

Adipogenesis Induced by Human Adipose Tissue-Derived Stem Cells

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Adipose tissue-derived stem cells (ASCs), including preadipocytes, may play an important role in *de novo* adipogenesis and are expected to be a useful external source of cells for adipose tissue engineering. In this study, we examined *in vivo* adipogenesis up to 24 weeks after implantation, induced by human ASCs that were isolated from adipose tissues and expanded *in vitro*. ASCs proliferated *in vitro* in the presence of basic fibroblast growth factor (bFGF), and the number of cells increased by more than 1000-fold at the fourth passage. The ability to differentiate into mature adipocytes was maintained up to the third passage. We incorporated designated numbers of third-passage-expanded cells into a type I collagen scaffold and implanted them into the back of nude mice with or without controlled-release bFGF. After the implantation of 2×10^6 ASCs with controlled-release bFGF, the greatest cross-sectional surface area of adipose tissue in the scaffold was 1.19 mm^2 at 12 weeks and 2.14 mm^2 at 24 weeks. About 2×10^6 ASCs with controlled-release bFGF was the best condition for total adipogenesis. Immunohistochemical analysis with antihuman vimentin antibody showed that the area of human-origin adipose tissue was maximum in the group with 8×10^6 ASCs incorporated in a scaffold at both 12 and 24 weeks. The amount of human-origin adipose tissue increased in all groups with implanted ASCs from 12 to 24 weeks. Only trace of human-origin adipose tissue was observed in other groups implanted ASCs. Our results show that human ASCs not only function as progenitor cells for *in vivo* adipogenesis, but also induce *de novo* adipogenesis for long period.

Introduction

BREAST CANCER IS THE MOST COMMON CANCER in women, and surgery remains one of the main treatments. Breast surgery results in deformity of the breast and negatively affects patients' quality of life. Perforator flaps or silicone implants have been used for breast reconstruction, but each has advantages and disadvantages. Several trials of autologous adipose tissue transplantation for breast reconstruction resulted in a 40–60% reduction in adipose tissue volume because of insufficient vascularization.^{1–6}

Recent studies in tissue engineering indicate that cell proliferation requires an appropriate cell source, scaffold, and microenvironment, including growth factors.^{7,8} The ideal cell source for tissue engineering must have self-renewal capability and immunocompatibility.⁹ Mesenchymal stem cells (MSCs) isolated from bone marrow stroma can differentiate into adipogenic, osteogenic, myogenic, and chondrogenic lineages. However, the procurement of cells from bone marrow that are suitable for clinical use has several drawbacks,

including severe pain, morbidity, and a low yield.¹⁰ Adipose tissue-derived stem cells (ASCs) can be isolated from collagenase digests of adipose tissue. Various kinds of term have been used for this cell population, for example, adipose-derived stem/stromal cells, adipose-derived adult stem cells, preadipocytes, processed lipoaspirate cells, and adipose mesenchymal stem cells. The International Fat Applied Technology Society reached a consensus to adopt the term “adipose-derived stem cells” to identify the isolated, plastic-adherent, multipotent cell population.¹¹ ASCs also can differentiate into adipogenic, osteogenic, myogenic, and chondrogenic lineages similar to MSCs.¹² Moreover, a comparison of MSCs and ASCs from the same patient showed no significant differences in the yield of adherent stromal cells, growth kinetics, cell senescence, multilineage differentiation capacity, or gene transduction efficiency.¹³ Gene array analysis revealed that less than 1% of genes were differentially expressed between ASCs and MSCs. ASCs were superior to MSCs with respect to maintenance of proliferating ability.¹⁴ The fraction of preadipocytes contributing to adipogenesis

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differs among species and ages. In humans the fraction is 1% in children and less than 0.1% in adults.¹⁵ Human adipose tissue is abundant and can be obtained more safely and easily under local anesthesia or at breast surgery than bone marrow cells.

During differentiation into mature adipocytes, preadipocytes express several types of extracellular matrix (ECM) proteins, including fibronectin, laminin, and types I, III, IV, V, and VI collagen. A fibronectin network develops initially, and a type I collagen network is formed last. These ECMs allow preadipocytes to differentiate into mature adipocytes.¹⁶ In response to adipogenic stimulation, ASCs form tissue sheets harboring lipid-filled adipocytes embedded into an abundant human ECM.¹⁷ *In vivo* adipogenesis depends on the type of ECM.¹⁸ Type I collagen has been widely used as a scaffold for adipose tissue engineering^{19–22} because of its porous structure. Preadipocytes readily adhere to and grow in type I collagen scaffolds.²³ An *in vitro* study reported a higher rate of adipogenic differentiation with type I collagen than with fibronectin.²⁴

In vitro proliferation of ASCs is enhanced by basic fibroblast growth factor (bFGF).²⁵ Although it remains controversial whether bFGF has direct adipogenic activity,^{26–29} bFGF has been shown to promote adipogenesis *in vitro* and *in vivo*.^{30–32} We found that the controlled-release bFGF more effectively promoted adipose tissue regeneration than aqueous bFGF.²⁰ We have reported that controlled-release 1 µg of bFGF/site was the most effective concentration on adipogenesis 6 weeks after implantation into nude mice, and a high dose of bFGF caused the inflammatory response in the collagen scaffold.²⁰

In a previous study, we implanted up to 5×10^5 human ASCs into nude mice and obtained newly formed adipose tissue 6 weeks after implantation. However, quantitative and qualitative differences in the implanted ASCs were not examined, and not examined for long time. The present study was therefore investigated the optimal passage number *in vitro* and the effects of the number of implanted ASCs on adipogenesis *in vivo* over the period up to 24 weeks.

