Pancreatic Lineage Cell Differentiation of Bone Marrow Mesenchymal Stromal Cells on Acellular Pancreatic Bioscaffold

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Objectives: We evaluated the potential differentiation ability of bone mesenchymal stem cells (BMSCs) into pancreatic lineage cells on a rat acellular pancreatic bioscaffold (APB) and the effect of differentiated BMSCs in vivo.

Methods: The BMSCs were dynamically or statically cultured with or without growth factor in both culture systems. We assessed the cytological behavior and differentiation. We also evaluated the pancreatic fibrosis and pathological scores.

Results: The proliferation rates of BMSCs were significantly higher in the APB groups. The APB induced BMSCs to express mRNA markers at higher levels. All tested pancreatic functional proteins were also expressed at higher levels in the APB group. The secretion of metabolic enzymes was higher in the APB system. The ultrastructure of BMSCs in the APB group further revealed the morphological characteristics of pancreatic-like cells. For the in vivo study, the pancreatic fibrosis and pathological scores were significantly lower in the differentiated BMSCs group. In addition, in both the in vitro and the in vivo study, growth factor significantly improved proliferation, differentiation, and pancreatic cell therapy.

Conclusions: The APB can promote BMSC differentiation toward pancreatic lineage and pancreatic-like phenotypes, giving it the potential for use in pancreatic cell therapies and tissue engineering.

Key Words: pancreas, acellular pancreatic bioscaffold, bone mesenchymal stem cells, proliferation, differentiation, chronic pancreatitis

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Severe pancreatic diseases, such as severe acute pancreatitis and pancreatic cancer, may cause pancreatic failure, which has an increasingly high global mortality rate.1–4 Because effective therapies are lacking, pancreas transplantation has been proposed as a potential therapeutic alternative for the treatment of organ defects or tissue injury. However, organ transplantation is greatly limited by the imbalance between the demand and supply of suitable donor organs; thus, regenerative medicine (RM), as an interdisciplinary and attractive field of research, has sought to overcome the limitations of replacement and transplantation treatment by facilitating the natural development of tissue.6

One of the roadblocks to success in the RM field is the identification of cells that can be used to regenerate bioengineered organs.7–9 Stem cells and their descendants or committed progenitors are capable of proliferating and differentiating into specialized cells.9 Because of their ability to self-renew and indefinitely maintain a population with identical properties through symmetric and asymmetric cell divisions, stem cell therapies for diseased solid organs are an important potential modality of RM.10

Mesenchymal stem cells (MSCs) can be isolated from tissues, such as bone marrow, adipose tissue, umbilical cord tissue, and amniotic fluid. Because of characteristics, such as self-renewal and multilineage differentiation capability into osteogenic, adipogenic, chondrogenic, and myogenic- and neurogenic-like lineages,11–15 MSCs offer great therapeutic potential and have been developed to treat a wide range of disorders.

The most extensively studied MSCs are bone mesenchymal stromal cells (BMSCs), characterized as fibroblast-like cells, which are isolated from bone marrow mesenchymal cellular populations. Bone mesenchymal stromal cells are renowned in RM for their multilineage differentiation potential and easy acquisition.15,16 Aside from their remarkable proliferative and multilineage differentiation and regenerative potential, BMSCs can affect the surrounding microenvironment by their multiple paracrine functions.17,18 In addition, they have immunomodulatory and antioxidant properties.19 Since the introduction of cell therapy as a strategy for the treatment of many diseases, BMSCs have emerged as ideal candidates for regenerative therapies.

Bone mesenchymal stromal cells can differentiate into cells of pancreatic lineages under certain culture conditions.20 However, such an inducing strategy cannot provide the conditions for BMSCs to proliferate rapidly with high viability. Also, these kinds of induced BMSCs do not maturely express all important pancreatic lineage cell markers. Moreover, they exhibit a loss of stem cell characteristics and functions after expansion in vitro.21,22

The acellular matrix (ACM) can serve as an ideal 3-dimensional (3D) platform for RM because it is biocompatible and preserves a 3D geometric and spatial architecture.23 The ACM has physiological levels of biochemical components, matrix-bound growth factors (GFs), and cytokines.24 Furthermore, it has intact and patent vascular structures that can transport nutrition and oxygen for seeding cells to attach and localize to specific topographical positions.25

A previous study showed that certain kinds of whole-organ ACM could support BMSCs to differentiate into mature cells and express functional markers.22,26–28 Furthermore, this type of induced cells has potential applications in regenerative therapy and tissue repair.21 Little is known, however, about the stimulatory effects of the whole-organ ACM on BMSC differentiation into pancreatic lineage cells.

Past work from our laboratory showed the biological utility of the acellular pancreatic bioscaffold (APB) as a whole-organ
ACM, which could support and enhance the proliferation and differentiation of AR42J pancreatic acinar cells for RM. Therefore, in this study, we investigated the ability of the APB to promote BMSC proliferation and differentiation, which may improve regenerative therapy.

