo* results demonstrated that the fat engraftment rate was significantly improved in the mixed group of ADSCs co-cultured with RE-01 cells. The number of blood vessels and fat quality of the transplanted adipose tissue were also increased in this group, suggesting that ADSCs co-cultured with RE-01 cells were highly effective in fat transplantation.

Conclusions: ADSCs co-cultured with RE-01 cells may be useful for improving the engraftment rate of fat grafts. However, further studies are required to verify the mechanisms.

Abbreviations: ADSCs, adipose-derived mesenchymal stem cells; PBMNCs, peripheral blood mononuclear cells; VEGF, vascular endothelial growth factor; HUVECs, human umbilical vein endothelial cells; iNOS, inducible nitric oxide synthase; *IGF-1*, insulin-like growth factor 1; *IL-1β*, interleukin-1 beta; *ADAMTS1*, A Disintegrin and Metalloproteinase with Thrombospondin Motifs 1; *FABP4*, fatty acid binding protein 4; *PPARγ*, peroxisome proliferator-activated receptor gamma; *PECAM1*, platelet endothelial cell adhesion molecule 1.

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1. Introduction

Breast cancer remains the leading malignancy among women worldwide, with approximately 2.3 million new cases diagnosed in 2020 according to the World Health Organization [1]. This trend is mirrored in Japan, with 97,142 new cases reported in 2019 [2]. Surgical treatments for breast cancer primarily include mastectomy and breast-conserving surgery, and in Japan, approximately 40 % of patients require mastectomy [3]. Breast loss has a significant psychological impact; as such, breast reconstruction surgery plays a crucial role in alleviating psychological distress and improving the quality of life (QOL) of patients [4]. Breast reconstruction primarily involves autologous tissue reconstruction and artificial implants, each with its own challenges. Autologous tissue reconstruction is highly invasive, whereas artificial implants have declined reliability, with reports of breast implant-associated anaplastic large cell lymphoma [5].

Fat grafting has attracted considerable attention as an alternative therapeutic approach. However, the retention rate of transplanted fat varies widely between 25 % and 80 %, often necessitating multiple treatments [6,7]. Various methods have been studied to improve the fat engraftment rate. For example, hypoxic preconditioning has been shown to enhance the angiogenic ability of adipose-derived mesenchymal stem cells (ADSCs), thereby improving the engraftment rate of transplanted fat tissue [8]. The addition of growth factors, ADSCs, and platelet-derived components to fat tissue also improves the engraftment rate and long-term stability of fat grafts [9].

ADSCs are stem cells from fat tissue that have the ability to promote angiogenesis; accordingly, they have been widely studied in regenerative medicine. However, ADSC transplantation alone is insufficient to achieve complete regeneration of fat tissue, including angiogenesis [10], because ADSCs cannot provide all the necessary cell types and fail to offer an appropriate environment to support effective angiogenesis and tissue regeneration [11]. To address this challenge, approaches such as combining cell populations that include angiogenesis-promoting cells have been considered. We previously reported on RE-01 cells, a cell population derived from peripheral blood mononuclear cells, which include several angiogenesis-promoting cells. RE-01 cells are characterized by high angiogenic capacity, minimal invasiveness during collection, and low-cost production [12]. *In vitro* experiments confirmed an increase in M2 macrophages and angiogenic T cells, a decrease in inflammatory cells, and antifibrotic effects. *In vivo* experiments in a mouse model of ischemic limbs also showed that RE-01 cell transplantation improved blood flow and promoted angiogenesis.

The combination of RE-01 cells, which have high angiogenic capabilities, and ADSCs, which excel in fat regeneration, is expected to have complementary effects. RE-01 cells may promote early vascular formation, whereas ADSCs support fat tissue regeneration, potentially supplementing insufficient vascular formation in conventional fat grafting and improving engraftment rates. Additionally, this combination may reduce the number of cells required for each cell type. However, the specific effects of this novel approach still need to be elucidated. Thus, this study aimed to evaluate the impact of combining RE-01 cells and ADSCs on the engraftment rate of fat grafts and explore the potential of this approach as a new option for breast reconstruction surgery. We hypothesized that combining RE-01 with ADSCs might promote angiogenesis and improve the fat grafting rate, consequently reducing the number of ADSCs required.

2. Methods

2.1. Culturing RE-01 cells

This study was approved by the Ethics Committee of Juntendo University (approval number: 16–102) and was conducted in accordance with the ethical principles embodied in the Declaration of Helsinki. Written informed consent was obtained from all participants prior to their enrollment in the study.

RE-01 cells were produced as previously reported [2]. Briefly, peripheral blood samples were collected from healthy volunteers were used to isolate mononuclear cells via Ficoll density gradient centrifugation with Histopaque-1077 (Sigma, St. Louis, Missouri). Residual blood cells were lysed with an ammonium-chloride-potassium lysis buffer. After two washes, cells were resuspended in phosphate-buffered saline (PBS). Peripheral blood mononuclear cells were cultured in Iscove's modified Dulbecco's medium containing GlutaMAX (Gibco, Thermo Fisher Scientific, USA) supplemented with 0.5 % fetal bovine serum (FBS, Cytiva, Tokyo, Japan), 0.5 % human serum albumin (Japan Blood Products Organization, Tokyo, Japan), human recombinant vascular endothelial growth factor (50 ng/mL), human recombinant thrombopoietin (20 ng/mL), human recombinant Fms-related tyrosine kinase-3 ligand (100 ng/mL) (all from PeproTech, New Jersey), and antibiotics (penicillin-streptomycin). The cells were incubated at 37 °C in a 5 % CO_2 humidified atmosphere for 5 days without changing the media. After 5 days, the RE-01 cells were harvested by pipetting and washed with ethylenediaminetetraacetic acid-PBS.

2.2. ADSC isolation and culture

Adipose tissue from the abdomen and thighs discarded during breast reconstruction surgery or liposuction for esthetic purposes was used. The adipose tissue was washed with PBS, mixed with 0.1 % collagenase IA solution, and agitated for 30 min. An ADSC medium (Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % FBS and 1 % antibiotics) was added, and cells were centrifuged (1200 *g*, 10 min, 24 °C) to isolate ADSCs. The isolated ADSCs were cultured in ADSC medium, and passages 4–6 were used for the experiments.