Materials and Methods

Human ASCs

This study was approved by the Kyoto University ethics committee. Informed consent was obtained from all patients. All patients were women 29–76 years of age. Samples of human adipose tissues were obtained as surgical waste tissue at breast surgery in Kyoto University Hospital (Kyoto, Japan). Donor samples for *in vitro* study were obtained from patients 30–76 years of age with a mean age of 58.6 years ($n = 14$). We allocated seven donors individually to with or without bFGF treatment group. There was no significance of age between with and without bFGF treatment groups. Donor samples for *in vivo* study were obtained from patients 29–70 years of age with a mean age of 50.8 years ($n = 8$). All donor samples were divided equally. ASCs were isolated from the adipose tissue samples as soon as possible after resection by a modification of the procedure described by Bjorntorp *et al.*³³ Briefly, the adipose tissue samples were washed with phosphate-buffered saline (PBS, pH 7.4) to remove blood cells, minced, and digested with collagenase (2 mg/mL; Wako Pure Chemical, Osaka, Japan) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) and 20 mg/mL bovine serum albumin at 37°C for 40 min while shaking. The digested

tissue was suspended in DMEM:Nutrient Mixture F-12(Ham) (1:1) (DMEM/F-12) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (0.1 mg/mL) (basal medium). The suspension was filtered through a 250-µm nylon mesh and centrifuged at 400 g for 10 min at 20°C. The sediment was suspended in basal medium, placed in 10-cm tissue culture dishes (Falcon; Falcon, New York, NY), and cultured in a humidified atmosphere of 95% air and 5% CO₂ at 37°C for 1 day. The dishes were gently washed with PBS to remove nonadherent cells and filled with the basal medium and cultured until the adherent ASCs became confluent (P0: Passage 0). Then ASCs were detached with 1% trypsin-EDTA solution (Sigma, St. Louis, MO).

Proliferation ability of ASCs

ASCs were suspended in the basal medium, placed in 10-cm tissue culture dishes or 96-well microtiter plate (Falcon) at the density of 1.0×10^4 cells/cm², and cultured with 100 ng/mL bFGF or without bFGF for 1 week.³⁴ To evaluate proliferative activity, viable cells in each dish were counted by the trypan blue dye exclusion method at the end of culture. These numbers represent the proliferation of undifferentiated ASCs. The proliferation of ASCs was also evaluated by MTT assay.³⁵ Using a commercially available kit for MTT assay (Chemicon International, Temecula, CA), we spectrophotometrically measured the absorbance of the solution mixture in each well at 570–630 nm.

Differentiation ability of ASCs

ASCs suspended in basal medium were placed in 24-well plates (Falcon) at a density of 1.0×10^5 cells/cm² and cultured for 1 day. The medium was then changed to DMEM/F-12 medium (1000 µL/well) containing 0.05 µM insulin, 0.2 nM 3,5,3-triiodothyronine, 100 nM transferrin, 17 µM calcium pantothenate, 33 µM biotin, and 100 nM dexamethasone (ITT medium)³⁶ and cultured for 21 days. To evaluate adipogenic differentiation of ASCs, glycerol-3-phosphate dehydrogenase (GPDH) activity was measured using a commercially available kit (GPDH activity measurement kit, JFL003; Hokudo, Hokkaido, Japan).³⁷ ASCs were washed twice with PBS and homogenized in the buffer solution included with the kit, using a handy sonic homogenizer (UR-20; Tomy Seiko, Tokyo, Japan) on ice. After mixing, the absorbance of the solution mixture was spectrophotometrically measured at 340 nm.

Materials

We prepared a disc form of type I collagen scaffold (diameter, 20 mm; height, 2.5 mm) and gelatin microspheres (isoelectric point, 5.0), as described in our previous report.^{20,38} An aqueous solution of human recombinant bFGF was kindly supplied by Kaken Pharmaceutical, Tokyo, Japan. Other chemicals were purchased from Wako Pure Chemical Industries, Kyoto, Japan, and used without further purification. To prepare controlled-release bFGF, 2 mg of gelatin microspheres was swollen with an aqueous solution of bFGF (20 µL, containing 1 µg of bFGF) and allowed to stand at 37°C for 1 h.

Implantation of ASCs

Animal experiments were reviewed by the Committee on the Ethics of Animal Experiments (Faculty of Medicine,

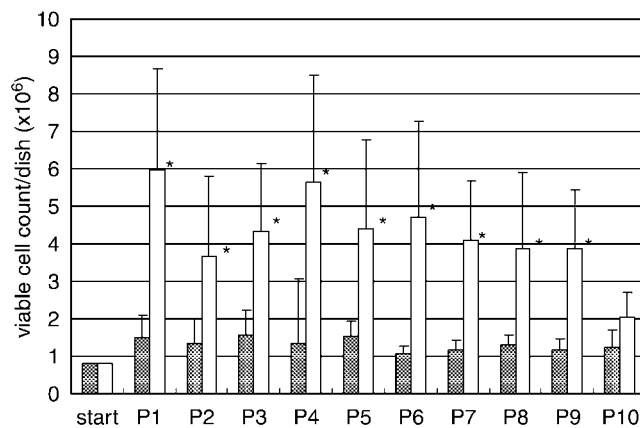


FIG. 1. Proliferation of ASCs *in vitro* assessed on the basis of the viable cell count. The number of viable ASCs per 10 cm of the tissue culture dish after culture with 100 ng/mL bFGF (open bars) or without bFGF (closed bars) for 1 week was measured by trypan blue dye exclusion assay. *: $p < 0.05$ versus without bFGF group.

Kyoto University, Kyoto, Japan) and were carried out in accordance with the Guidelines for Animal Experiments of the Faculty of Medicine, Kyoto University.

The designated number of third-passage ASCs (0: A group and B group; 5×10^5 : C group and D group; 2×10^6 : E group and F group; 8×10^6 : G group and H group) were incorporated into the collagen scaffolds with (B, D, F, and H groups) or without controlled-release bFGF (A, C, E, and G groups), and implanted subcutaneously into the back of 6-week-old female BALB/c nude mice (Shimizu Laboratory Supply, Kyoto, Japan) under general anesthesia. Twelve-week group consisted of five mice. Twenty-four-week group consisted of three mice. Twelve and 24 weeks after implantation, the mice were euthanized with an overdose of anesthesia, and the implanted sites including the skin (approximately $2 \times 2 \text{ cm}^2$) were carefully removed for subsequent histological examinations.