MATERIALS AND METHODS

Isolation and Identification of BMSCs

All animal experiments were approved by the Institutional Animal Care of China and performed in accordance with the Animal Welfare Act Institutional Guidelines. We collected BMSCs from the bone marrow of adult Sprague-Dawley rats (weighing approximately 250 g, between 6 and 7 weeks old). The rats were killed using chloroform, and the femur and tibia were removed. To collect BMSCs, a 23-gauge syringe was inserted into the bone cavity and flushed with serum-free Dulbecco’s modified Eagle's medium (DMEM). After centrifuging at 1200 rpm for 10 minutes, the bone marrow cells were resuspended in DMEM (HyClone, Logan, Utah) that was supplemented with 10% fetal bovine serum (FBS), 10 U/mL penicillin, 10 μg/mL streptomycin (Gibco, Sydney, Australia), and 1% L-glutamate (Sigma-Aldrich, St. Louis, Mo). Finally, the number of viable cells was checked and transferred to culture dishes at a density of 5 × 10^5 cells per cm² in high-glucose DMEM containing 10% FBS for incubation at 37°C in a 5% CO₂ atmosphere. The medium was changed every 2 to 3 days. Only low-passage (55) cells were used in experiments. Cells were passaged every 7 to 10 days at a 1:3 ratio. The BMSCs were characterized by flow cytometry for detection of CD90, CD29, and CD45 expressions.

Decellularization of Rat Pancreas and Reseeding of APB

Adult Sprague-Dawley rats weighing approximately 250 g, aged 6 to 7 weeks, were killed. As previously described, the pancreata were decellularized by Easy-Load Digital Drive peristaltic pumps, and the biocompatibility of APB was assessed. Bone mesenchymal stromal cells were reseeded onto the APB and cultured in the biomimetic bioreactor system made by our laboratory for 7 days to evaluate replication and further differentiation. Cells were seeded on the APB by a multistep infusion with 2.5 × 10^6 cells of 2 mL each through the hepatic portal vein and the pancreatic duct. The medium was changed every 2 to 3 days. We observed the ultrastructure of seeded BMSCs on the APB by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). In addition, to assay the optimal flow rate of the APB supporting the proliferation of BMSCs, the dynamic culture was perfused retrogradely at different speeds (0, 0.5, 1, 2, and 4, and 6 mL/min). We quantified the DNA content of BMSCs in the APB scaffold at each speed on days 4 and 7.

Analysis of BMSC Characteristics by Laser-Scanning Confocal Microscopy

Tissue samples were fixed in 4% formaldehyde (ThermoFisher, Waltham, Mass), cryoprotected with 30% sucrose, and cut into 5-μm-thick sections. For immunostaining, we used rabbit primary antibodies (1:200; Boersen, Shanghai, China). We used goat antirabbit AlexaFluor 488 (1:500; Invitrogen, Carlsbad, Calif) as a secondary antibody. For colabeling using antibodies from the same host species, we conducted sequential staining by GFP (BioHermes, Boston, Mass). After the first primary antibody staining, we included an additional blocking step before adding a secondary antibody. The slides were washed 3 times with 1 × phosphate-buffered saline (PBS) (5–10 minutes each) before being mounted with ProLong Gold Anti-fade Reagent with DAPI (Invitrogen). After washing, the cells were incubated with streptavidin-conjugated Texas red for 30 minutes at 37°C, washed 6 times (10 minutes each) with PBS, and mounted on glass slides using FITC-guard (Testog Inc, Springfield, Ill) as the mounting medium.

We then examined cells in a PHOIBOS 1000 laser scanning confocal microscope (Sarastro, Stockholm, Sweden). Texas red was excited with an argon laser. We collected and used the emitted signals to create 3D reconstructions of serial confocal sections using the Vanis program (Sarastro).

In Vitro Differentiation of BMSCs Into Pancreatic Lineage Cells

After BMSCs were reseeded on APB for 7 days, they were induced into pancreatic-like clusters in differentiation culture systems (ie, dynamic culture on the APB and static culture in tissue culture flasks [TCFs]) in 3 steps for another 21 days. We recorded the time at the end of day 7 of BMSC reseeding on the APB as time zero.

Step 1: BMSCs were cultured in high-glucose DMEM (25 mM) containing 2% FBS, 0.2 mM β-mercaptoethanol (Gibco), 10 ng/mL basic fibroblast GF (bFGF) (Peprotech, Rocky Hill, NJ), and 10 ng/mL epidermal GF on (EGF) (Peprotech) for 7 days.

Step 2: BMSCs were cultured in serum-free high-glucose DMEM (25 mM) containing 10 ng/mL bFGF, 10 ng/mL EGF, 2% B27 (Gibco), 0.5% BSA, 10 mM nicotinamide (Sigma-Aldrich), and 10 ng/mL exendin-4 (Sigma-Aldrich) for 7 days.

Step 3: The cells were cultured in serum-free high-glucose DMEM (25 mM) containing 10 ng/mL EGF, 10 ng/mL Activin A (Peprotech), 10 ng/mL betacellulin (Peprotech), 2% B27, and 0.5% bovine serum albumin for 7 days. The medium was changed every 2 days.