2.3. Flow cytometry

ADSCs and RE-01 cells were suspended in fluorescence-activated cell sorting (FACS) buffer (PBS supplemented with 2 mM ethylenediaminetetraacetic acid and 2 % fetal bovine serum). After treatment with an Fc receptor blocking reagent (Miltenyi Biotec, Auburn, California), RE-01 cells were stained with specific antibodies as follows: fluorescein isothiocyanate (FITC) anti-human CD19, peridinin chlorophyll protein/cyanine (Cy) 5.5 anti-human CCR2, brilliant violet (BV) 421 anti-human CD56, PE anti-human CD34, PE/Cy7 anti-human CD206, APC anti-human CXCR4, AlexaFluor-700 anti-human CD3, allophycocyanin (APC)/cyanine 7 (Cy7) anti-human CD14, FITC anti-human CD4, PerCP/Cy5.5 anti-human CD25, BV421 anti-human CD127 (IL-7Ra), PE/Cy7 anti-human CD31(all from BioLegend, San Diego, California) and appropriate isotype controls. Additionally, ADSCs were stained with the following antibodies: FITC anti-human CD73, BV421 anti-human CD90, and PE anti-human CD45, PE anti-human CD34, PE/Cy7 anti-human CD31 (all from BioLegend) and appropriate isotype controls. After 30 min of incubation at 4 C and washing with FACS

buffer, the cells were analyzed using a BD FACSCelesta Flow Cytometer (BD Biosciences) and FlowJo™ software.

2.4. ADSC and RE-01 cell co-culture

ADSCs (passages 4–6) were seeded in 6-well plates, and RE-01 cells were seeded in inserts (0.4 μm pore size) placed above them. The ratio of ADSCs to RE-01 cells was set at 1:0.5 and 1:0.1. After 3 days of co-culture in DMEM supplemented with 10 % FBS and 1 % antibiotics, the inserts were removed to separate the RE-01 cells. Only the ADSCs remaining in the 6-well plates were collected for subsequent assays.

2.5. PCR analysis of ADSCs after co-culture

After 3 days of co-culture, mRNA was extracted from ADSCs using an RNeasy Micro Kit (Qiagen, Hilden, Germany), and cDNA was synthesized using a high-capacity RNA-to-cDNA Kit (Applied Biosystems, Thermo Fisher Scientific). Gene expressions of insulin-like growth factor 1 (*IGF-1*), interleukin (*IL*)-*1β*, and A Disintegrin and Metalloproteinase with Thrombospondin Motifs 1 (*ADAMTS1*) were analyzed using the THUNDERBIRD qPCR probe mix (TOYOBO, Osaka, Japan) and StepOne Plus (Applied Biosystems). The Taqman probe and primer mixtures used were *18S-rRNA* (Hs03928990_g1), *IGF-1* (Hs00153126_m1), *IL-1β* (Hs01555410_m1), and *ADAMTS1* (Hs00199608_m1) (all obtained from Thermo Fisher Scientific).

2.6. Adipose differentiation of ADSCs

After 3 days of co-culture, the inserts were removed, and only ADSCs were cultured in adipogenic induction medium (DMEM supplemented with 10 % FBS, 1 μm dexamethasone, 0.5 mM IBMX, 60 μm indomethacin, and 10 μg/mL human recombinant insulin). After 3 days of induction, RNA was extracted, and gene expressions of fatty acid binding protein 4 (*FABP4*) (Hs01086177_m1) and peroxisome proliferator-activated receptor gamma (*PPARγ*) (Hs01115513_m1) were analyzed following the method described above. For adipose staining, after 2 weeks, ADSCs were fixed in 4 % PFA and dehydrated in 60 % isopropanol for 1 min. The cells were stained with Oil Red O staining solution (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) at 20–25 °C for 20 min and washed with H_2O. Subsequently, nuclear staining was performed using Mayer's hematoxylin stain. Images were obtained using a fluorescence microscope (BZ-X710; Keyence Corp., Osaka, Japan).

2.7. Tube formation assay of ADSCs and human umbilical vein endothelial cells (HUVECs) after co-culture

HUVECs were purchased from Lonza (Switzerland) and cultured in EGM-2 MV medium (Lonza) according to the manufacturer's protocol. The tube formation assay was conducted using two methods. The first method involved adjusting the ratio of ADSCs to RE-01 cells to 1:0.1 and 1:0.5 and directly mixing them with HUVECs. The second method involved co-culturing ADSCs and RE-01 cells at ratios of 1:0.1 and 1:0.5, followed by mixing the co-cultured ADSCs with HUVECs. In both methods, HUVECs (20,000 cells/well) were incubated with either naïve ADSCs and RE-01 cells (total of 4500 cells/well) or co-cultured ADSCs (4500 cells/well) on Matrigel-coated 24-well plates at 37 °C in 5 % CO_2. Tube formation was observed and quantified every hour using a time-lapse microscope (IX83; Olympus, Tokyo, Japan).

2.8. Fat grafting and tissue harvesting using nude mice

Animal experiments were approved by the Juntendo Animal Care and Ethical Committee (approval number: 1319) and were performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Eight-week-old female BALB/cJJcl nu-nu mice (CLEA Japan Inc., Tokyo, Japan) were divided into three groups: fat-only group (fat grafts alone), ADSC group (fat grafts with ADSCs), and ADSC-co group (fat grafts with ADSCs co-cultured with RE-01 cells). For the ADSC-co group, ADSCs were seeded into 6-well plates, and RE-01 cells were seeded into inserts placed above them, adjusted to a ratio of 1:0.1. After co-culturing for 3 days, the inserts were removed to separate the RE-01 cells, and only the remaining ADSCs were added to the fat grafts. Human liposuction fat tissue was mixed with either ADSCs or RE-01-co-cultured ADSCs suspended in 20 μL PBS at a concentration of $2 \times 10^{\wedge}4$ cells per 200 μL of fat tissue. The mixture was then transplanted into the dorsal region of mice. Each group consisted of four to six mice with identical grafts transplanted bilaterally on the back ($n = 2$ per mouse).

2.9. CT image analysis

Computed tomography (CT) was performed weekly after transplantation using a LaTheta LCT-200 scanner (Hitachi, Tokyo, Japan). Graft volume was calculated by measuring the area of each slice using ImageJ software (version 1.53, National Institutes of Health, Bethesda, MD, USA). The grafted fat was harvested 4 weeks after transplantation for further evaluation.

2.10. Histological analysis

The harvested tissues were fixed in 10 % neutral buffered formalin, paraffin embedded, and cut into 5-μm-thick sections. The sections were then subjected to antigen retrieval in citrate buffer (pH 6.0) using an autoclave at 120 °C for 10 min, followed by endogenous peroxidase blocking with 3 % hydrogen peroxide. After blocking with 5 % normal rabbit serum, the specimens were incubated with a primary antibody against perilipin (PLIN1, N-terminus-specific, GP29, 1:100; PROGEN, Heidelberg, Germany). The secondary antibody used was HRP-rabbit anti-guinea pig (DAKO no. PO141, 1:250; Dako, Glostrup, Denmark). A separate set of specimens was blocked with 2.5 % normal goat serum, followed by incubation with primary antibodies against CD31 (ab28364, 1:50; Abcam, Cambridge, UK) and inducible nitric oxide synthase (iNOS) (NB300-605, 1:20; Novus Biologicals, Littleton, CO, USA). The secondary antibody used was Histofine Simple Stain Mouse MAX-PO(R) (Nichirei Biosciences, Tokyo, Japan).