De novo adipogenesis and human ASC-derived adipogenesis

One half of each tissue specimen was fixed in 10% neutralized formalin solution and embedded in paraffin. Sections (thickness, $2 \mu\text{m}$) of the specimens were stained with hematoxylin and eosin (H-E). The other half of the specimen was frozen, and sections were stained with oil red O to confirm the presence of mature adipose tissue. Paraffin sections were stained with a monoclonal antibody against human vimentin (mahv, clone V9, Code Nr. M 0725 Lot 057; DAKO, Glostrup, Denmark). The antibody was used at a dilution of 1:25. The positive control was human adipose tissue, and the negative control was murine adipose tissue. Adipose tissue area of the scaffolds and the human vimentin-positive area were measured and analyzed with the computer program Image-Pro Plus (Media-Cybernetics, Bethesda, MD). We took pictures of H-E sections with Axio Vision (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) software, and opened these pictures with Image-Pro Plus. We measured the area of scaffolds and newly formed adipose tissue with the manual

measurement function. Scaffolds were stained with H-E, but lipid droplets were not stained. We excluded fibroblastic capsules around the scaffolds. Morphologically, collagen scaffolds have a net-like structure, adipose tissue has a granulated structure, and fibroblastic capsules have a layered structure. The average area of the scaffolds was 2.14 mm^2 and did not differ significantly among the groups.

Statistical analysis

The Mann-Whitney U-test (Microsoft Excel, Statcel2) was employed for statistical analysis, and $p < 0.05$ was considered to indicate statistical significance.

Results

Proliferative activity of ASCs

The proliferative activity of the ASCs *in vitro* was retained through the 10th passage as assessed by the viable cell count (Fig. 1) and MTT assay (Fig. 2). The proliferative activity of the ASCs was 1.6–4.0-fold higher in the presence of bFGF than in the absence of bFGF (Fig. 1). The number of ASCs increased by more than a 1000-fold at the fourth passage of ASCs cultured with bFGF. Statistically significance was seen in the presence of bFGF (Figs. 1 and 2). There was no correlation between the proliferative activity of the ASCs and age of donors.

Differentiation of ASCs to mature adipocytes

Differentiation of ASCs to mature adipocytes as assessed by GPDH activity assay was observed at all passages (Fig. 3). The extent of differentiation of ASCs in the presence of bFGF was greater than that in the absence of bFGF from the first to third passages, and decreased from the fourth passage onward. From first to third passage, differentiation of ASCs in the presence of bFGF was significantly greater than any other groups. Open and closed bars at start show intrinsic GPDH. There was no correlation between the extent of differentiation and age of donors.

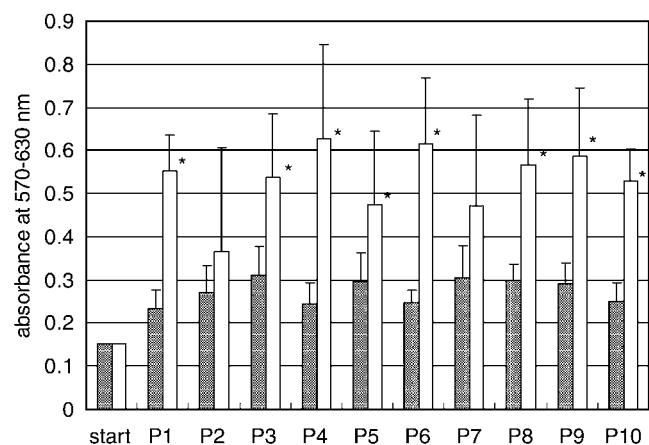


FIG. 2. Proliferation of ASCs *in vitro* as assessed by MTT assay. Proliferation of ASCs in 96-well microtiter plates cultured with 100 ng/mL bFGF (open bars) or without bFGF (closed bars) for 1 week was measured by MTT assay. *: $p < 0.05$ versus without bFGF group.

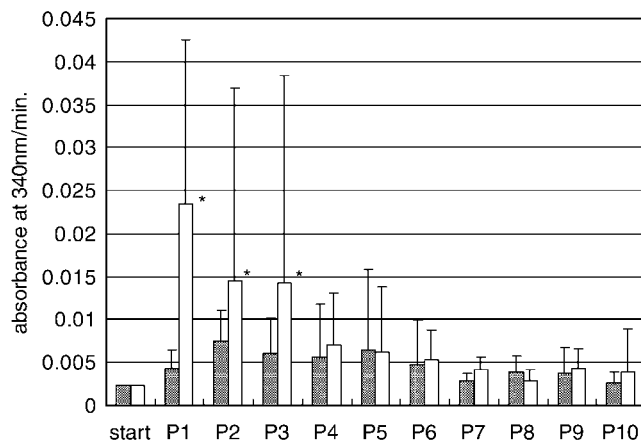


FIG. 3. Adipogenic differentiation of ASCs. GPDH activity of ASCs at each passage after culture with 100 ng/mL bFGF (open bars) or without bFGF (closed bars) for 1 week and incubation in DMEM/F-12 medium containing insulin, 3,5,3-triiodothyronine, transferrin, calcium pantotenate, biotin, and dexamethasone for 21 days was measured using a commercially available GPDH activity measurement kit and a spectrophotometer (absorbance of the solution mixture at 340 nm per minute). *: $p < 0.05$ versus without bFGF group and the group passages 4–10.

Adipogenesis of implanted human ASCs

On the basis of the *in vitro* data described above, we implanted the designated number of third-passage ASCs cultured with bFGF into the back of 6-week-old female BALB/c nude mice. The ASCs were incorporated into the collagen scaffolds with or without controlled-release bFGF. In all the groups, adipogenesis was induced in the scaffold and maintained up to 24 weeks after implantation (Figs. 4-1 and 4-2). The greatest cross-sectional surface area of adipose tissue in the scaffold was 1.19 mm² 12 weeks after implantation, which was equivalent to 68.84% of the scaffold area. In contrast, the adipose tissue area was 0.58 mm² in the group receiving a scaffold alone (without ASCs) with controlled-release bFGF (B group) (Fig. 5-1). In the group receiving 2×10^6 ASCs incorporated in a scaffold without controlled-release bFGF (E group), the adipose tissue area was 0.40 mm². The area of adipose tissue was 0.08 mm² in the group receiving a scaffold alone (A group). The extent of adipogenesis in the group receiving 2×10^6 ASCs incorporated in a scaffold without controlled-release bFGF (E group) was similar to that in the group receiving a scaffold alone with controlled-release bFGF (B group). In the group receiving 8×10^6 ASCs in a scaffold without controlled-release bFGF (G group), adipogenesis in the scaffold decreased to the level of the group receiving a scaffold alone (A group). At 24 weeks, the total adipose tissue area increased in most of the groups (Fig. 5-2). The greatest cross-sectional surface area of adipose tissue in the scaffold was 2.14 mm² in the F group, which was almost twice as that at 12 weeks. In the group receiving 8×10^6 cells without controlled-release bFGF (G group), total adipogenesis was greater than *de novo* adipogenesis (A and B groups). Controlled-release bFGF thus had statistically an additive effect on adipogenesis in most of the groups at both 12 and 24 weeks.

