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Human extracellular matrix (ECM) powders for injectable cell delivery and adipose tissue engineering

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ABSTRACT

Here, we present extracellular matrix (ECM) powders derived from human adipose tissue as injectable cell delivery carriers for adipose tissue engineering. We postulate that human adipose tissue may provide an ideal biomaterial because it contains large amounts of ECM components including collagen. Fresh human adipose tissue was obtained by a simple surgical operation (liposuction). After removing blood and oil components, the tissue was homogenized, centrifuged, freeze-dried, and ground to powders by milling. In an *in vitro* study, the human ECM powders were highly effective for promotion of cell attachment and proliferation for three-dimensional (3D) cell culture. In *in vivo* studies, suspensions of human ECM powders containing human adipose-derived stem cells (hASCs) were subcutaneously injected into nude mice. At eight weeks post-injection, numerous blood vessels were observed and the newly formed tissue exhibited adipogenesis with accumulated intracellular small lipid droplets. Overall, the grafts showed well-organized adipose tissue constructs without any signs of tissue necrosis, cystic spaces, or fibrosis. We believe that human ECM powders could act as efficient injectable biomaterials for tissue engineering and have great potential for meeting clinical challenges in regenerative medicine, particularly in relation to adipose tissue engineering.

1. Introduction

Scaffolds for tissue engineering are fabricated into different types, including implantable and injectable types, using different manufacturing technologies. Injectable tissue engineering is attractive not only because it minimizes the risk of infections, scarring, and high costs, but also because it may be used to fill irregularly sized defect sites [1–3]. Injectable tissue engineering scaffolds may be in the form of solutions, pastes, gels, microparticles, and microbeads. The local, direct delivery of cells attached to scaffolds is performed by simple injection, thereby circumventing the need for surgery [4,5]. The scaffolds are fabricated from either natural materials, including collagen, chitosan, and alginate, or synthetic materials, including poly(glycolide) (PGA), poly (lactide) (PLA), and poly(caprolactone) (PCL) [6–8].

In the body, cells are supported by the intricate extracellular matrix (ECM), which contains various protein fibers interwoven in a hydrated gel composed of a network of glycosaminoglycan (GAG) chains [9–11]. The ECM influences cellular responses, such as survival, development, shape, migration, polarity, and behavior of cells, by interacting with cellular adhesion molecules, growth regulators, binding proteins, proteolytic enzymes, and enzyme inhibitors [12]. Tissue engineering scaffolds composed of ECM derived from intact mammalian tissues have been shown to facilitate the reconstruction of various tissues in both clinical and animal studies [11]. The ECM scaffolds have been derived from a variety of tissues, including small intestinal submucosa (CuffPatch™, Durasis®, Restore®) [13–15], heart valves [16,17], blood vessels [18], skin (AlloDerm, Graft Jacket®) [19], nerves [20], skeletal muscle [21], placenta [22], bladder [23] and liver [24]. ECM scaffolds have many biological advantages, such as biocompatibility, biodegradability, and bio-inductive properties. However, it should be noted that most ECMs are isolated from animals or cadavers, and as a result, concerns have been raised regarding immunogenicity and pathogen transmission.

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Here, we describe ECM powders derived from human adipose tissue. Adipose tissue is a highly specialized connective tissue found ubiquitously in the body that serves unique functions such as energy storage, cushioning, and insulation [25]. The major cellular component in adipose tissue is lipid-filled adipocytes that are held in place by collagen fibers. Other cellular components contained in adipose tissue are stromal–vascular cells including smooth muscle cells, endothelial cells, fibroblasts, blood cells, and adipose-derived stem cells (ASCs). Indeed, adipose tissue not only contains various ECM components, such as collagen, reticular fibers, elastin fibers, nerve fibers, vascular stroma, and lymph nodes, but also contains many endocrine and paracrine actors commonly referred to as adipokines [26,27]. More importantly, adipose tissue can be safely obtained in large quantities using liposuction techniques [28,29]. Hence, we hypothesize that adipose tissue could provide an ideal material for cell delivery and tissue engineering. Herein, we have developed injectable human ECM powders derived from adipose tissue and explored their possible applications to stem cell delivery and adipose tissue engineering.