Each graft was analyzed using a fluorescence microscope (BZ-X710, Keyence Corp.) at 10 × magnification, with 15–30 images taken to cover the entire graft cross-section. Immunohistochemical staining was then performed. First, the total graft area was measured using ImageJ software. Then, for perilipin staining, the percentage of perilipin-positive adipocytes relative to the total graft area was calculated. For CD31 staining, the number of CD31-positive cells per unit graft area was counted. The number of iNOS-positive cells was counted using iNOS staining.

2.11. PCR analysis of fat grafts

RNA extraction and polymerase chain reaction (PCR) were performed on grafts preserved at −30 °C in TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA). RNA was extracted using the RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany) according to the

manufacturer's protocol. cDNA was synthesized from the purified RNA using a high-capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). The expression levels of human platelet endothelial cell adhesion molecule 1 (*PECAM1*) (Hs01065279_m1) and mouse *PECAM1* (Mm01242576_m1) (all obtained from Thermo Fisher Scientific) were assessed using the THUNDERBIRD Probe qPCR Mix (TOYOBO) on a StepOnePlus Real-Time PCR system (Applied Biosystems). Gene expression was determined using the comparative Ct (ΔΔCt) method. The data were normalized to the housekeeping gene mouse 18S rRNA (Mm03928990_g1; Thermo Fisher Scientific). Normalized target quantities in the experimental groups were compared with those in the control group, with the results presented as fold changes relative to the control.

2.12. Statistical analysis

Data were expressed as mean ± standard deviation. One-way ANOVA, two-way ANOVA, and Kruskal-Wallis test, were used for statistical analysis, followed by appropriate multiple comparison tests. Tukey's multiple comparisons test or Dunn's multiple comparison test was applied for post hoc comparisons between groups. The specific statistical methods applied to each analysis are detailed in the figure legends. All statistical analyses were performed using GraphPad Prism 9 (version 9.5.1; GraphPad Software LLC). Statistical significance was set at p-values <0.05.

3. Results

3.1. Expression profiles of key cell surface markers in ADSCs after Co-culture

The profile of RE-01 cells is depicted in Fig. 1A. As previously reported, the presence of larger cells was observed. The RE-01 cells included 15.5 ± 4.8 % CD206-positive M2 macrophages, 30.0 ± 10.3 % CD3+/CXCR4+/CD31+ angiogenic T cells, and 0.437 ± 0.436 % CD34-positive stem cells. Conversely, the inflammatory cell population consisted of 0.37 ± 0.19 % CCR2+ M1 macrophages, 4.2 ± 1.2 % CD19-positive B cells, and 10.3 ± 3.6 % CD56-positive NK cells.

The expression levels of key cell surface markers (CD73, CD90, CD34, CD31, and CD45) in the ADSCs after co-culture with the RE-01 cells showed no significant changes than those of the naïve ADSCs

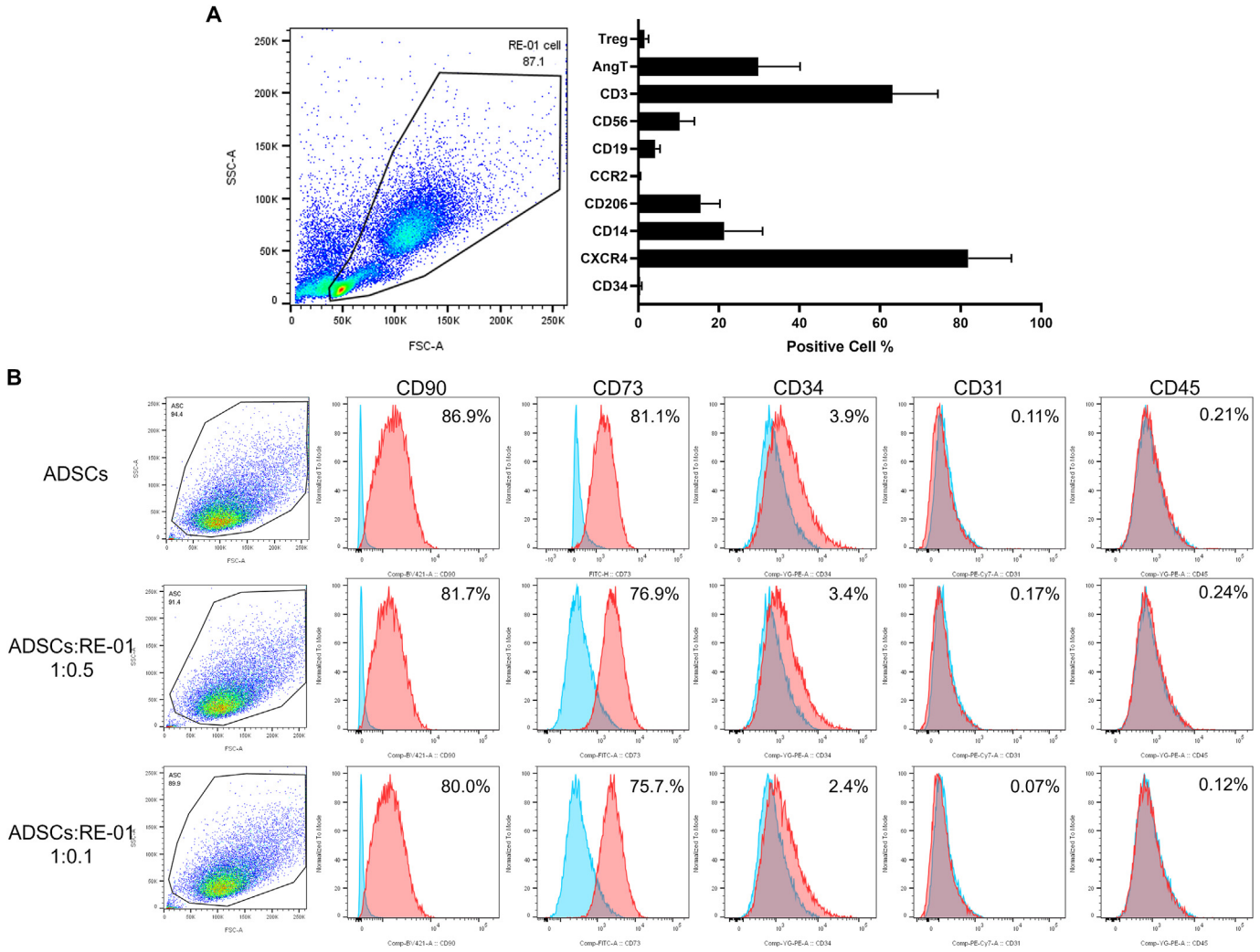

Fig. 1. Characteristics of RE-01 cells and ADSCs after co-culture with RE-01 cells.
A. The representative image of FACS scattergrams of RE-01 cells. The expression of Treg, AngT, CD3, CD56, CD19, CCR2, CD206, CD14, CXCR4 and CD34 in RE-01 cells were analyzed by flowcytometry (n = 6). B, C. The expressions of surface marker on ADSCs and ADSCs after co-culture with RE-01 cells (at a ratio of 1:0.5 and 1:0.1) were analyzed by flowcytometry. In all cases, CD90 and CD73 were positive, while CD34, CD31, and CD45 were negative (red). Blue lines indicate isotype control (n = 1). ADSCs, adipose-derived stem cells; RE-01, regenerative endothelial cells.