Human ASC-derived adipogenesis

Human vimentin-positive cells were found in all the groups receiving ASCs. Most of these cells were not mature adipocytes. However, at 12 weeks, in the group receiving 8×10^6 ASCs in a scaffold with controlled-release bFGF (H group), human ASC-derived mature adipocytes were observed (Fig. 6). The extent of human ASC-derived adipogenesis in the H group 12 weeks after implantation was equivalent to 14.6% of total adipogenesis in the scaffold. In contrast, the extent of human ASC-derived adipogenesis in other groups was less than 1% (Fig. 7-1). At 24 weeks, human ASC-derived adipose tissue area was increased in all the groups receiving ASCs (Fig. 7-2). The greatest cross-sectional surface area of human-derived adipose tissue in the scaffold was 0.35 mm² in the group receiving 8×10^6 ASCs in a scaffold without controlled-release bFGF (G group), and this was 43.8% of total adipose tissue area. In the H group, human-derived adipogenesis was 0.17 mm², which was equivalent to 44.4% of total adipogenesis in the scaffold.

Discussion

ASCs are similar to MSCs¹³ and proliferate considerably when cultured with bFGF.^{39,40} We isolated 5×10^5 ASCs from 5 g of fresh adipose tissue obtained during breast cancer surgery. We considered 5×10^5 ASCs were scarce as a starting material for adipogenesis *in vivo*, and proliferated with bFGF *ex vivo*. Heimburg *et al.* showed that 8×10^4 to 3.5×10^5 pre-adipocytes can be harvested from 1 g of fresh adipose tissue, depending on the donor and the method used for cell retrieval.⁴¹ ASCs are isolated from excised or aspirated adipose tissue. Lipoaspiration from the abdominal wall requires another incision site and is associated with further pain. Moreover, informed consent would have to be additionally received for this procedure. It is therefore not suitable for our study. We used excised adipose tissue obtained during breast surgery in this study. The adipose tissue is usually discarded after operation, and informed consent is readily obtained. Viable cells from adipose tissue decrease with preservation conditions and time periods after resection.⁴² We usually preserve resected adipose tissue at 4°C and isolate ASCs as soon as possible after resection to minimize cell damage. We had probatively isolated ASCs 72 h after surgery and obtained about half the number of ASCs from 5 g of adipose tissue.

In this study, we did not exclude patients with axillary lymph node metastasis or advanced stage, and cancer cells might remain in breast or axillary adipose tissue. However, we did not see any cancer cells in ASCs cultured *in vitro* for 10 weeks and in the adipose tissues *in vivo* for 24 weeks after implantation. In the future, the procedure should be clinically performed in carefully selected patients to avoid cancer cell dissemination.

When cultured with 100 ng/mL of bFGF, ASCs proliferated more than 1000-fold at the fourth passage, whereas the ability of ASCs to differentiate into mature adipocytes decreased subsequently. Two reasons may account for this phenomenon. First, the "stem cell" population decreases and other populations of cells, such as fibroblasts, increase after the fourth passage. Stem cells are characterized by self-renewal capacity, long-term viability, and multilineage potential. The stem cell-associated marker CD34 was at peak levels in the stromal vascular fraction cells and early passage ASCs throughout the

