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Fabrication of Porous Extracellular Matrix Scaffolds from Human Adipose Tissue

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Adipose tissue is found over the whole body and easily obtained in large quantities with minimal risk by a common surgical operation, liposuction. Although liposuction was originally intended for the removal of undesired adipose tissue, it may provide an ideal material for tissue engineering scaffolds. Here we present novel, porous scaffolds prepared from human adipose tissues. The scaffolds were fabricated in a variety of macroscopic shapes such as round dishes, squares, hollow tubes, and beads. The microscopic inner porous structure was controlled by the freezing temperature, with a decrease in pore size as the freezing temperature decreased. The scaffold prepared from human adipose tissue contains extracellular matrix components including collagen. Preliminary *in vitro* studies showed that human adipose-derived stem cells attached to a human extracellular matrix scaffold and proliferated. This scaffold based on human adipose tissue holds great promise for many clinical applications in regenerative medicine, particularly in patients requiring soft-tissue regeneration.

Introduction

Scaffolds play a central role in tissue engineering by preserving tissue volume, providing temporary mechanical function, and guiding the complex multicellular processes of tissue formation and regeneration.[1,2] Although many synthetic, biodegradable materials have been extensively explored as a scaffold material, there are certain limitations on those materials used in tissue engineering.[3] Synthetic materials, for example polyester-based biodegradable polymers such as poly(lactic-co-glycolic acid) and polycaprolactone, show good mechanical properties of controlled biodegradability and sophisticated architecture design,[3,4] but usually cause foreign body reactions and integrate poorly with the host tissue.

Most animal cells secrete proteins and proteoglycans that form the extracellular matrix (ECM). The ECM is nature's ideal biologic scaffold material: it helps cells hold together in tissues, and performs protective and supportive functions. The structural and functional molecules of the ECM have not been fully characterized; however, individual components, such as collagen, laminin, fibronectin, and hyaluronic acid, can be isolated and used for a variety of therapeutic applications including tissue engineering and regenerative medicine.[5] Intact ECM has been harvested from various tissues including the small intestine, skin, liver, pancreas, and urinary bladder,[5–7] and one of the most widely studied ECM scaffolds is derived from porcine small intestinal submucosa and is available under the trade names Oasis®, CuffPatch™, Surgisis®, Durasis®, Stratasis®, and Restore™. Small intestinal submucosa have been exhaustively studied and used in more than one million human patients to reconstruct a variety of tissues including the integument, body wall, urinary bladder, rotator cuff, intestine, urethra, ureter, and diaphragm.[5,6,8,9] Intact ECM from human skin has been used for soft-tissue repair and reconstruction[6,10–12] under the trade names AlloDerm, Graft Jacket®, and Axis™ dermis. The widespread use of intact ECM and ECM components is attributed to their excellent biocompatibility, biodegradability, and bioinductive properties. However, it should be noted that most intact ECMs and ECM components are isolated from animals or cadavers, and as a result, many concerns have been raised regarding immunogenicity and pathogen transmission.

Here we describe human ECM scaffolds derived from adipose tissue. Adipose tissue is the most prevalent tissue in the human body and performs several essential functions, such as energy storage, cushioning, and insulation.[13–15] Adipose tissue is a type of loose connective tissue and the main cellular component is lipid-filled adipocytes. Other

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cellular components include fibroblasts, smooth muscle cells, endothelial cells, immune cells (leukocytes and macrophages), and adipose-derived stem cells (ASCs). In addition, adipose tissue contains various ECM components, such as collagen, reticular fibers, elastin fibers, nerve fibers, vascular stroma, and lymph nodes.[16,17] Adipose tissue secretes a variety of peptides, cytokines, and complement factors, such as adiponectin, leptin, inflammation cytokine, chemokines, and angiogenesis factors, which regulate numerous cellular processes including insulin action, energy homeostasis, inflammation, and cell growth.[18] The structural and functional molecules of adipose tissue provide the means by which cells communicate with each other and with the external environment.

Adipose tissue can be easily obtained with minimal risk by liposuction, a common surgical procedure.[15,19] Liposuction is the most commonly performed aesthetic operation in the world now: in the United States alone, almost half a million elective liposuction surgeries were performed in 2004.[15] We have developed novel, porous human ECM scaffolds from adipose tissue obtained by liposuction, leading to a new tissue engineering concept of autologous tissue engineering, in which both scaffold material and cells are derived from the same patient.

Materials and Methods

Preparation of human ECM scaffolds from adipose tissue

Human adipose tissue was obtained with informed consent from eight healthy female donors aged between 20 and 40 who had undergone liposuction at the Kangnam Plastic Surgery Clinic (Seoul, Korea). Infiltration of saline, liposuction, and centrifugation was performed by a single combined machine (Lipokit; Medikan, Seoul, Korea).[20] The adipose tissue obtained by liposuction (20 mL) was washed three times with distilled water to remove blood components. Distilled water (10 mL) was added to the adipose tissue and the tissue–water mixture was homogenized at 12,000 rpm for 5 min under room temperature using a homogenizer (T 18 basic ULTRA-TURAX, IKA®; Werke, KG Staufen, Germany). The tissue suspension was centrifuged at 3000 rpm for 5 min and the upper layer containing oil components was discarded. This process was repeated several times. The final gel-like, thick tissue suspension (~5 mL) was washed three times by addition of ~25 mL of distilled water to the gel-like tissue suspension, gentle mixing by pipetting, and centrifugation at 3000 rpm for 5 min. The final gel-like tissue suspension was gently poured into molds with different shapes, frozen at −10°C, −40°C, −70°C, or −196°C (liquid nitrogen temperature), and freeze dried (Fig. 1).