(cultured alone) (Table 1). Notably, CD73 and CD90 maintained consistently high expression levels even after co-culture. Additionally, variations in the expression levels attributed to the co-culture ratios (1:0.5 and 1:0.1) were minimal, and other markers, such as CD34 and CD45, exhibited very low expression levels (Fig. 1B).

3.2. Effects of RE-01 cells on tube formation and angiogenesis-related gene expression in ADSCs

The number of tubular structures formed by HUVECs was significantly higher in the group mixed with ADSCs and RE-01 cells at ratios of 1:0.5 (1.72 ± 0.22 vs 0.99 ± 0.42, $p < 0.05$) and 1:0.1 (1.76 ± 0.23 vs 0.99 ± 0.42, $p < 0.01$) compared to the ADSC group. (Fig. 2A and B). Further, the number of tubular structures formed by HUVECs was significantly higher in the group co-cultured with ADSCs and RE-01 at ratios of 1:0.5 (1.63 ± 0.14 vs 1.14 ± 0.18, $p < 0.01$) and 1:0.1 (1.51 ± 0.22 vs 1.14 ± 0.18, $p < 0.01$) compared to the ADSC group (Fig. 2C and D). Real-time PCR showed that the mRNA expression levels of *IGF-1* and *IL-1B*, which are known as pro-angiogenic cytokines, were significantly higher in the ADSC-co group than in the ADSC group (*IGF-1*: at a ratio of 1:0.5, 2.7-fold, $p < 0.01$; at a ratio of 1:0.1, 3.1-fold, $p < 0.01$. *IL-1B*: at a ratio of 1:0.5, 5.7-fold, $p < 0.01$; at a ratio of 1:0.1, 5.6-fold, $p < 0.05$). The mRNA expression level of the anti-angiogenic gene *ADAMTS1* was slightly higher at a ratio of 1:0.1 (at a ratio of 1:0.5, 1.3-fold, $p = 0.05$; at a ratio of 1:0.1, 1.4-fold, $p < 0.05$) (Fig. 2E). However, no significant differences in the effect based on the cell ratio during co-culture were observed.

3.3. Promotion of adipogenic differentiation in ADSCs co-cultured with RE-01 cells

The mRNA expression levels of *FABP4* and *PPARγ* were significantly increased in ADSCs co-cultured with RE-01 cells at a ratio of 1:0.1 (1.4-fold, $p < 0.01$; 1.3-fold, $p < 0.01$) (Fig. 3A). A similar trend was observed at a ratio of 1:0.5; however, the increase did not reach statistical significance (1.2-fold, $p = 0.10$; 1.1-fold, $p = 0.26$) (Fig. 3A). Furthermore, the lipid droplet area was increased in ADSCs co-cultured with RE-01 cells at a ratio of 1:0.1 compared to ADSCs cultured alone (1.55-fold, $p < 0.05$), and a similar trend was observed at a ratio of 1:0.5, although it did not reach statistical significance (1.37-fold, $p = 0.095$) (Fig. 3B and C).

3.4. Fat engraftment rate in nude mice with adipose tissue transplantation supplemented with ADSCs co-cultured with RE-01 cells

In the fat-only and ADSC groups, the volume of transplanted fat tissue decreased from day 7 onward. In contrast, the volume was consistently significantly higher on day 21 (89.56 ± 7.96 vs 79.91 ± 8.20, $p < 0.05$; 89.56 ± 7.96 vs 73.15 ± 10.55, $p < 0.01$) and day 28 (84.58 ± 10.90 vs 70.44 ± 13.70, $p < 0.05$; 84.58 ± 10.90 vs 69.01 ± 14.02, $p = 0.05$) in the ADSC-co group than in the fat-only and ADSC groups (Fig. 4A–C).

Table 1
Fluorescence-activated cell sorting analysis results of the ADSCs after co-culture with the RE-01 cells.

	ADSCs	ADSCs:RE-01 1 : 0.5	ADSCs:RE-01 1 : 0.1	P value
CD90+ %	96.70 ± 3.270	95.48 ± 3.913	94.57 ± 6.213	0.731
CD73+ %	92.25 ± 4.195	90.81 ± 4.494	91.20 ± 4.585	0.170
CD34+ %	10.71 ± 9.967	16.76 ± 11.26	16.30 ± 10.59	0.560
CD31+ %	0.115 ± 0.075	0.135 ± 0.080	0.197 ± 0.257	0.666
CD45+ %	15.65 ± 13.16	17.22 ± 13.74	16.23 ± 13.40	0.979

Notes: Values are expressed as mean ± SD. n = 6. Ordinary one-way ANOVA and Tukey's multiple comparisons test were used. ADSCs, Adipose-derived mesenchymal stem cells.

3.5. Enhanced fat graft quality and microvessel formation with reduced inflammation by ADSCs co-cultured with RE-01 cells

The proportion of perilipin-positive viable fat cells was significantly higher in the ADSC-co group than in the fat-only group (11.93 ± 5.96 vs 6.67 ± 2.40, $p < 0.05$) and ADSC group (11.93 ± 5.96 vs 6.09 ± 3.28, $p < 0.05$) (Fig. 5A, D). Moreover, the number of CD31-positive microvessels was significantly higher in the ADSC-co group than in the fat-only group (72.95 ± 16.98 vs 34.19 ± 22.60, $p < 0.01$) and ADSC group (72.95 ± 16.98 vs 46.67 ± 22.46, $p < 0.05$) (Fig. 5B, D). Additionally, the number of iNOS-positive cells was significantly lower in the ADSC-co group than in the fat-only group (14.08 ± 8.90 vs 28.67 ± 9.93, $p < 0.01$) and ADSC group (14.08 ± 8.90 vs 26.00 ± 12.05, $p < 0.05$) (Fig. 5C and D).

3.6. Angiogenesis-related gene expression in transplanted fat grafts suggests mouse origin

When human-specific probes were used, angiogenesis-related gene expression in the ADSC and ADSC-co groups did not show a significant difference when compared with that in the fat-only group (ADSC group: 0.3-fold, $p = 0.27$; ADSC-co group: 0.5-fold, $p = 0.46$, respectively) (Fig. 6A). However, when mouse-specific probes were used, *PECAM1* expression was significantly higher in the ADSC-co group (ADSC group: 0.7-fold, $p = 0.90$; ADSC-co group: 2.4-fold, $p < 0.05$, respectively) (Fig. 6B).