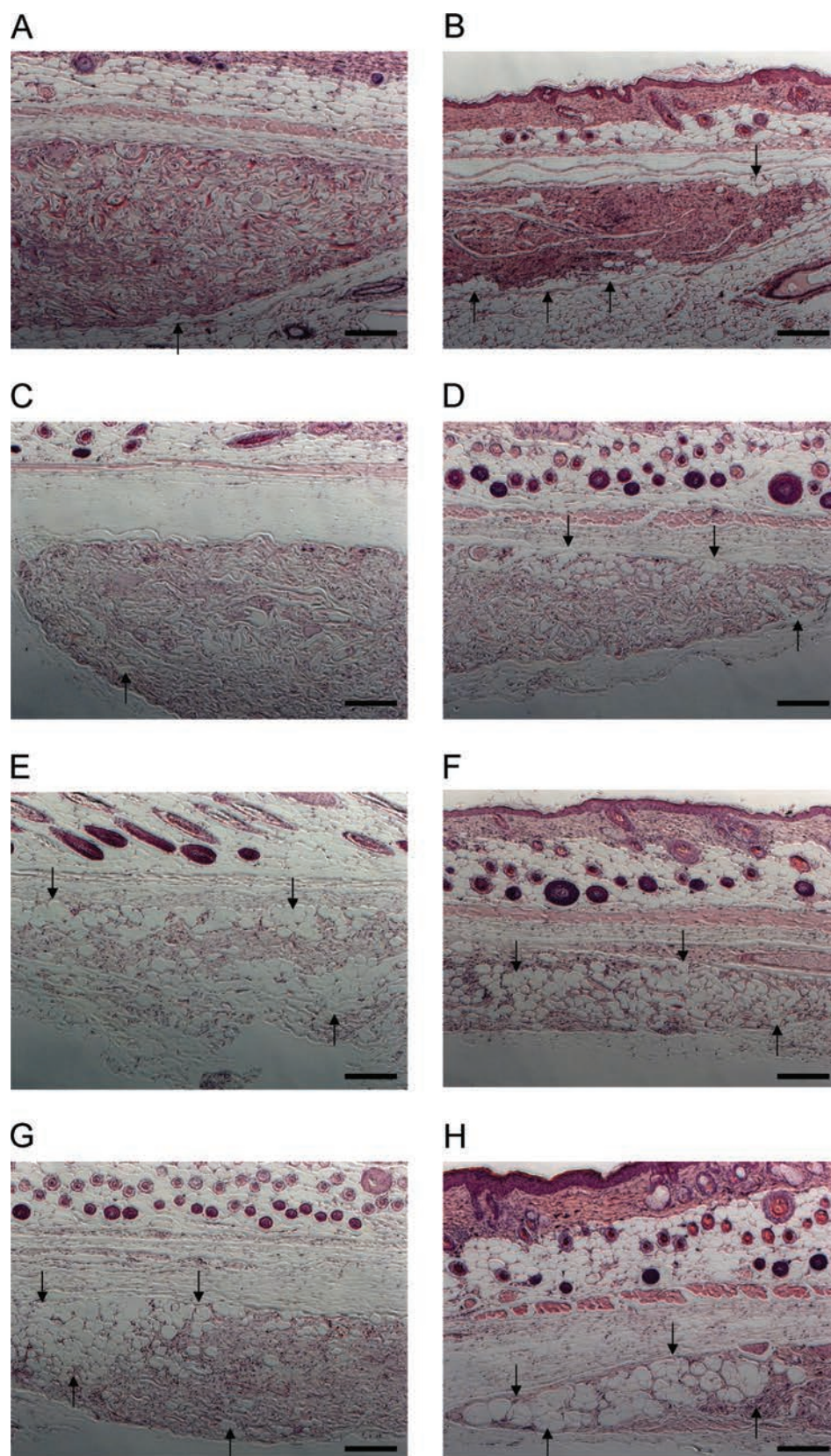


FIG. 4-1. Adipogenesis of implanted human ASCs 12 weeks after implantation. The designated number of third-passage ASCs cultured with bFGF (0: **A, B**; 5×10^5 : **C, D**; 2×10^6 : **E, F**; 8×10^6 : **G, H**) and incorporated into collagen scaffolds with (**B, D, F, H**) or without controlled-release bFGF (**A, C, E, G**) were implanted into the back of 6-week-old female BALB/c nude mice. Formation of adipose tissue in the implanted scaffold 12 weeks after implantation is indicated by the arrows (magnification $\times 100$). Scale bar = 100 μ m. Color images available online at www.liebertonline.com/ten.

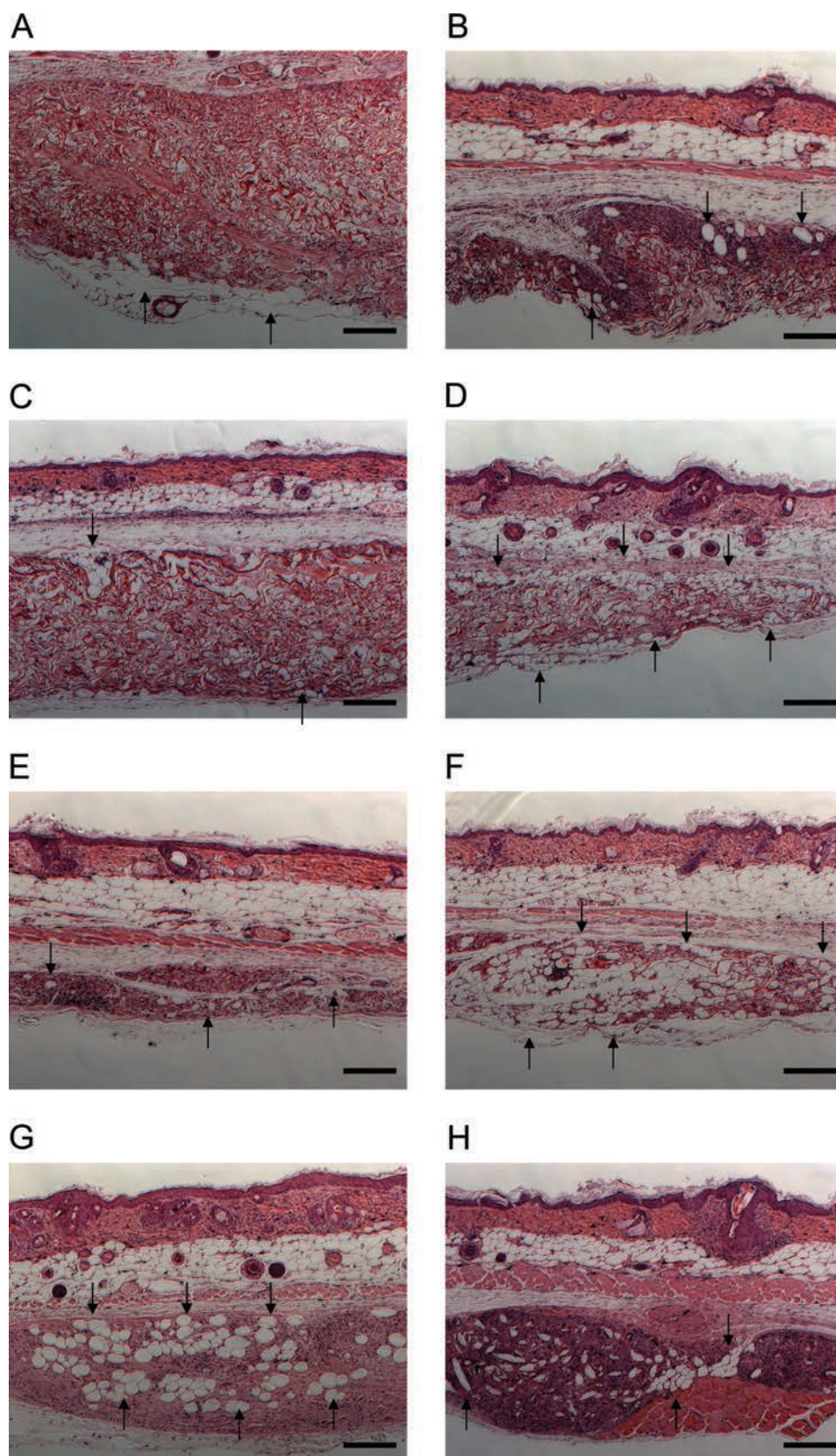


FIG. 4-2. Adipogenesis of implanted human ASCs 24 weeks after implantation. The designated number of third-passage ASCs cultured with bFGF (0: **A, B**; 5×10^5 : **C, D**; 2×10^6 : **E, F**; 8×10^6 : **G, H**) and incorporated into collagen scaffolds with (**B, D, F, H**) or without controlled-release bFGF (**A, C, E, G**) were implanted into the back of 6-week-old female BALB/c nude mice. Formation of adipose tissue in the implanted scaffold 24 weeks after implantation is indicated by the arrows (magnification $\times 100$). Scale bar = 100 μ m. Color images available online at www.liebertonline.com/ten.