Isolation of human ASC from adipose tissue

Human ASC was isolated from subcutaneous adipose tissue of eight healthy female donors aged between 20 and 40 who had undergone liposuction at the Kangnam Plastic Surgery Clinic. According to the liposuction procedure,[21–23] human adipose tissue was dissected and digested in phosphate-buffered saline supplemented with 0.01% (w/v) Type II collagenase (Gibco-BRL–Life Technologies, Carlsbad, CA) for 1 h at 37°C. The digested tissue was filtered through a 100 μm mesh to remove undigested debris and capillary filaments. The filtered cell suspension was centrifuged at 1000 rpm for 7 min and the pellet was washed in phosphate-buffered saline.

Cell culture

Disc-shaped human ECM scaffolds (diameter 1 cm and height 0.5 cm) were placed in 24-well plates. A suspension of human ASCs or murine 3T3-L1 containing 1×10^5 cells/scaffold was seeded onto the ECM scaffolds using a syringe

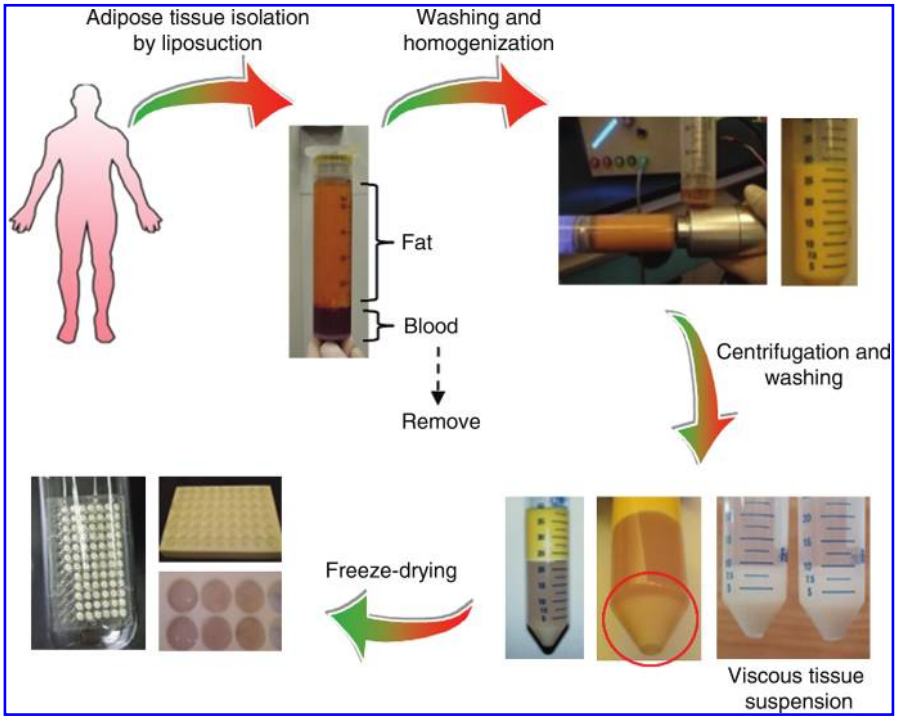

FIG. 1. Schematic representation of the fabrication procedure for human ECM scaffolds. ECM, extracellular matrix. Color images available online at www.liebertonline.com/ten.

needle. The medium was supplemented with Dulbecco's modified Eagle's medium (Gibco-BRL–Life Technologies, Carlsbad, CA), 10% fetal bovine serum (Gibco-BRL–Life Technologies, Carlsbad, CA), and 1% antibiotics (Gibco-BRL–Life Technologies, Carlsbad, CA). The cell-seeded scaffolds were incubated at 37°C with 5% CO_2. Cell number and viability were measured by an automatic cell counter (Nucleocounter™; ChemoMetec, Allerud, Denmark).

Scanning electron microscopy

The inner structure of human ECM scaffolds was observed using scanning electron microscopy (SEM) (Hitachi S-4800 FE-SEM, Tokyo, Japan). The scaffolds were fixed to metal stubs and coated with platinum by a sputter at an accelerating voltage of 15 kV.

Pore size distribution

The through pore size distribution of human ECM scaffolds was characterized on sheet specimens (diameter 2.5 cm and thickness 1.5 cm) by capillary flow porometry (CFP-1100-AEX; Porous Materials, Ithaca, NY). The capillary flow porometry measures only the throat diameter of each through pore, and blind pores are excluded.

Mechanical properties

The compressive strength of scaffolds was measured on cylindrical specimens (diameter 1.3 cm and height 1.5 cm) using an Universal Testing Machine (STM-10E; United Calibration, Huntington Beach, CA) at a crosshead speed of 1 mm/min. The scaffolds were compressed to 80% of their original length. The compressive modulus was obtained from the initial slope of the stress–strain curve.

Analysis of ECM components

Tissue specimens were fixed in 4% paraformaldehyde, embedded in paraffin, and sliced using a microtome. Sections were deparaffinized and dehydrated in ethanol. For collagen fiber staining, sections were first stained by a rapid trichrome method.[24,25] The samples were fixed in Bouin's solution for 1 h at 56°C and stained with Wiegert's iron hematoxylin for 10 min. Samples were washed and stained with Gomori's trichrome solution for 20 min, and then differentiated in a 0.5% acetic acid solution. Gomori's silver impregnation technique was used to stain reticulum fibers.[26,27] The samples were oxidized with an acidified 0.5% potassium permanganate solution and differentiated with a 2% potassium metabisulfite solution. Samples were impregnated with a working ammoniacal silver solution then toned with a gold chloride solution. Fullner and Lillie's Orcinol-new fuchsin method[28,29] was used to stain elastic fibers in the scaffolds. Samples were stained at 37°C with an Orchinol-new fuchsin working solution for 15 min and dehydrated.