4. Discussion

This study addresses a critical challenge in post-mastectomy breast reconstruction: the inconsistent success rates of conventional fat grafting techniques. While fat grafting offers a natural approach to breast reconstruction—a procedure of profound psychological significance for breast cancer survivors—current methods demonstrate variable engraftment efficiency, limiting their reliability and predictability. To overcome these limitations, we investigated a novel approach in our research: the combination of angiogenesis-promoting RE-01 cells with ADSCs. We hypothesized that this combination would enhance the survival and integration of fat grafts, potentially offering a more dependable solution for breast reconstruction that addresses the shortcomings of traditional methods. Our findings indicated that co-culturing ADSCs with RE-01 cells for 3 days induced significant changes in the gene expression and function of ADSCs. Specifically, the expression of angiogenesis-related genes (*IGF*-1 and *IL-1β*) increased, and the angiogenic potential was enhanced. Additionally, the adipogenic differentiation potential of ADSCs was promoted. Flow cytometry results suggest that co-culturing with RE-01 cells is unlikely to have a significant impact on the cell profile of ADSCs, indicating that they remain almost identical to naïve ADSCs. These results suggest that the interaction between RE-01 cells and ADSCs influences their physiological functions. Importantly, the findings of this study can significantly contribute to improving the QOL of patients with breast cancer.

The increased expression of angiogenesis-related genes, namely, *IGF*-1 and *IL-1β*, is noteworthy as these genes play crucial roles in angiogenesis. *IGF*-1 promotes cell proliferation and tissue repair while inducing the migration and proliferation of endothelial cells [13,14]. *IL-1β*, a key regulator in inflammatory responses, also plays an important role in the process of angiogenesis [15,16]. In this

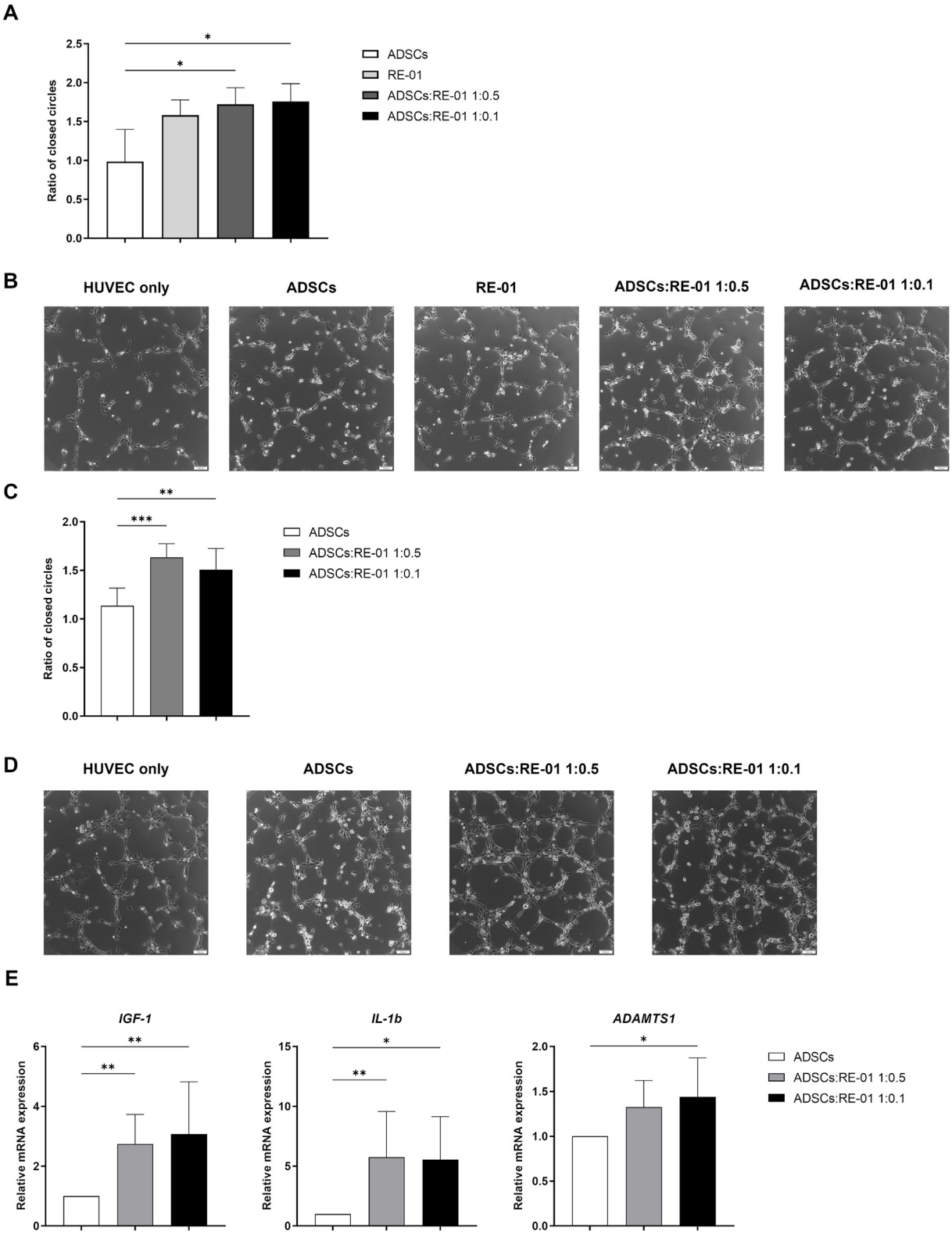

Fig. 2. Analysis of angiogenic potential of ADSCs.

A. The ratio of closed circles formed in the tube formation assay is measured after directly mixing HUVECs with ADSCs and RE-01 cells at ratios of 1:0.1 and 1:0.5 (n = 3). B. Representative images of tube formation assay in each group. C. The ratio of closed circles formed in the tube formation assay is measured after mixing HUVECs with ADSCs that have been co-cultured with RE-01 cells at ratios of 1:0.1 and 1:0.5 for 3 days (n = 6). D. Representative images of each group from the tube formation assay. Data in A and C are presented as mean ± SD. Means are compared using Ordinary one-way ANOVA and Tukey's multiple comparisons test. E. The mRNA expression levels of angiogenesis-related genes (*IGF-1*, *IL-1B*, and *ADAMTS1*) are analyzed using real-time PCR in ADSCs co-cultured with RE-01 cells for 3 days (n = 6). Data are presented as mean ± SD. Mean values are compared using Kruskal-Wallis test and Dunn's multiple comparison test. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant

ADSCs, adipose-derived mesenchymal stem cells; HUVECs, human umbilical vein endothelial cells; *IGF-1*, insulin-like growth factor 1; *IL-1β*, interleukin-1 beta; *ADAMTS1*, A Disintegrin and Metalloproteinase with Thrombospondin Motifs 1.