2. Materials and methods

2.1. Fabrication of human ECM powders from adipose tissue

Human adipose tissue was obtained from healthy donors between 20 and 40 years of age who had undergone liposuction with a single combined machine (Lipokit, Medikan Inc., Seoul, Korea) at the Kangnam Plastic Surgery Clinic (Seoul, Korea) [30]. The adipose tissue obtained by liposuction (~20 ml) was washed several 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. 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 frozen at −70 °C, freeze-dried, and crushed using a manual mill.

2.2. Isolation of human adipose-derived stem cells from adipose tissue

hASCs were isolated from subcutaneous adipose tissue of healthy female donors between 20 and 40 years of age who had undergone liposuction at the Kangnam Plastic Surgery Clinic (Seoul, Korea). According to the modified procedure for the isolation of hASC [31,32], the adipose tissue was washed with PBS containing 5% penicillin/streptomycin (P/S) (Gibco-BRL, USA). After the removal of red blood cells, the human adipose tissue was digested in PBS supplemented with 0.01% (w/v) collagenase type II (Gibco-BRL) for 1 h at 37 °C. The digested tissue was filtered through a 100-μm mesh to remove aggregated tissue and debris. The filtered suspension was centrifuged at 200 ×g for 7 min and the pellet was washed several times with PBS. The isolated cells were incubated in DMEM (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL) and 1% P/S at 37 °C under 5% CO_2.

2.3. Scanning electron microscopy (SEM)

The structures of human ECM powders were observed using scanning electron microscopy (SEM, Hitachi S-4800 FE-SEM, Japan). The human ECM powders were fixed to metal stubs and coated with platinum by sputtering at an accelerating voltage of 15 kV.

2.4. In vitro studies

The human ECM powders were sterilized by ethylene oxide (EO) gas, washed with PBS, and pre-wetted with serum-free DMEM medium. For a conventional two-dimensional (2D) cell culture, hASCs were plated in a tissue culture plate at a density of 1×10^5 cells/100 mm^2 in DMEM supplemented with 10% FBS and 1% P/S and grown for 20 days at 37 °C with 5% CO_2. For three-dimensional (3D) cell culture, hASCs were seeded into a suspension of human ECM powders in DMEM. The hASC-seeded human ECM powders were incubated for 12 h to allow cell attachment and were then transferred to a spinner flask containing a magnetic stir bar. The spinner flasks were filled with 50 ml of culture medium and put on a magnetic stir plate (Bellco Glass Inc., USA) at 80 rpm in an incubator. The culture medium was half removed and replaced by fresh medium every three days. Cells were maintained for 20 days and cell number was measured by an automatic cell counter (Nucleocounter™, ChemoMetec, Denmark). The cell viability analysis was performed using a commercially available Live/Dead® Viability/Cytotoxicity kit (Molecular Probes™, Eugene, OR, USA). The human ECM powder suspensions containing cells were transferred to new 12-well plates and washed with PBS, and were then stained for 20 min with 100 μl of the combined Live/Dead® reagents at 37 °C under darkness. After staining, the human ECM powders were observed using a fluorescence microscope (IX81, OLYMPUS CORPORATION, Tokyo, Japan) equipped with a digital camera.

2.5. In vivo experiments

Suspensions of human ECM powders alone or human ECM powders containing hASCs (0.5 ml, 1×10^5 cells) were injected subcutaneously into the backs of female mice (BALB/cAnNCrj-nu/nu, six weeks old) using an 18-gauge needle. At eight weeks after the injections, the grafts were explanted, weighed, and fixed with 4% paraformaldehyde. Ten mice were analyzed per experimental group.