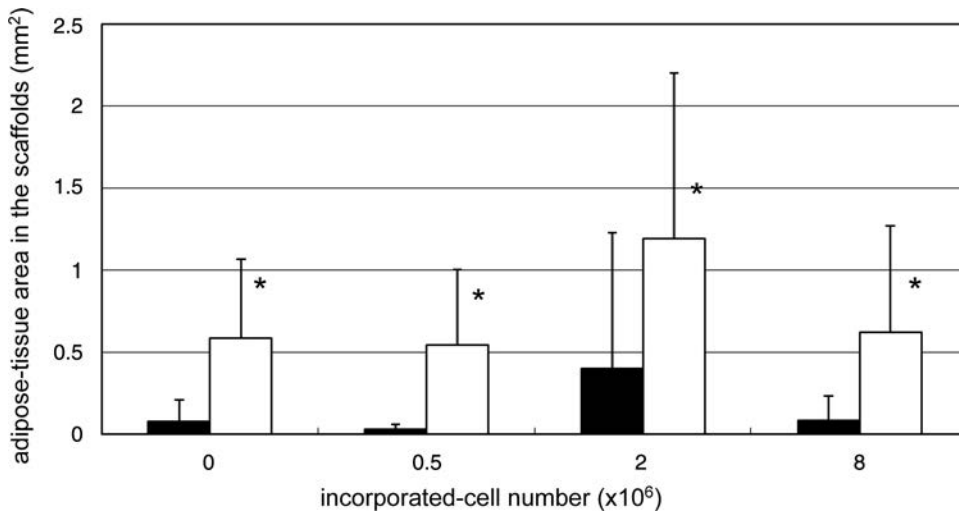


FIG. 5-1. Adipose tissue area in the scaffolds 12 weeks after implantation. The designated numbers of third-passage ASCs incorporated into scaffolds with (open bars) or without controlled-release bFGF (closed bars) were implanted into the back of 6-week-old female BALB/c nude mice. The adipose tissue area in the scaffolds was measured and analyzed with

the computer program Image-Pro Plus. The average area of the scaffolds was 2.14 mm² and did not differ significantly among the groups. *: $p < 0.05$ versus without controlled-release bFGF and group A.

culture period.^{43,44} Second, because the ASCs were cultured in medium containing 10% FBS, bFGF expression was progressively lost, leading to impaired self-renewal ability.⁴⁵ To obtain ASCs that retain the ability to differentiate into mature adipocytes, ASCs should optimally be cultured with bFGF until the third passage. A previous study has reported that human adipose tissue-derived MSCs retain their capacity to differentiate into mature adipocytes (GPDH activity) for least 15 passages.⁴⁶ However, peak GPDH activity is at the fifth passage. These findings are consistent with our results.

De novo adipogenesis occurs without implanting ASCs,^{19,31,32,47-49} because preadipocytes are recruited from surrounding adipose tissue and differentiate into mature adipocytes. Ideally, adipose tissue engineering techniques would simulate this phenomenon after breast surgery in the future. In women with breast cancer, however, less ASCs would survive at the implantation site of ASCs incorporated

in a collagen scaffold with controlled-release bFGF, because the conserved breast usually receives radiation therapy after breast conserving surgery.⁵⁰ Because *de novo* adipogenesis is unlikely, we require exogenous progenitor cells. Implanted progenitor cells are expected to differentiate into mature adipocytes. Cell implantation therapy has some benefits, and ASC-assisted lipotransfer has been used for cosmetic breast augmentation.⁵¹ Clinically, the implantation of ASCs including preadipocytes is prerequisite to a successful outcome of adipose tissue engineering.

One of our objectives was to confirm how many mouse-derived cells can be recruited and how many human-derived cells must be implanted. We immunohistochemically distinguished human-origin from mouse-origin adipose tissue with the use of antihuman vimentin antibody. Vimentin is not specific for preadipocytes or adipocytes, but it is expressed on these cells. Franke *et al.* concluded that lipid droplets are

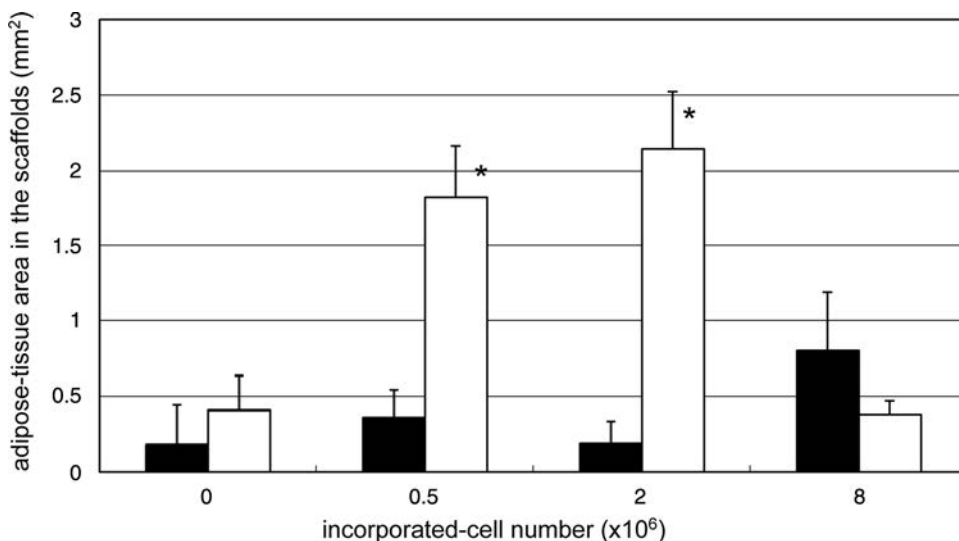
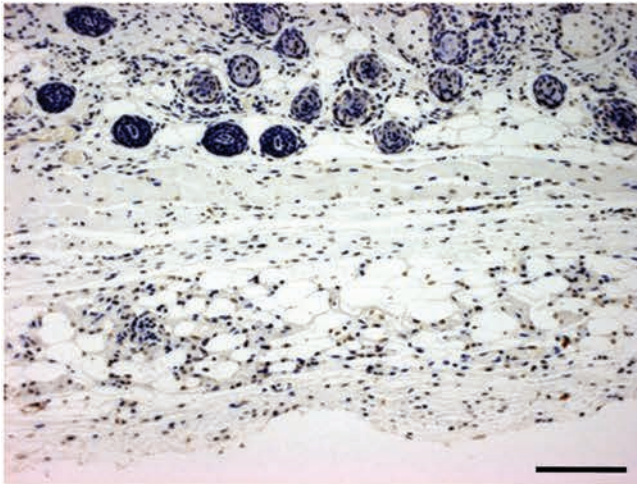
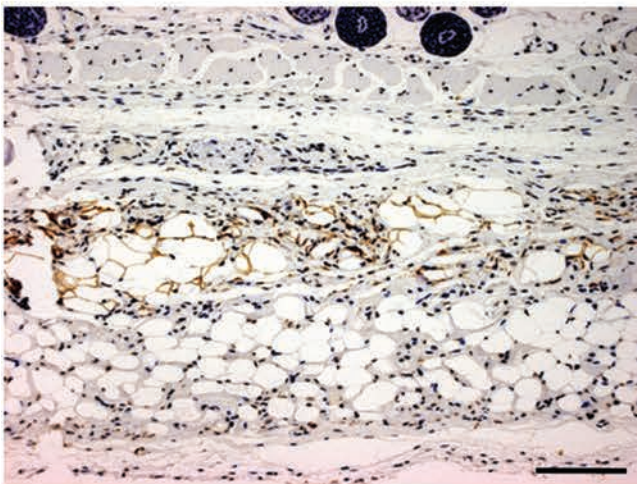