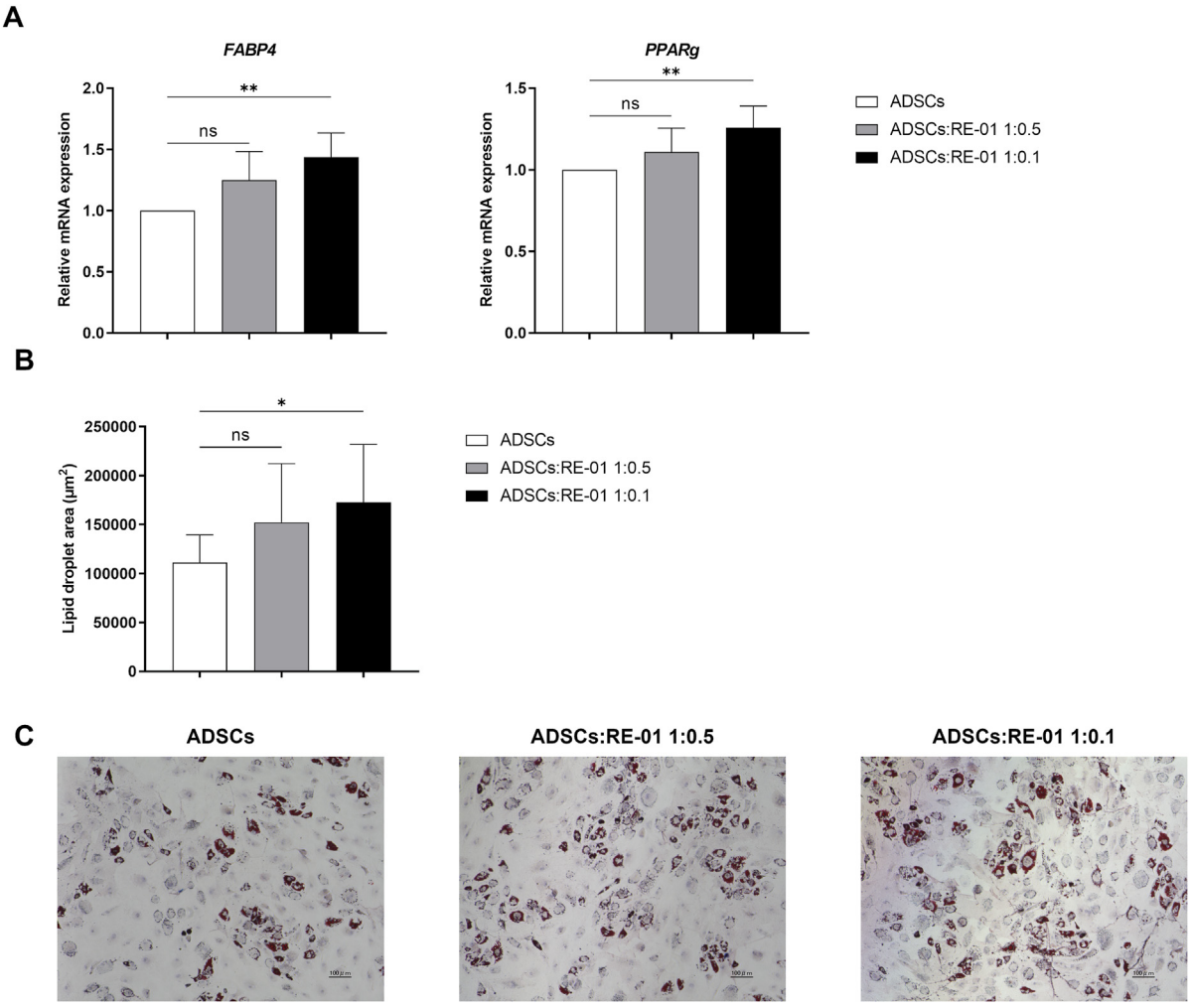

Fig. 3. Analysis of adipogenic differentiation potential of ADSCs.
A. The expression levels of adipogenic differentiation-related genes (*FABP4* and *PPARγ*) are analyzed using real-time PCR in ADSCs co-cultured with RE-01 cells for 3 days and induced for adipogenic differentiation (n = 6). Values are compared using Kruskal-Wallis test and Dunn's multiple comparison test. B. Quantitative analysis of Oil Red O staining was performed to evaluate lipid accumulation in ADSCs after 2 weeks of adipogenic differentiation induction. The results are shown as the mean intensity of staining for each group (n = 4). Values are compared using repeated measures one-way ANOVA, and Tukey's multiple comparisons test. C. Representative images of Oil Red O staining for each group are shown. Lipid droplets in differentiated adipocytes appear red. Data are presented as the mean ± SD. *p < 0.05; **p < 0.01; ns, not significant
ADSCs, adipose-derived mesenchymal stem cells; *FABP4*, fatty acid binding protein 4; *PPARγ*, peroxisome proliferator-activated receptor gamma.

study, the expression levels of *ADAMTS1,* an anti-angiogenic gene, showed a slight increase. However, the expression levels of pro-angiogenic genes, such as *IGF-1* and *IL-1β,* increased significantly, outweighing the effects of *ADAMTS1* and suggesting an overall promotion of angiogenesis. Our findings suggest that RE-01 enhances the angiogenic potential of ADSCs by upregulating the expression of these genes. Regarding the promotion of adipogenic differentiation, ADSCs are known to be multipotent stem cells capable of differentiating into adipocytes, osteocytes, and chondrocytes [17]. Adipogenic differentiation is essential for the formation and function of adipose tissue. The significant increase in mRNA expression of *FABP4* and *PPARγ* in ADSCs co-cultured with RE-01 cells indicates that RE-01 cells may influence the differentiation trajectory of ADSCs [18,19]. This suggests that ADSCs differentiate into adipose tissue cells, a crucial process in fat tissue formation and repair [17]. It is possible that RE-01 cells transmit adipogenic differentiation signals to ADSCs through specific secretions, resulting in increased expression of *FABP4* and *PPARγ*.

Furthermore, the more red-stained lipid droplets on Oil Red O staining in the ADSC-co group is considered to be the result of enhanced adipogenic differentiation owing to co-culture. The enhanced adipogenic differentiation of ADSCs by RE-01 suggests that RE-01 cells are involved in inducing adipogenic differentiation [20,21]. It was previously considered that the co-transplantation of RE-01 cells and ADSCs could achieve effective fat transplantation with high angiogenic and fat regeneration capabilities. However, the results of the current study suggest that it may be possible to use only ADSCs stimulated by co-culturing with RE01 cells for transplantation. The advantage of transplanting ADSCs alone is that they can be used for allogeneic transplantation, whereas the RE-01 cells used for stimulation can also be used for autologous transplantation. Additionally, the volume of transplanted fat tissue in the ADSC-co group is significantly maintained, and the number of CD31-positive microvessels is significantly higher, suggesting enhanced angiogenesis [22]. This indicates that newly formed blood vessels provide appropriate nutrients and oxygen to the transplanted fat tissue, thereby improving the engraftment rate [23].