2.6. Histological analysis

The grafts were assessed by hematoxylin–eosin and oil red O stainings of frozen sections. The tissue samples were fixed in 10% sucrose. After being embedded in OCT compound, samples were frozen at -70 °C. The frozen samples were sliced into 10-μm sections using a cryostat, washed with distilled water and 30% isopropanol to remove the OCT compound, and then stained with the hematoxylin–eosin and oil red O working solutions.

2.7. Reverse transcription polymerase chain reaction (RT-PCR)

Total mRNA was isolated from cells and tissues using a Trizol reagent (Invitrogen, USA). The complementary DNA (cDNA) was synthesized using moloney murine leukemia virus reverse transcriptase (M-MLV RT, Invitrogen, USA). The cDNA was used as a template for PCR analysis with primers specific for human peroxisome proliferating activated receptor γ (PPARγ), adipocyte fatty acid-binding protein (aP2), adiponectin, and β-actin (Table 1). 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.

3. Results

3.1. Preparation and characterization of human ECM powders

Human adipose tissues were obtained from healthy donors who had undergone liposuction. The human ECM powders were prepared using simple physical treatments without the addition of chemicals or enzymatic factors. Human adipose tissue isolated after liposuction was washed with distilled water to remove blood components and then homogenized. The tissue suspension was centrifuged and washed several times to remove oil components including lipids. The gel-like product was freeze-dried and then ground to powders by milling (Fig. 1). SEM images of human ECM powders are shown in Fig. 2. These

Table 1
Sequences of PCR primers.

Gene	Forward and reverse primer sequences	Annealing temperature (°C)	Product size (bp)	Origin	Gene bank accession no.
PPARγ	5′-AGA CAA CAG ACA AAT CAC CAT-3′	50	401	Human	NM015869
	5′-CTT CAC AGC AAA CTC AAA CTT-3′				
	5′-ACT GCC TAT GAG CAC TTC AC-3′	50	448	Mouse	NM011146
	5′-CAA TCG GAT GGT TCT TCG GA-3′				
aP2	5′-TGC AGC TTC CTT CTC ACC TTG A-3′	55	256	Human	NM001442
	5′-TCC TGG CCC AGT ATG AAG GAA ATC-3′				
	5′-GAA TTC GAT GAA ATC ACC GCA-3′	55	94	Mouse	NM024406
	5′-CTC TTT ATT GTG GTC GAC TTT CCA-3′				
Adiponectin	5′-TGG TGA GAA GGG TGA GAA-3′	50	221	Human	NM004797
	3′-AGA TCT TGG TAA AGC GAA TG-3′				
	5′-CAA GGG AAC TTG TGC AGG-3′	50	400	Mouse	NM009605
	3′-CGT GAT GTG GTA AGA GAA GTA G-3′				
Leptin	5′-TCT TGT GGC TTT GGC CCT ATC T-3′	55	181	Human	NM000230
	3′-CCA GTG TCT GGT CCA TCT TGG ATA-5′				
	5′-TGC TGC AGA TAG CCA ATG AC-3′	55	142	Mouse	NM008493
	3′-GAG TAG AGT GAG GCT TCC AGG A-5′				
β-actin	5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′	60	663	Human	NM001101
	5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′				
	5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′	60	349	Mouse	NM007393
	5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′				

powders appeared to be suitable for 3D culture of adherent cells because they were highly diverse in size and shape. They have both rugged and smooth surfaces with an extensive surface area, which could be favorable for cell adhesion and proliferation.