FIG. 5-2. Adipose tissue area in the scaffolds 24 weeks after implantation. The designated numbers of third-passage ASCs incorporated into scaffolds with (open bars) or without controlled-release bFGF (closed bars) were implanted into the back of 6-week-old female BALB/c nude mice. The adipose tissue area in the scaffolds was measured and analyzed with the computer program Image-Pro Plus. *: $p < 0.05$ versus without controlled-release bFGF and group A.

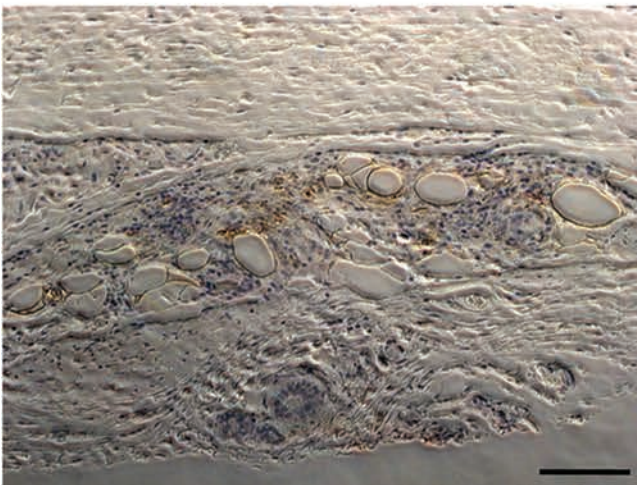
F (12 weeks)



H (12 weeks)



H (24 weeks)



encaged in a vimentin-containing structure.⁵² The antihuman vimentin antibodies used to confirm newly formed adipose tissue were human derived in many *in vivo* studies.^{20,22,53,54} We previously reported that human-derived adipocytes were distinguishable from *de novo* adipocytes with antihuman vimentin staining.²⁰ In our study, structures around lipid vacuoles were stained with antihuman vimentin antibody, indicating the presence of human-derived mature adipocytes. No human vimentin-positive cells were found in the groups receiving a scaffold alone, with or without controlled-release bFGF (A and B groups). At 12 weeks, in the group receiving 2×10^6 ASCs with controlled-release bFGF (F group), the human vimentin-positive area was accounted for less than 1% of newly formed adipose tissue. And in the group receiving 8×10^6 ASCs with controlled-release bFGF (H group), the human vimentin-positive area was equivalent to about 15% of newly formed adipose tissue. At 24 weeks, human-derived adipose tissue area increased in every group. In the F group, the human vimentin-positive area was 2.8% of newly formed adipose tissue. In the H group, the human vimentin-positive area was equivalent to 44.4% of newly formed adipose tissue. Both human adipose tissue area and percentage of the scaffold were increased from 12 to 24 weeks in the H group. Human ASCs take longer time to differentiate into mature adipocytes than mouse ASCs. Implanted human ASCs differentiate into mature adipocytes in the host and continue to differentiate for a long time. At 24 weeks in the G group, human-derived adipogenesis was 0.35 mm^2 , while *de novo* adipogenesis was 0.45 mm^2 and greater than the A group. From this result, implanted human ASCs not only differentiate into mature adipocytes but also promote *de novo* adipogenesis. Implanted human ASCs function for a long time as progenitor cells for *in vivo* adipogenesis and induce *de novo* adipogenesis.

An optimal cell seeding concentration for scaffold formation may exist. Our results suggested that 2×10^6 ASCs/site was the best concentration at both 12 and 24 weeks. At 12 weeks, in the group receiving 2×10^6 ASCs incorporated in a scaffold without controlled-release bFGF (E group), the area of newly formed adipose tissue was similar to that in the group receiving the scaffold alone with controlled-release bFGF (B group). Although bFGF has an obvious effect on adipogenesis, the number of implanted cells is also an important factor. Heimburg *et al.* seeded 10^6 preadipocytes cultured in the medium supplemented with epidermal growth factor onto collagen sponges, which were then implanted into mice. They found that implantation of a large number of preadipocytes is important for the promotion of adipogenesis.²² Torio-Padron *et al.* injected human ASCs in fibrin into nude mice and also concluded that an increased cell concentration enhances the formation of adipose tissue.⁵⁵ However,

FIG. 6. Human ASC-derived adipogenesis. Immunohistochemical sections of newly formed adipose tissue 12 and 24 weeks after implantation. Human-origin adipose tissue is stained by antihuman vimentin antibody. Group receiving 2×10^6 ASCs with controlled-release bFGF at 12 weeks (F), group receiving 8×10^6 ASCs with controlled-release bFGF at 12 weeks (H), and group receiving 8×10^6 ASCs with controlled-release bFGF (H) at 24 weeks (magnification $\times 200$). Scale bar = $100 \mu\text{m}$. Color images available online at www.liebertonline.com/ten.