Collagen quantification

Specimens were fixed in Bouin's solution and stained with 0.1% Sirius Red F3BA (Sigma, St. Louis, MI) solution in

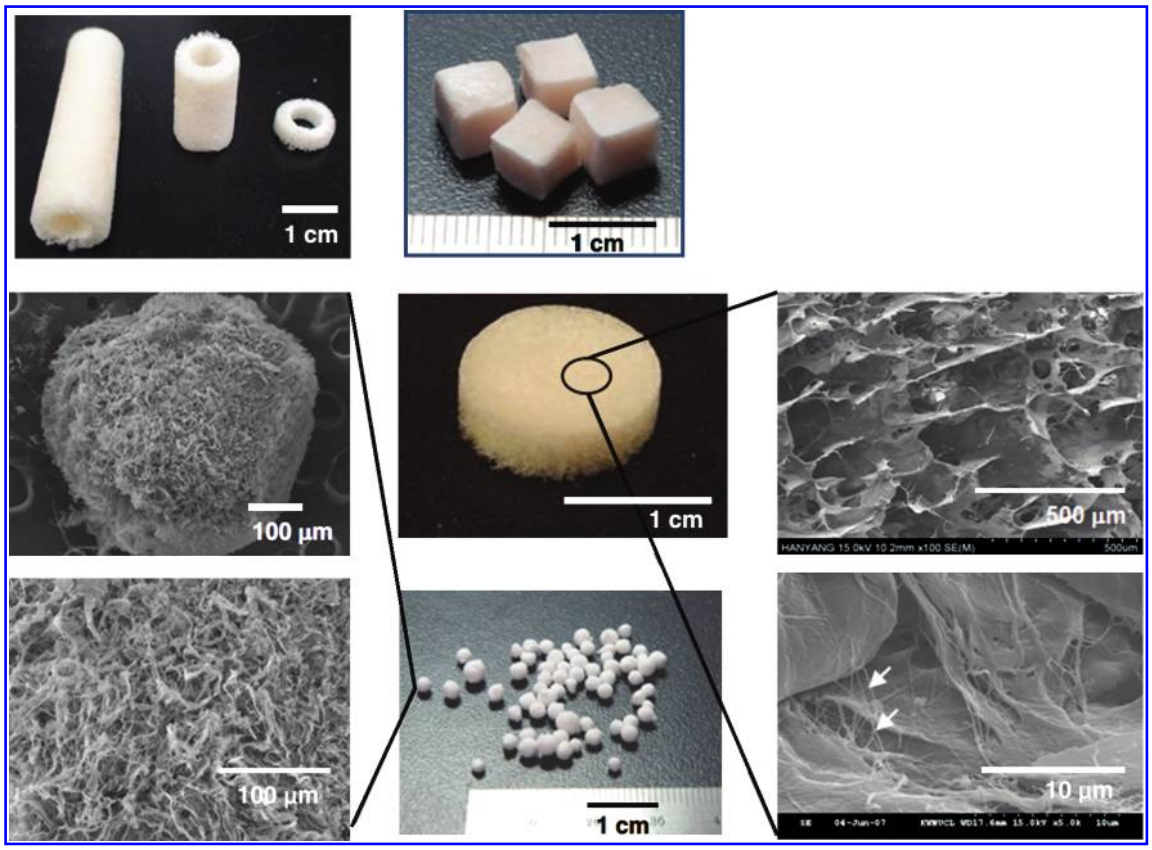

FIG. 2. Human ECM scaffolds with a variety of macroscopic shapes and their microscopic images obtained by scanning electron microscopy. Color images available online at www.liebertonline.com/ten.

saturated picric acid. After staining, specimens were washed in running tap water and again in 0.01 N HCl to remove the nonbound dye. Then 0.1 N NaOH was added to elute the dye boned on human ECM under gentle shaking for 10 min at room temperature. The dye/NaOH solution was transferred to a cuvette, and the absorbance was measured at 540 nm using a spectrophotometer (Ultrospec 2100 *pro*; Amersham Biosciences, Piscataway, NJ). Human collagen type I isolated from tendon was used as a control. The amount of collagen was normalized by total protein concentration.[30–32]