3.2. In vitro cell adhesion and proliferation studies

Cell adhesion and proliferation properties of human ECM powders were compared to those of a general two-dimensional (2D) culture method. For a cell adhesion study, a cell suspension containing 1×10^5 cells in DMEM was gently mixed with a suspension of human ECM powders in DMEM. The cells were allowed to attach on the human ECM powders for 12 h. For a cell proliferation study, the cells were incubated for 20 days in a spinner flask. As shown in Fig. 3A, the percentage of cell attachment to the human ECM powders was slightly lower than that in 2D culture. However, hASCs proliferated much better in 3D culture using human ECM powders than in 2D culture (Fig. 3B). The number of hASCs on human ECM powders significantly increased for 20 days, resulting in a 12-fold expansion in cell number. In addition, hASCs in human ECM powders showed good spreading and distribution, as confirmed by Live/Dead assays (Fig. 3D,E). Most cells attached onto the human ECM powders expressed green fluorescence, indicating that they were still viable 20 days post-seeding. Red fluorescence indicating dead cells was hardly observed.

3.3. In vivo study

To assess *in vivo* biocompatibility, mechanical stability, cell ingrowth and adipogenic differentiation, a suspension of human ECM powders with hASC (Fig. 4C) or without hASC (Fig. 4B) was subcutaneously injected into nude mice. As a control, fresh human adipose tissue isolated from liposuction was also subcutaneously injected into nude mice (Fig. 4A). The injected human ECM powders were easily identified throughout the experimental period of eight weeks, as shown in Fig. 4. The human ECM powders adhered to surrounding tissues and the surface of grafts revealed new vessel formation. The graft of hASC-loaded human ECM powders was slightly larger in volume than other grafts.

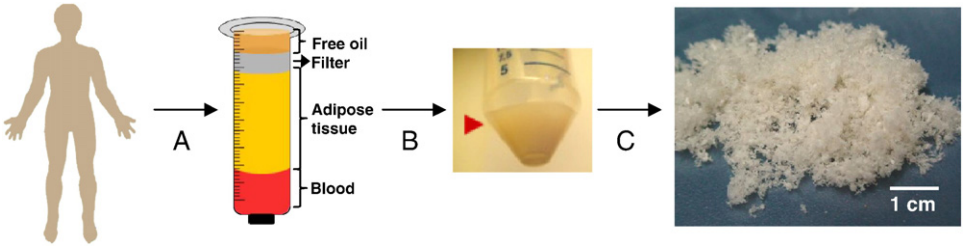

Fig. 1. Schematic representation of the preparation procedure for human ECM powders. (A) Human adipose tissue was obtained by liposuction and washed several times with distilled water to remove blood and oil components. (B) The adipose tissue was mixed with distilled water and homogenized at 12,000 rpm for 5 min. The homogenized adipose tissue was centrifuged at 3000 rpm for 5 min and washed several times with distilled water. (C) The final gel-like products were freeze-dried and ground to powders by milling.

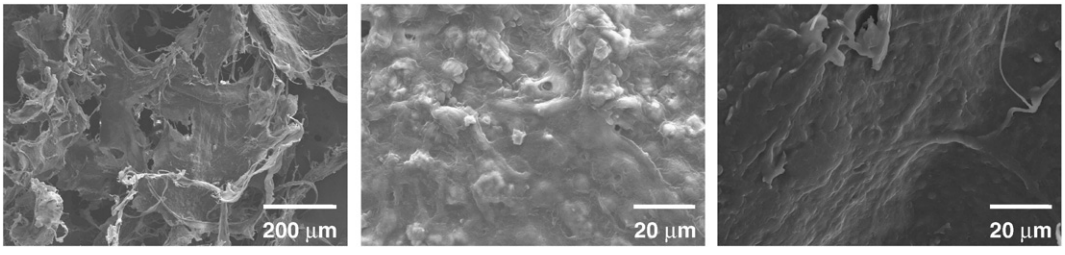

Fig. 2. SEM images of human ECM powders. Milled human ECM powders showed both rugged and smooth surfaces.