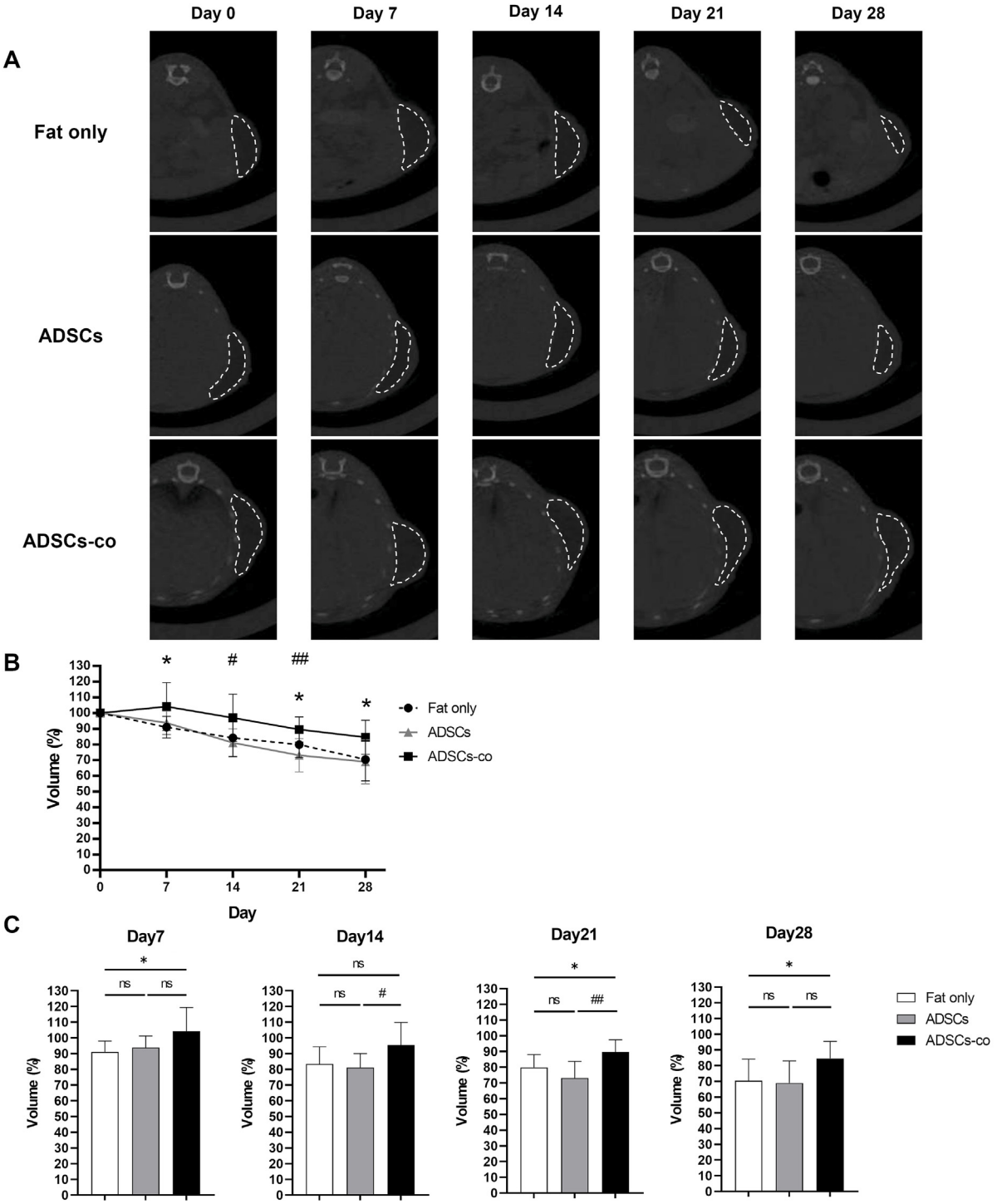

Fig. 4. Changes in graft volume in fat transplantation in nude mice.
The day of fat transplantation in nude mice is designated as day 0, and CT scans are performed on days 0, 7, 14, 21, and 28. A. Representative CT images in each group at each time point. The area enclosed by the white dotted line represents the transplanted adipose tissue. B, C. Graphs showing the percentage change in graft volume relative to day 0 (fat-only group, n = 12; ADSC-co group, n = 12; ADSC group, n = 8). Data are presented as the mean ± SD. Statistical analysis was performed using two-way ANOVA and Tukey's multiple comparison test. *$p < 0.05$ for fat-only group vs ADSC-co group; $^{\#}p < 0.05$ for ADSC group vs ADSC-co group; and $^{\#\#}p < 0.01$ for ADSC group vs ADSC-co group; ns, not significant ADSCs, adipose-derived mesenchymal stem cells.

Moreover, the reduced number of iNOS-positive cells, a marker of inflammatory macrophages, in the ADSC-co group, which showed the highest engraftment rate, suggests that suppression of the inflammatory response may have contributed to the improved engraftment rate, consistent with the results of previous reports [24–26]. Inflammatory macrophages hinder tissue regeneration [27–29], and the lower number of iNOS-positive cells in the ADSC-co group indicates that the inflammatory response in the transplanted tissue is suppressed, promoting the tissue regeneration process. Further, the significantly increased mRNA expression of