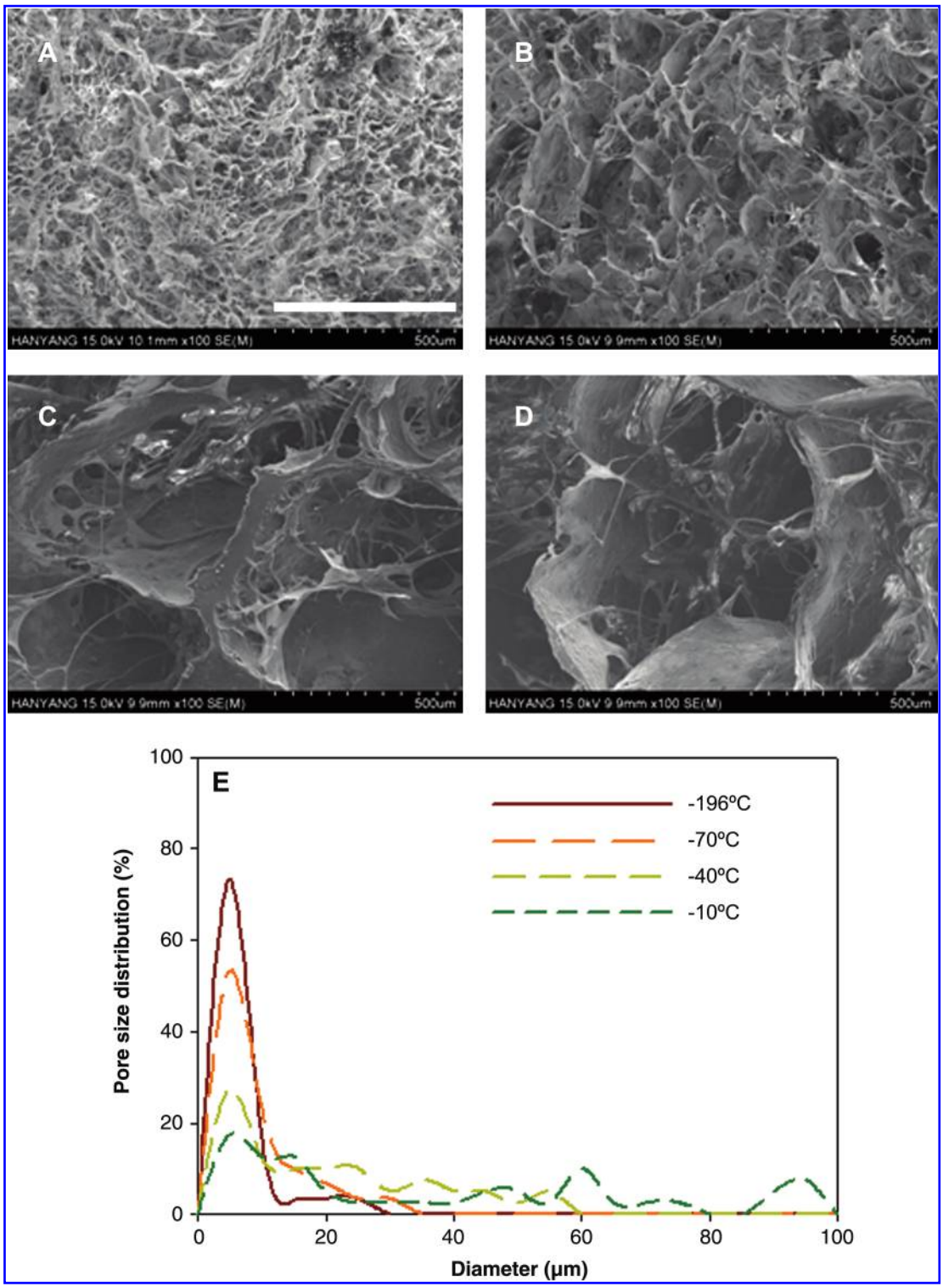

FIG. 3. Effect of freezing temperature on the pore size of a human ECM scaffold. Freezing temperatures were −196°C (**A**), −70°C (**B**), −40°C (**C**), and −10°C (**D**). The scale bar represents 500 μm. Pore size distribution (**E**) measured by capillary flow porometry. Color images available online at www.liebertonline.com/ten.

In vivo *experiments*

A human ECM scaffold was transplanted subcutaneously into the back of each 6- to12-week-old nude mouse. Mice were housed with free access to water and a standard chow diet. Animals were killed at a predetermined time and the grafts were explanted. Five mice were analyzed for each experimental group. Tissue specimens were fixed in 4% paraformaldehyde, embedded in paraffin, and sliced into 10 μm sections using a microtome. Samples were deparaffinized and hydrated through graded ethanol, and then stained with a hematoxylin–eosin solution.

Reverse transcription–polymerase chain reaction

mRNA was isolated from cells and tissues using Trizol reagents (Invitrogen, Karlsruhe, Germany). cDNA was synthesized using M-MLV RT (Invitrogen) following the manufacturer's instructions. The cDNA was used as a template for polymerase chain reaction (PCR) analysis with primers specific for peroxisome proliferative–activated receptor gamma (PPARγ) (NM011146, forward: 5′-ACT GCC TAT GAG CAC TTC AC-3′, reverse: 5′-CAA TCG GAT GGT TCT TCG GA-3′), leptin (NM008493, forward: 5′-TGC TGC AGA TAG CCA ATG AC-3′, reverse: 5′-GAG TAG AGT GAG GCT TCC AGG A-3′), and β-actin (NM007393, forward: 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′, reverse: 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′). Amplification was performed using *Taq* polymerase as follows: initial denaturation for 5 min at 94°C, followed by 35 cycles of 1 min at 94°C for denaturation, 1 min at 50–60°C for annealing, and 7 min at 72°C for extension. The expression of a housekeeping gene, β-actin, was used as an internal control. PCR products were separated by electrophoresis in 2% agarose gels and stained with ethidium bromide.

Results

Fabrication of human ECM scaffolds

The procedure for preparation of human ECM scaffolds from adipose tissue is shown in Figure 1. The scaffold fabrication process is quite simple, and the human ECM scaffolds were prepared without addition of any chemicals. The volume of the human ECM components extracted from adipose tissue was approximately 5% of the original adipose tissue volume. The gel-like viscous human ECM suspension was gently poured into molds with different shapes, such as round dishes, square molds, and hollow tubes. Porous ECM beads were prepared by dripping into liquid nitrogen using a syringe needle. The three-dimensional (3D) macroscopic shape was manipulated depending on the mold, as shown in Figure 2.