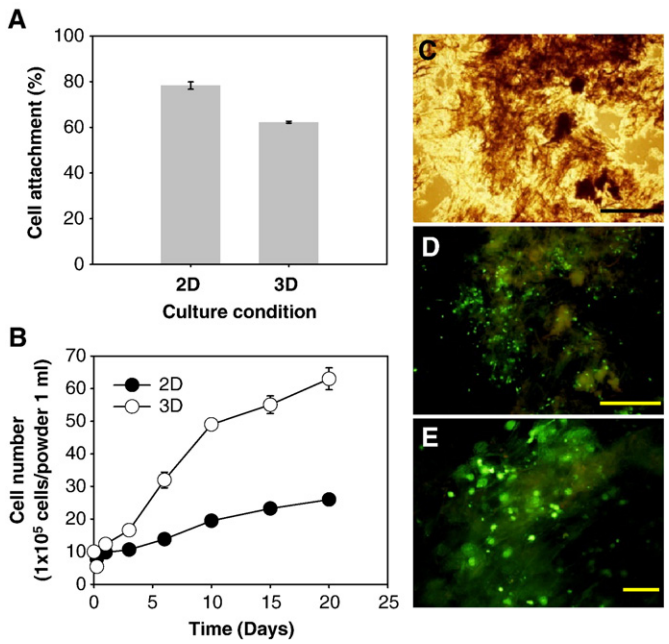

Fig. 3. (A) Cell attachment on human ECM powders after 12 h. (B) Cell proliferation on human ECM powders for 20 days. Each point represents the mean and standard deviation of $n=3$ independent replicates. The number of cells was measured by an automatic cell counter. A suspension containing 1×10^5 cells was seeded on human ECM powders and cultivated in a spinner flask. As a control group (2D culture), hASCs were grown in a 100-mm culture dish. (C) An optical photograph of cells attached on hECM powders on day 20. (D and E) Fluorescence micrographs of cells attached on human ECM powders, stained by calcein-AM (green = live) and ethidium homodimer (red = dead) on day 20. Scale bars represent 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The grafts were histologically examined by hematoxylin–eosin staining (Fig. 5). Both grafts of hASC-loaded human ECM powders and human ECM powders alone showed new well-organized tissue

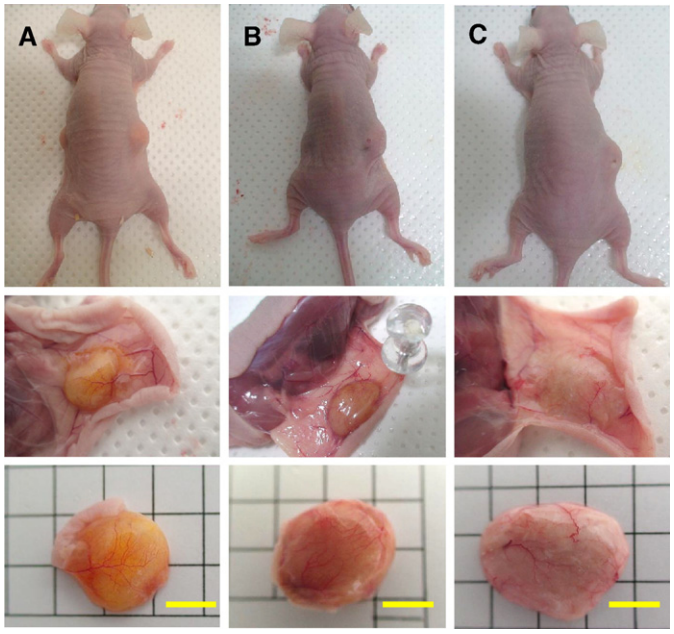

Fig. 4. Macroscopic appearance of grafts in nude mice. Fresh human adipose tissue (A) which was isolated from liposuction, a suspension of human ECM powders without cells (B), or a suspension of human ECM powders containing hASC (C) was individually injected into the back of each nude mouse. At eight weeks after subcutaneous injection, the mice were sacrificed and the grafts were carefully explanted. The yellow scale bar represents 5 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