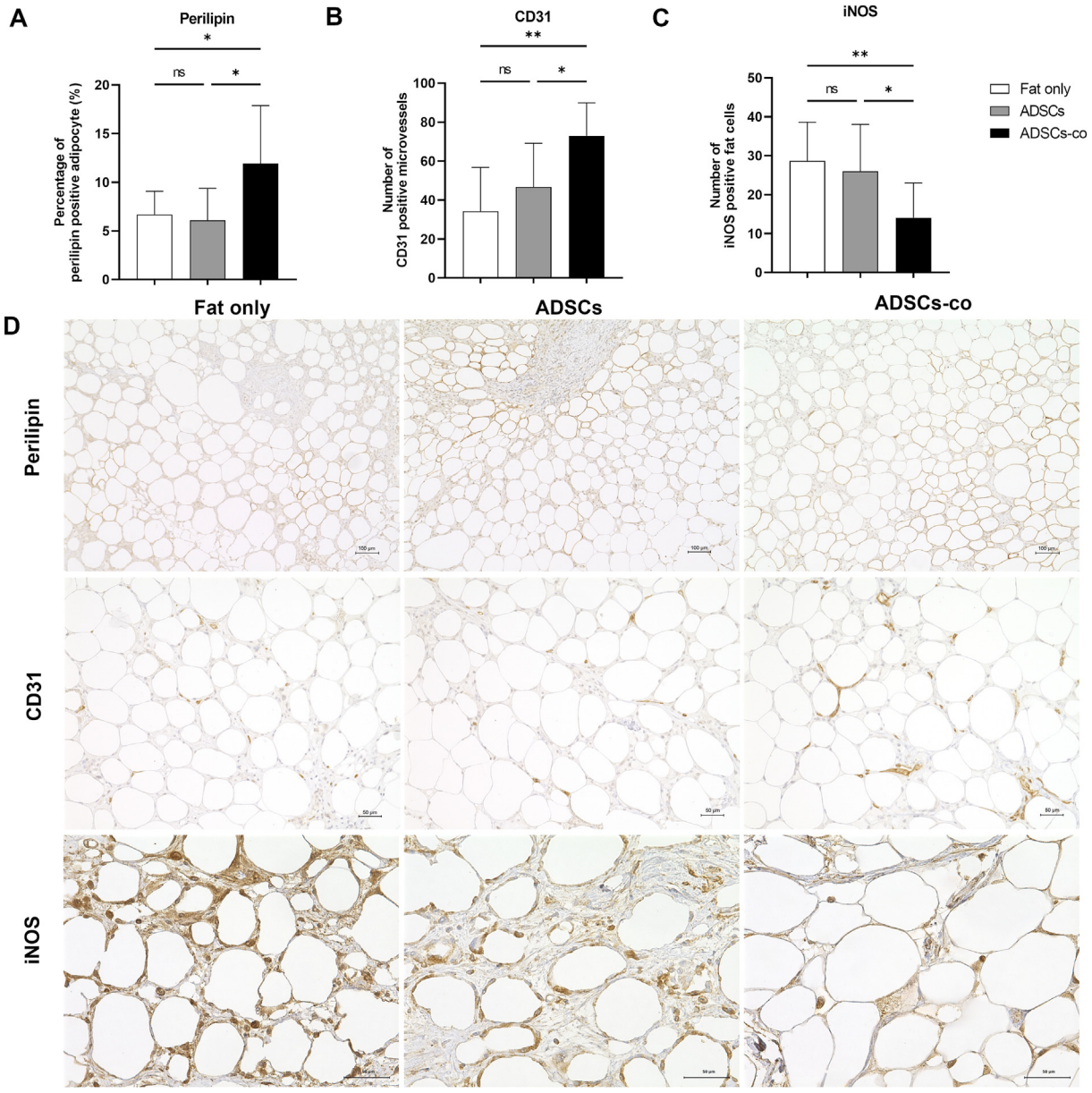

Fig. 5. Tissue analysis of fat grafts with RE-01-co-cultured ADSCs.
Fat grafts are harvested from nude mice 28 days after fat transplantation, and paraffin sections are prepared. Immunohistochemical staining is performed, and the results are quantified. A. Graph showing the percentage of perilipin-positive adipocytes. B. Graph showing the number of CD31-positive microvessels. C. Graph showing the number of iNOS-positive fat cells. D. Representative images of perilipin, CD31, and iNOS staining in each group (perilipin, CD31: n = 8, iNOS: n = 12). Data are presented as the mean ± SD. Mean values are compared using ordinary one-way ANOVA and Tukey's multiple comparison test.
*p < 0.05; **p < 0.01; ns, not significant
ADSCs, adipose-derived mesenchymal stem cells; iNOS, inducible nitric oxide synthase.

PECAM1 in the ADSC-co group supports that angiogenesis is promoted, resulting in an improved engraftment rate. The lack of significant differences in angiogenesis-related gene expression when human probes were used and the significant increase in the expression levels when mouse probes were used suggested that new blood vessels were formed in the transplanted human fat tissue [30,31]. The engraftment rate of fat grafts using ADSCs typically range from 70 % to 80 %, but maintaining this high engraftment rate requires 1000–10,000 ADSCs/μL [32–34]. In this study, an equivalent effect was obtained using 100 ADSCs/μL. Given that isolating ADSCs requires liposuction, the effectiveness of a smaller number of ADSCs indicates the utility of ADSCs co-cultured with RE-01 cells.

However, a limitation of this study is that the specific mechanism by which RE-01 cells affect ADSCs is not elucidated. The proteins and cytokines secreted by RE-01 cells should be identified using mass spectrometry or proteomic analysis to clarify how these factors influence gene expression and function in ADSCs [35,36]. Additionally, the effect of RE01 cells on breast cancer cells is currently unknown, and there is concern regarding the risk of cancer development. Further verification using breast cancer cell lines and mouse models is required to address the

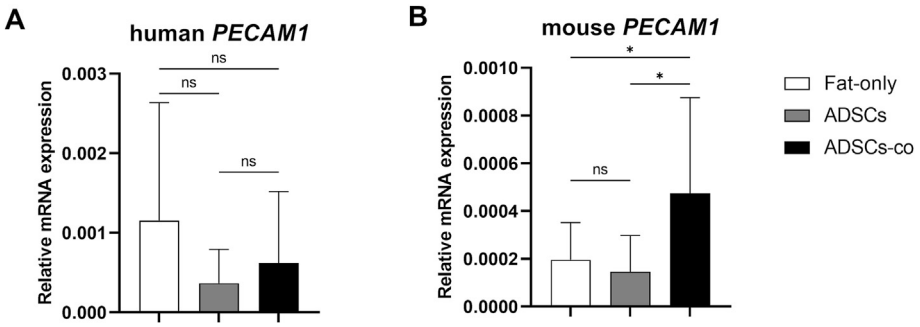

Fig. 6. Analysis of vascular-related genes in transplanted adipose tissue grafts.
The transplanted fat grafts are harvested 28 days after fat transplantation into the dorsal region of nude mice, and changes in the expression of the angiogenesis-related gene *PECAM1* are analyzed using real-time PCR. A. Human-specific *PECAM1* probes. B. Mouse-specific *PECAM1* probes (fat-only group, n = 12; ADSC-co group, n = 12; ADSC group, n = 8). Data are presented as the mean ± SD. Mean values are compared using the ordinary one-way ANOVA and Tukey's multiple comparison test. *p < 0.05; ns, not significant
ADSCs, adipose-derived mesenchymal stem cells; *PECAM1*, platelet endothelial cell adhesion molecule 1.

potential for recurrence or new cancer development after transplantation [37].

5. Conclusion

Co-culturing ADSCs with RE-01 cells, a population with angiogenic and wound healing capabilities, enhances both the angiogenic potential and adipogenic differentiation capacity of ADSCs. In the fat grafting experiments using a mouse model, the volume of transplanted fat tissue is maintained in the ADSC-co group. The improved angiogenesis and suppression of inflammatory responses contributed to an improved engraftment rate. Notably, the use of RE-01-stimulated ADSCs alone in fat transplantation improved the engraftment rate, suggesting that the amount of adipose tissue required for ADSC isolation can be reduced. Our findings indicate that co-culturing ADSCs with RE-01 cells represents a promising new approach to enhancing the efficacy of fat transplantation. This method can potentially improve the outcomes of breast reconstruction and other fat grafting procedures. However, further research is necessary to elucidate the precise mechanisms by which RE-01 cells influence ADSCs and to verify the long-term safety of this approach. In conclusion, the use of RE-01-stimulated ADSCs in fat transplantation offers a novel strategy for enhancing fat graft survival and quality. Although these results are encouraging, additional studies are required to fully understand the underlying mechanisms and ensure the safety and efficacy of this approach before its clinical application.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors did not use generative AI or AI-assisted technologies in writing this manuscript.

Declaration of competing interest

Rica Tanaka is a Chief Executive Officer of ReEir Inc. ReEir Inc. is the startup company to industrialize RE-01 cells.

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