Characterization of human ECM scaffolds

Scaffolds for tissue engineering must have a highly porous structure within arbitrary and complex 3D shapes.[1,2] A porous scaffold structure allows mass transport for cell nutrition, channels for cell migration, and surfaces for cell attachment. 3D shapes provide temporary mechanical function. A material suitable for tissue engineering should be biocompatible and biodegradable for integration into surrounding host tissues. The human ECM scaffold possesses a highly porous structure, as shown in Figures 2 and 3. Since ice crystal growth is a function of the freezing temperature, the pore size of a scaffold could be controlled.[33,34] The human ECM scaffolds were fabricated using different freezing temperatures, −10°C, −40°C, −70°C, and −196°C (liquid nitrogen), and the internal porous structure was viewed by SEM. All scaffolds had a highly porous structure, and the pore size was greatly affected by the freezing temperature, with a decrease in pore size as the freezing temperature decreased from –10°C to –196°C.

Figure 4 shows representative stress–strain curves of human ECM scaffolds that were subjected to compression testing. These curves did not differ significantly between scaffolds that were fabricated at different freezing temperatures. The Young's modulus values of human ECM scaffolds were 0.12–0.38 MPa at 1% of the initial strain value, indicating that the scaffolds were soft.

Composition analysis of human ECM scaffolds

To analyze ECM components in the scaffolds, specimens were specifically stained with Gomori's one-step trichrome for collagen fibers (Fig. 5A), Gomori's silver impregnation for reticulum fibers (Fig. 5B), and Fullner and Lillie's Orcinol-new fuchsin for elastic fibers (Fig. 5C). A large number of collagen fibers were observed as green staining by Gomori's method (Fig. 5A), indicating that the major ECM component in the scaffolds was collagen. Reticulum fibers were also observed as deep brown or black staining (Fig. 5B), but few elastic fibers were detected in the human ECM scaffold (Fig. 5C). The amount of collagen in human ECM derived from adipose tissue was measured by Sirius Red–based colorimetric microassay. The amount of collagen in human ECM did not greatly differ from that of fresh adipose tissue (Fig. 5E).

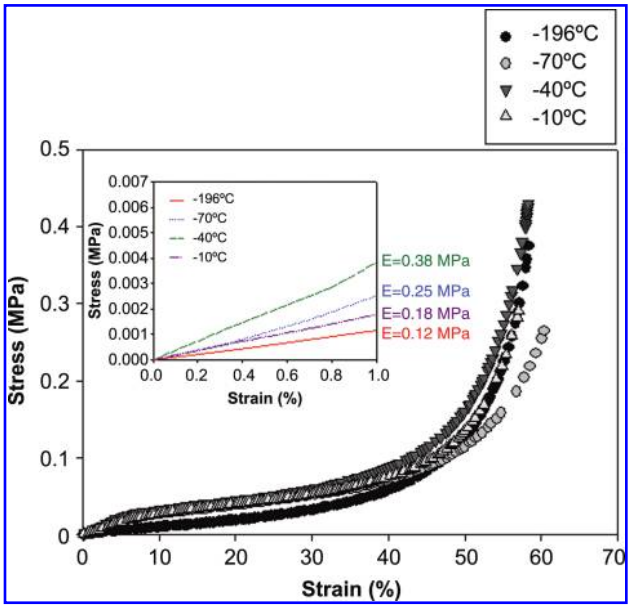

FIG. 4. Compressive stress–strain curves of human ECM scaffolds (average height 1.5 cm, diameter 1.3 cm). Color images available online at www.liebertonline.com/ten.