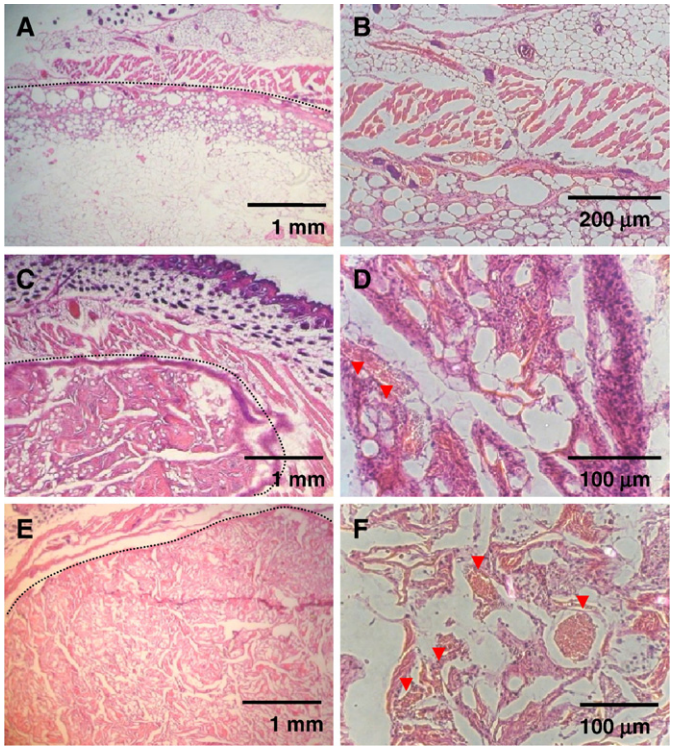

Fig. 5. Histological examination of the grafts, stained by hematoxylin–eosin, eight weeks after injections of fresh human adipose tissue (A and B), a suspension of human ECM powders without hASCs (C and D), and a suspension of human ECM powders with hASCs (E and F). Newly formed adipose tissue and blood vessels were observed in the grafts of human ECM powders without cells and human ECM powders with cells. The black dotted lines indicate implantation sites and the red arrows indicate newly formed blood vessels. Magnification: left, 40×; right, 400×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

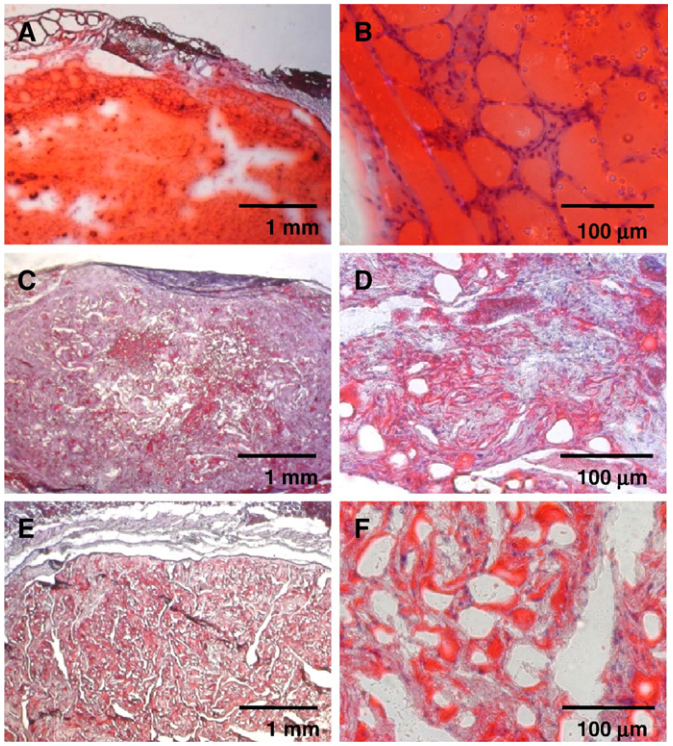

Fig. 6. Histological examination of grafts, stained by oil red O, eight weeks after injection of fresh human adipose tissue (A and B), a suspension of human ECM powders without hASCs (C and D), and a suspension of human ECM powders with hASCs (E and F). Numerous oil drops were observed in the grafts of human ECM powders with hASCs (E and F). Magnification: left 40×; right, 400×.