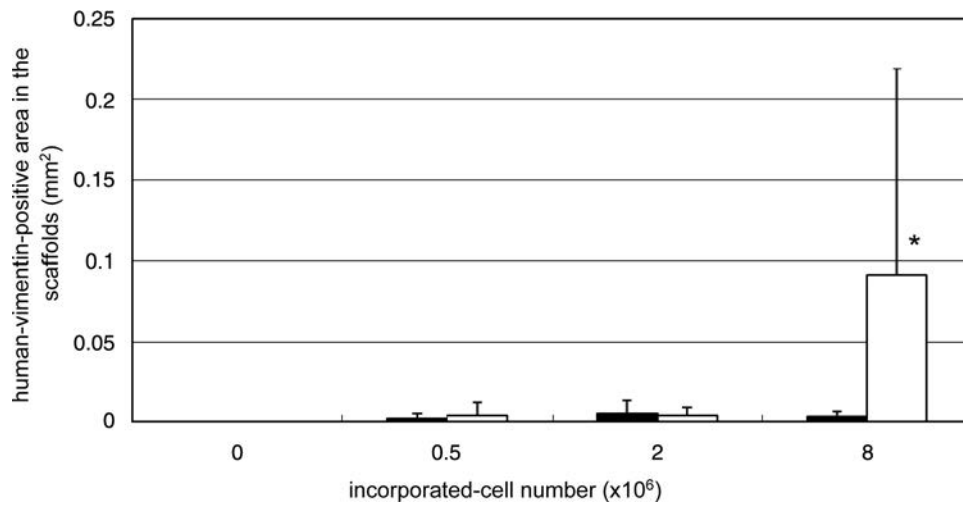


FIG. 7-1. Human vimentin-positive area in the scaffolds 12 weeks after implantation. The designated number of third-passage ASCs cultured with bFGF incorporated into the scaffolds with (open bars) or without controlled-release bFGF (closed bars) were implanted into the back of 6-week-old female BALB/c nude mice. The human vimentin-positive area in the scaffolds was measured and analyzed with Image-Pro Plus. *: $p < 0.05$ versus any other group.

from our data, there might be the best cell concentration for total adipogenesis. Both 12 and 24 weeks after implantation, maximum adipose tissue area was observed in the group receiving 2×10^6 ASCs with controlled-release bFGF (F group), not in the group receiving 8×10^6 ASCs (G and H groups). Implanted ASCs not only differentiate into mature adipocytes, but also secrete ECM to promote maturation. The amount of ECM or cytokines secreted by ASCs may increase with an increased number of implanted ASCs and produce an appropriate micromilieu for proliferation and differentiation of ASCs themselves. Stillaert *et al.* reported that ASCs secrete additional ECM components and that these ECM components were able to act as inductive factors to further enhance adipogenesis *in vivo*.⁵⁶ In our study, 2×10^6 ASCs with controlled-release bFGF (F group) might be the optimum condition in terms of secreted ECM, cytokines, and succeeding cell survival. Implanting 8×10^6 ASCs (G and H groups) was not good condition for total adipogenesis. The number of ASCs that can survive in the scaffold might be limited.

Since there are many inflammatory cells in the specimens of the G and H groups, some of 8×10^6 ASCs are supposed to be dead. The dead cells might cause inflammation and inhibit differentiation into mature adipocytes. Only in the H group at 24 weeks, bFGF did not have additive effects for both total and human-derived adipogenesis. Total adipogenesis decreased, but human-derived adipogenesis increased in the H group. Too many ASCs might inhibit *de novo* adipogenesis.

In summary, our results indicate that the implantation of optimum number of ASCs with controlled-release bFGF is the key for a successful outcome of functional adipose tissue engineering for long term. Newly formed adipose tissue induced by human ASCs fully matured and functioned for a long time. There are few papers that report *in vivo* human-derived adipogenesis for such a long period as our paper. This study is baseline for future clinical practice. Further studies are needed to discover the most efficient ways of generating adipose tissue for clinical practice.

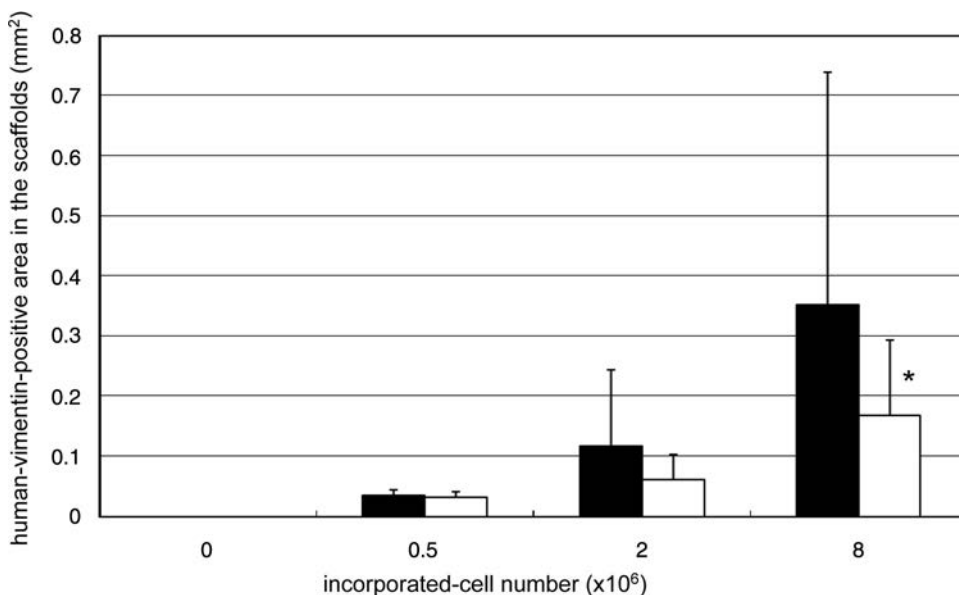


FIG. 7-2. Human vimentin-positive area in the scaffolds 24 weeks after implantation. The designated number of third-passage ASCs cultured with bFGF incorporated into the scaffolds with (open bars) or without controlled-release bFGF (closed bars) were implanted into the back of 6-week-old female BALB/c nude mice. The human vimentin-positive area in the scaffolds was measured and analyzed with Image-Pro Plus. *: $p < 0.05$ versus groups C and D.

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