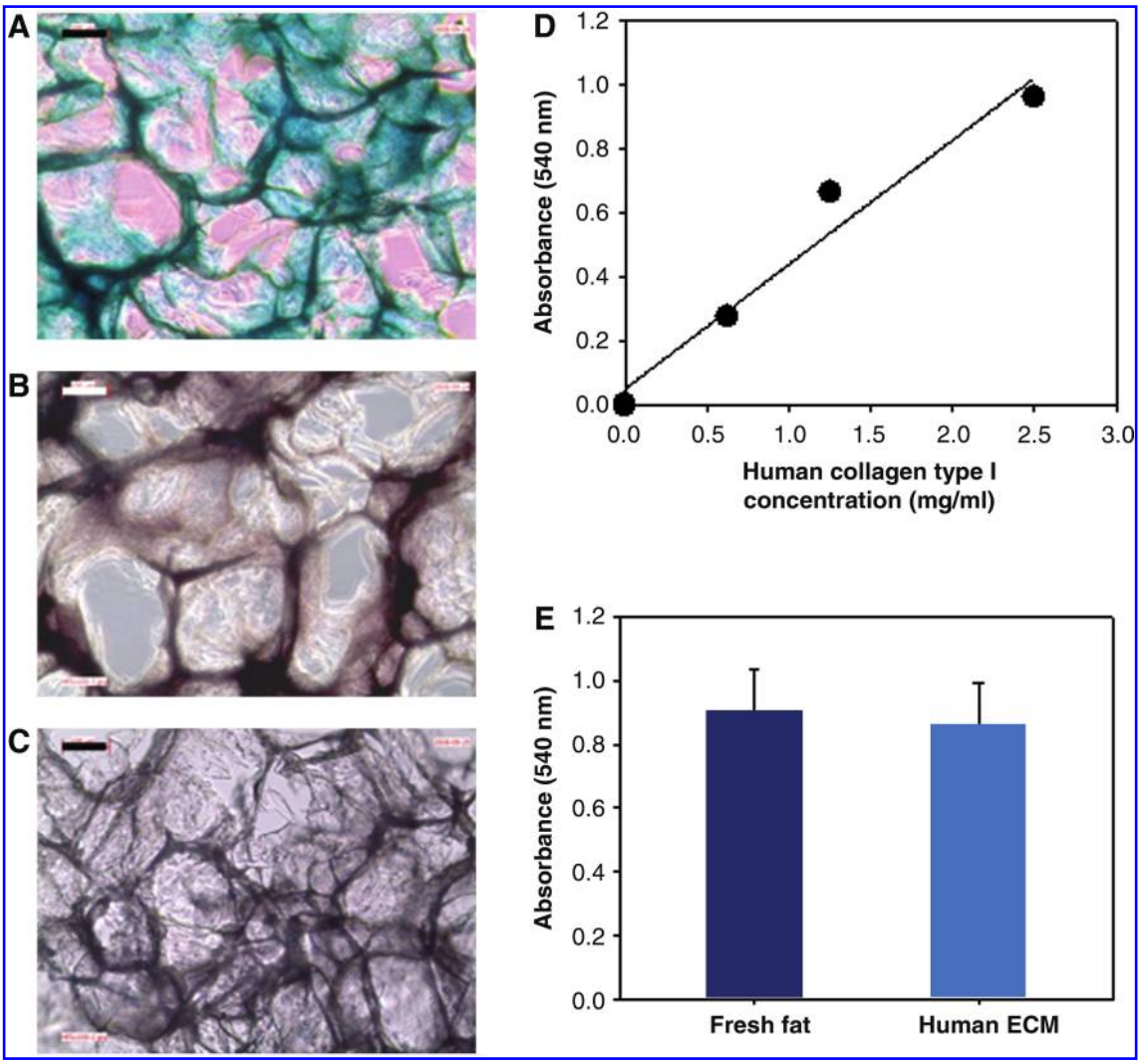

FIG. 5. Analysis of ECM components of scaffolds. Sections of specimens were stained with specific agents as follows: (**A**) Gomori's one-step trichrome for collagen (light blue or green); (**B**) Gomori's silver impregnation for reticulum fibers (dark brown/black); and (**C**) Fullner and Lillie's Orcinol-new fuchsin for elastic fibers (deep violet). (**D**) A standard curve of optical absorbance at 540 nm was plotted as a function of collagen concentration by Sirius red colorimetric microassay. (**E**) The collagen contents of human ECM and fresh human adipose tissue. The scale bars represent 100 μm. Color images available online at www.liebertonline.com/ten.

In vitro *study*

Cell attachment and proliferation in human ECM scaffolds were examined using murine 3T3-L1 and human ASCs. Figure 6A shows cell attachment onto scaffolds fabricated at different temperatures. A suspension containing 1×10^5 cells was seeded on a disc-shaped scaffold and incubated for 6 h. The freezing temperature, which determined the pore size of the scaffold, greatly influenced cell attachment. The percentage of cell attachment on the scaffold with the largest pore size (fabricated at -10°C) was approximately 10 times higher than that on the scaffold with the smallest pore size (fabricated under liquid nitrogen). There was no significant difference in cell attachment between murine 3T3-L1 and human ASCs. The scaffold with the highest cell attachment was selected for the cell proliferation test (Fig. 6B). The number of murine 3T3-L1 and human ASCs in the scaffolds significantly increased with culture time. The proliferation of murine 3T3-L1 on the scaffolds was greater than that of human ASC. The cell attachment and proliferation assays suggested that human ASC could proliferate into a mass of cells on human ECM scaffolds.

In vivo *study*

To assess *in vivo* stability and adipogenic differentiation, a human ECM scaffold was transplanted into a nude mouse (Fig. 7). The grafted scaffold was easily identified throughout the experimental period of 8 weeks (Fig. 7C), implying high mechanical stability *in vivo*. At the end of this period, the human ECM scaffold had adhered to the surrounding tissues and was carefully separated as shown in Figure 7D. The gross shape of the scaffold was virtually unchanged. The top of the graft was covered by a thin layer of macroscopically yellow tissue and new vessels, which was tightly connected to the scaffold. There were fine ligamentous contacts to the