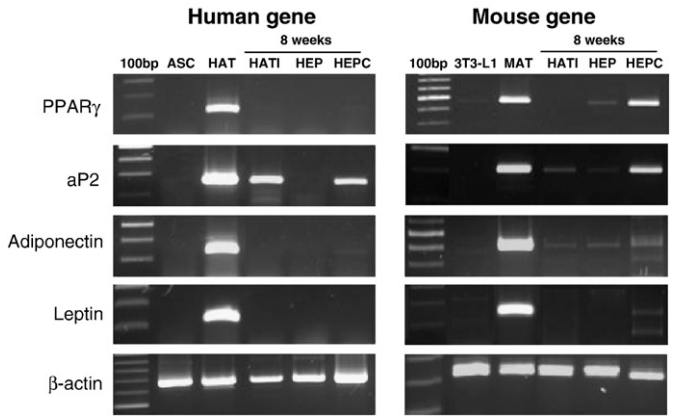

Fig. 7. RT-PCR analysis of gene expression for adipogenic differentiation in the grafts. hASCs and mouse 3T3-L1 undifferentiated cells were used as a negative control. Fresh human and mouse adipose tissues were used as a positive control. Each gene was analyzed using human and mouse-origin primers designed for peroxisome proliferative activated receptor gamma (PPARγ), adipocyte fatty acid-binding protein (aP2), adiponectin, and leptin. β-actin was used as an internal control. HAT, human adipose tissue; MAT, mouse adipose tissue; HATI, human adipose tissue injection; HEP, human ECM powders only; HEPC, human ECM powders with cells.

without any signs of tissue necrosis, cystic spaces, or fibrosis. Overall, the neovascularization, ingrowth of mouse cells from the neighboring host tissue, and new tissue formation were more prominent in the grafts of hASC-loaded human ECM powders than those of human ECM powders alone. However, it is important to note that without pre-seeding with hASC, human ECM powders still induced the formation of new blood vessels and ingrowth of mouse cells.

The *in vivo* adipogenesis was analyzed by oil red O staining (Fig. 6) and by RT-PCR (Fig. 7). Both grafts showed adipogenesis with accumulated intracellular small lipid droplets, but there was more significant tissue formation and adipogenesis in the grafts of hASC-loaded human ECM powders than in the grafts without hASC. The expression of adipogenic genes such as PPARγ, aP2, adiponectin, and leptin was evaluated by RT-PCR. Fresh human and mouse adipose tissues were depicted for comparison. The expression of the human gene for adipogenesis, aP2, was clearly observed in the grafts of fresh human adipose tissue and hASC-loaded human ECM powders. Other adipocyte-specific human genes were not detected in the grafts. The expression of mouse genes for adipogenesis such as PPARγ, aP2, and adiponectin was weakly detected in the grafts of fresh human adipose tissue and human ECM powders alone. However, in the grafts of hASC-loaded human ECM powders, two mouse adipogenic genes, PPARγ and aP2, were strongly expressed, which suggests that the pre-seeded hASCs could induce adipogenesis of host tissue. Thus, RT-PCR analysis of the grafts suggested the ingrowth, migration and adipogenic differentiation of mouse cells from neighboring host tissues.

4. Discussion

Biological scaffold materials composed of ECM derived from cadavers, animals or plants have been used in human clinical applications [10,11]. While the composition and structure of these ECMs may positively influence a variety of cellular responses to the surrounding environment, they often lack sufficient mechanical strength and can lead to adverse immune responses and pathogen infections [12,33]. We developed a new biological scaffold material that utilizes ECM derived from human adipose tissue. Human adipose tissue contains a variety of cells, and therefore is rich in ECM components and releases a wide variety of cytokines [25,27]. More importantly, human adipose tissue is easily available due to a common surgical operation, liposuction.

An important advantage of human ECM powders is that they can pass through an 18-gauge needle. The use of injectable scaffolds is of clinical significance as it minimizes patient discomfort, risk of infection, scar formation, and the cost of treatment [4,34]. The use of an injectable cell delivery system is a very appealing option, as cell-filled matrices may be created by simple injection rather than surgical operations. Human ECM powders and accompanying cells can be injected directly into cavities of any size or shape.

We have shown that human adipose-derived stem cells (hASCs), when cultured on the human ECM powders, exhibited good adhesion and proliferation *in vitro* (Fig. 3). Generally, hASCs with multipotent differentiation potential appear promising as a source of autologous stem cells. The cells can be expanded *in vitro* up to 120 cell divisions without losing their stem cell potential. In order to obtain a sufficient number of cells for therapeutic purposes, extensive *in vitro* expansion would be required [35,36]. An important result of this study is the finding that human ECM powders are highly supportive of 3D stem cell culture. That is, the surface features and chemical and biological composition of human ECM powders could dramatically support the adhesion and proliferation of hASCs. The *in vitro* mass proliferation of stem cells could be achieved in a 3D culture system using human ECM powders to produce sufficient cells to engraft a patient.

The *in vivo* results provide evidence that human ECM powders support *in vivo* adipogenesis either alone or in combination with hASCs. The autologous adipose tissue transplantation has yielded poor results, with a 40–60% reduction in graft volume [29]. Human ECM powders alone or with hASC exhibited not only an increased graft volume, but also the formation of new adipose tissue with numerous blood vessels in the grafts without any signs indicating adverse immune responses (Fig. 5). As expected, prominent adipogenesis with a large number of oil drops was observed in the human ECM powders initially seeded with the hASCs (Fig. 6). Notably, we observed that adipogenesis was proceeding in grafts of human ECM powders alone. Local distributions of oil drops were found in these grafts, although the sizes of the oil drops were smaller than those in the grafts of hASC-seeded human ECM powders. These results suggest that human ECM powders induce ingrowth and differentiation of mouse cells such as preadipocytes from the neighboring host tissues. Adipogenic-specific genes, such as PPARγ, aP2, leptin, and adiponectin play a key role in regulating adipogenesis. They are involved in various functions of adipose tissue such as the transport or storage of lipids, glucose metabolism, and homeostasis [26,37]. Specifically, the adipogenic transcription factor, PPARγ, plays a central role in regulating adipocyte differentiation and fatty acid metabolism. Mouse adipocyte-specific mRNAs were expressed in both the grafts of human ECM powders alone and in combination with hASCs. These data support that the increased extent of adipogenesis cannot be attributed solely to direct differentiation of hASCs, but also to migration and/or adipogenic differentiation of host preadipocytes.

Although the exact mechanisms of how host cells migrated into grafts and influenced adipogenesis are not clear, many studies have documented the ability of adipocytes to secrete various paracrine factors, which may positively influence both the migration and differentiation of preadipocytes [25,38]. In this study, the expression of human and mouse adipogenic genes in human ECM powders in the presence of hASC implies that the hASC promotes the ingrowth and differentiation of host mouse cells. We also believe that human ECM powders containing a variety of adipokines themselves have the ability to induce the ingrowth, migration, and adipogenic differentiation of host cells. Human ECM powders could provide efficient biomaterial not only for scaffolds of 3D culture systems for stem cell expansion and differentiation, but also as injectable carriers for *in vivo* stem cell delivery and reconstruction of lost or damaged tissues.

5. Conclusions

We fabricated human ECM powders from adipose tissue for injectable tissue engineering. Human ECM powders with diversity in

size and shape provide excellent substrates for cell adhesion and growth, particularly for 3D culture of adherent cells. In an *in vivo* study using nude mice, human ECM powders were shown to support the formation of new adipose tissue. Our findings demonstrate that human ECM powders could act as efficient injectable cell delivery carriers and that long-term stable adipose tissue can be engineered *in vivo* by simple injection with cell-seeded human ECM powders, rather than surgical operation.

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