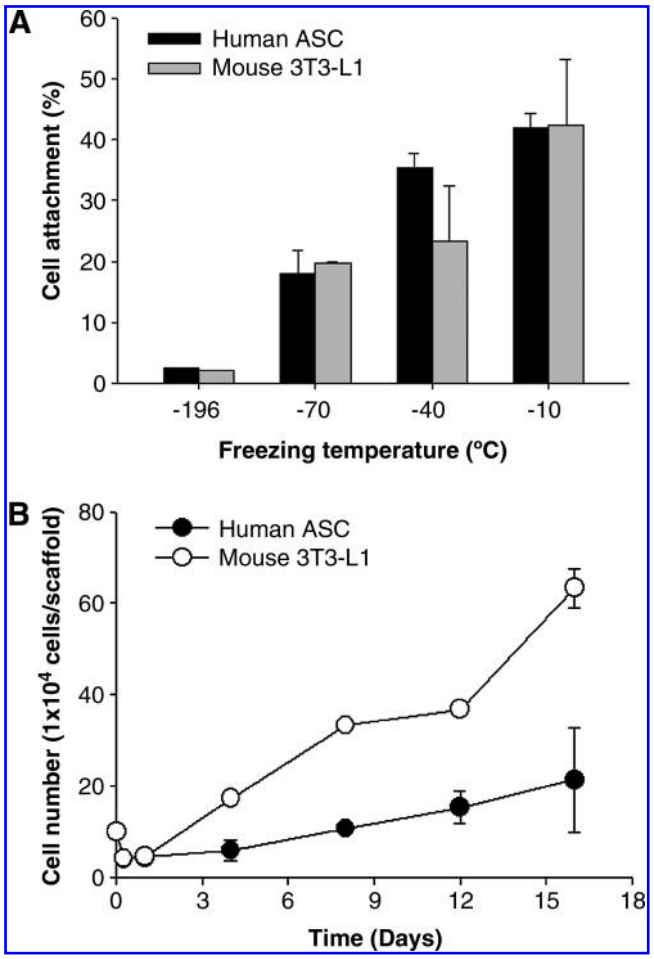

FIG. 6. (**A**) Cell attachment to human ECM scaffolds prepared under different freezing temperatures at 6 h after seeding. A suspension containing 1×10^5 cells was seeded onto disc-shaped human ECM scaffolds (diameter 1 cm and thickness 0.5 cm). After 6 h incubation, the number of attached cells was measured by an automatic cell counter. Each bar represents the mean% attached cells ± standard deviation, obtained from six identical samples. (**B**) Cell proliferation on human ECM scaffolds. A suspension containing 1×10^5 cells was seeded onto disc-shaped human ECM scaffolds (diameter 1 cm and thickness 0.5 cm). Cells were harvested after 1 to 16 days, and the number of cells was measured by an automatic cell counter. Each bar represents the mean cell number ± standard deviation, obtained from three identical samples.

surrounding tissues. Overall, the results demonstrated that the human ECM scaffold exhibited high mechanical stability and was highly compatible with the surrounding tissues (Fig. 7E).

The expression of the adipocyte genes PPARγ and leptin was evaluated by reverse transcription–PCR. Fresh mouse adipose tissues were used as a positive control for adipocyte-specific gene expression. PPARγ plays a key role in regulating several genes critical to adipogenesis and indicates terminal differentiation of adipocytes, and leptin is one of the most important adipose-derived hormones as a indicator of insulin metabolism.[35–37] Expression of PPARγ and leptin was detected in the graft. Thus, reverse transcription–PCR analysis of the graft suggested migration and adipogenic differentiation of the host mouse cells, as shown in Figure 7F.

Discussion

Tissue engineering uses 3D porous scaffolds to support cell adhesion and expansion. In general, the cells are isolated from patients, and the scaffolds are prepared from naturally occurring, synthetic, or hybrid materials. The isolated cells are seeded into the 3D porous scaffolds and temporarily cultured *in vitro*. The scaffolds with attached cells are implanted back into the patients and become incorporated into the body. Here we describe a new approach based on autologous scaffolds, in which tissue engineering scaffolds are derived from human tissues. Adipose tissue contains various kinds of cells including adipocytes, fibroblasts, smooth muscle cells, endothelial cells, immune cells (leukocytes and macrophages), and ASC.[16,17] Thus, adipose tissue is rich in ECM components such as collagen. Further, adipose tissue is a major endocrine and secretory organ and releases a wide variety of cytokines.[38] The fact that both cells and scaffold materials can be obtained from human adipose tissue is the key concept of this approach, and it may be termed autologous tissue engineering.

Recently, there has been considerable interest in tissue engineering scaffolds fabricated from various living tissues.[5] These natural scaffolds consisted mostly of ECM components such as collagen and contained various cytokines, but they were derived from animal tissues or isolated from cadavers and their usefulness may be limited by serious concerns regarding adverse immune responses and viral infection. It should be noted that our novel human ECM scaffold was fabricated using only physical stimuli, without addition of chemicals or enzymatic factors (Fig. 1). In addition, the porous human ECM scaffold can be fabricated in a variety of macroscopic shapes (Fig. 2) and the microscopic inner porous structure can be controlled by the freezing temperature (Fig. 3).

The major component of human ECM scaffolds is collagen (Fig. 5). Collagen has been extensively used as a biomaterial because it promotes cell adhesion and growth.[39] To date, most of this collagen has been extracted from animals,[40] although more recently human collagen has been extracted from donor cadavers, placentas, and aborted fetuses.[41] Undoubtedly, collagen isolated from the patient's own tissue is both safer and biocompatible.

Human ECM-derived scaffolds developed in this study have innovative potential for many clinical challenges in patients suffering from soft-tissue defects or needing soft-tissue augmentation. Adipose tissue engineering still presents a great challenge in plastic and reconstructive surgery.[42] Millions of plastic and reconstructive surgical operations are performed each year to repair soft-tissue defects that result from traumatic injury, tumor resections, and congenital defects.[43] In addition, over 10 million cosmetic procedures are performed, including various forms of soft-tissue augmentation.[44] Strategies used to repair adipose tissue defects include the transplantation of raw fat,[45–48] the use of fillers and implants,[49] and 3D porous scaffolds seeded with cells.[37,44,50–52] Although the transplantation of raw fat tissue is logical, it has not been consistently successful in patients.[42,45–48] When raw fat tissue is transplanted to a target location, a significant

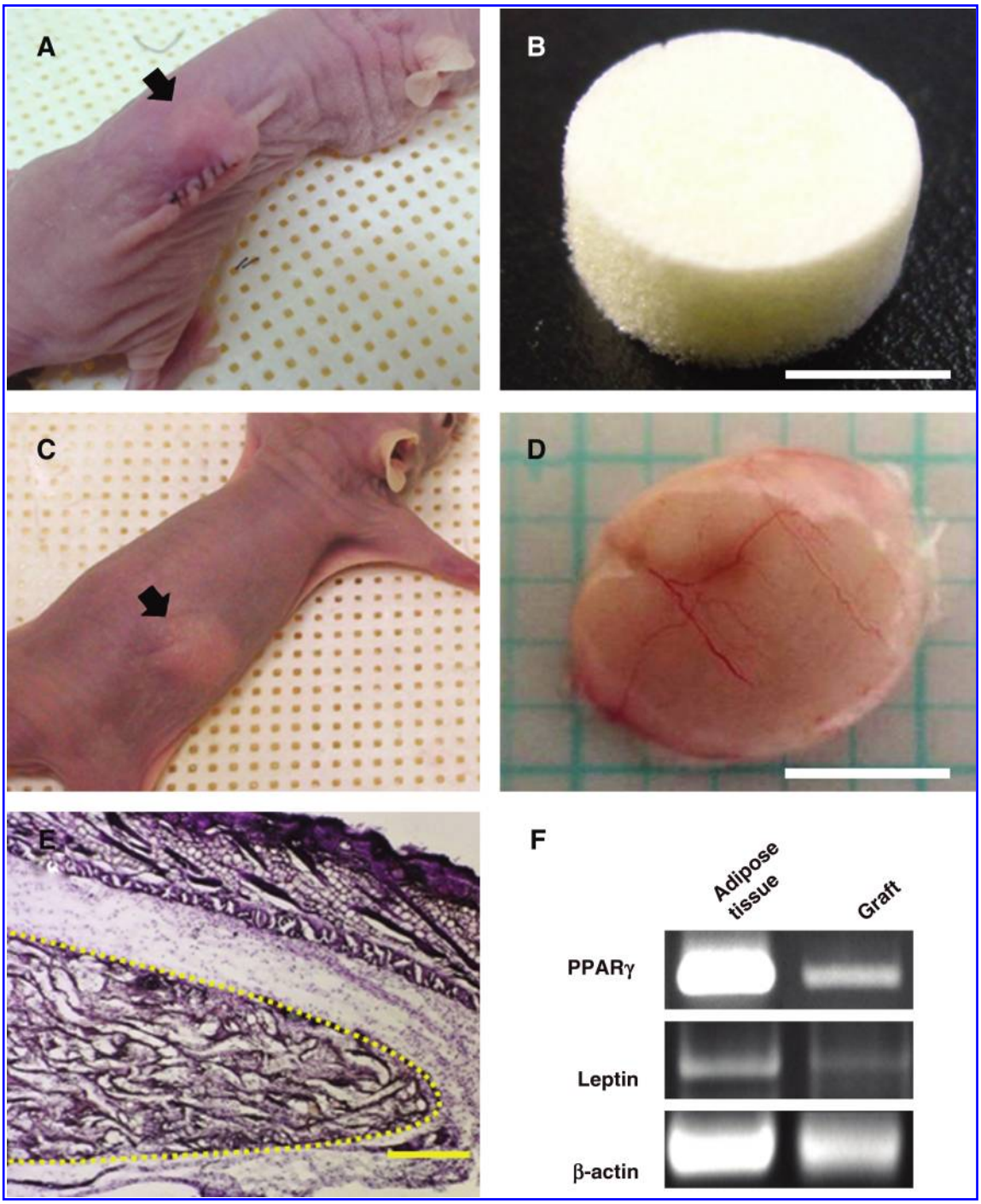

FIG. 7. Macroscopic appearance and histological examination of grafts in nude mice. A disc-shaped human ECM scaffold with a diameter of 1 cm and thickness of 0.5 cm (**B**) was transplanted subcutaneously into the back of each nude mouse (**A**). After 8 weeks (**C**), the mouse was killed and the graft was explanted (**D**). The grafts were stained with hematoxylin–eosin (**E**). Reverse transcription–polymerase chain reaction analysis of gene expression in the grafts (**F**). Fresh mouse fat tissue was used as a positive control. Each gene was analyzed using primers specific for peroxisome proliferative–activated receptor gamma, leptin, and β-actin. The white scale bars represent 50 mm and yellow scale bar represents 100 μm. The black arrows indicate implantation sites. Color images available online at www.liebertonline.com/ten.

amount of the transplanted fat tissues is frequently resorbed over time. Therefore, restoration of soft tissue might require a tissue engineering approach, for which autologous ECM scaffolds could be optimal. We believe that this new approach of using human ECM scaffold could find broad clinical utility in soft-tissue engineering, not only for aesthetic plastic surgery such as breast implants but also for reconstruction of tissues lost as a result of extensive deep burns, tumor resection, and hereditary and congenital defects.

Conclusions

The present study describes a new tissue engineering scaffold derived from human adipose tissue. Highly porous 3D scaffolds with a variety of shapes were fabricated from human adipose tissue. The scaffold possessed an open pore microstructure with a high degree of interconnectivity and the pores were fairly uniform. The human ECM scaffolds are supportive of the adhesion and proliferation of human ASCs. Our findings suggest that human adipose tissue could be one of the most abundant, expendable, and easily harvested biomaterials. Development of scaffolds made of human adipose tissue may open new opportunities for further advances in tissue engineering.

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Disclosure Statement

No competing financial interests exist.

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