In the static culture system, cells were cultured in the TCF system with a zero flow rate, whereas in the APB dynamic system, cells were cultured in the biomimetic bioreactor system on APB at the optimal flow speed. Depending on whether the differentiation was induced by GF in the culture system, we divided our study into 4 groups: BMSCs cultured in TCFs without GF (TCF-GF (–)), BMSCs cultured in TCFs with GF (TCF-GF(+)), BMSCs cultured on the APB without GF (APB-GF(–)), and BMSCs cultured on the APB with GF (APB-GF(+)).

Analysis of the Morphological Characteristics of Differentiating BMSCs by SEM and TEM

The samples were fixed in 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) for 60 minutes and washed thoroughly 3 times with 0.1 M PBS for 15 minutes each. Next, the samples were fixed in 1% OsO₄ in 0.1 M PBS for 60 minutes. This was followed by 3 more PBS washing cycles of 15 minutes each. The samples were then dehydrated in a gradient series of alcohol for 15 minutes each. In addition, samples were critical point dried and coated with Au/Pd using a Cressington Coater 108A sputter coater (Ted Pella, Sacramento, Calif). We took electron microscope images using a JEOL JSM-6335F (JEOL, Tokyo, Japan) field emission SEM.

For TEM, the samples were fixed in 2.5% glutaraldehyde in PBS, postfixed in 1% OsO₄ in PBS, dehydrated through a graded series of alcohols, and embedded in Epon. We cut thin (60 nm) sections using a Reichert Ultracut S, mounted on 200 mesh copper grids, and counterstained them with 2% aqueous uranylacetate for
7 minutes and 1% aqueous lead citrate for 2 minutes. We observed the samples with a JEOL 1011 TEM.

Microscopy Analysis of Morphological Changes in BMSCs During Differentiation

The morphologic changes of BMSCs were observed during the differentiation process, and the major and minor axes of the cells were computed from the moments up to the second order of the thresholded binary image of the cell using NIH Image J (V1.8.0.112; NIH, Bethesda, Md); The aspect ratio of the cell is the ratio of the major to minor axis.

Also, the identification of cell shape into round, branched, and spindle cell morphology was counted. Cell areas were also measured for each type of cell morphology, giving insights into surface cell spreading. The number of focal points was measured per cell for each type of cell morphology and normalized by the cell areas for each cell morphology. For each condition, a range of 80 to 300 cells was analyzed. A custom-made image analysis system Quantimet Q550 (Leica, Wetzlar, Germany) was used to measure the cell morphology parameters.

Cell Proliferation Assay

We analyzed bromodeoxyuridine (BrdU) incorporation immunohistochemically using a BrdU immunohistochemistry system. We assessed cell proliferation by measuring BrdU incorporation using a commercially available BrdU enzyme-linked immunosorbent assay (ELISA) kit (Abcam, Cambridge, UK).

FIGURE 1. The morphology of BMSCs isolated from bone marrow. A, The morphology of BMSCs in passage 1. B, The morphology of BMSCs in passage 3.

FIGURE 2. The morphology of BMSCs reseeded on APB by SEM and LSCM. A, The seeded BMSCs attached on APB (yellow arrows) and the adhesion among neighboring cells (white arrows) by SEM (scale bars = 1 μm). B, BMSCs not only attached on the surface of the bioscaffold, but also formed in cluster in the inner structure of APB indicated by LSCM (scale bars = 1 μm). Green, GFP-positive cells; red, component collagen; blue, DAPI. LSCM, laser-scanning confocal microscopy.
according to the manufacturer's protocol. Cells were fixed with a fixation solution and incubated with the anti-BrdU antibody for 90 minutes. After washing, we added tetramethyl-benzidine and measured the absorbance by a spectrophotometric plate reader at 405 nm.

**Assessment of the Expression of Pancreatic Lineage Gene Markers by Real-Time Reverse Transcription Polymerase Chain Reaction**

We extracted RNA using a NucleoSpin kit (Seebio Biotech, Shanghai, China) according to the manufacturer's protocol. We measured the absorbance at 280 and 260 nm using a BioRad Smart Spec spectrophotometer (BioRad Laboratories, Hercules, Calif) to evaluate the RNA concentration and quality. We performed reverse transcription using the ImProm II (Promega, Madison, Wis) reverse transcription kit according to the manufacturer's recommendations. We performed quantitative real-time polymerase chain reaction analysis for pancreatic acinar genes. Experiments were repeated in triplicate.

**Quantification of Pancreatic Functional Proteins by Western Blotting**

We prepared whole-cell lysates to evaluate important pancreatic proteins. We used antibodies at the following concentrations: anti-α-amylase (α-Amy), 1:1000; anticytokeratin 7 (CK7), 1:2500; anti-C-peptide, 1:2500; and antifetal liver kinase-1 (Flk-1), 1:2000. Membranes were incubated with the appropriate HRP-conjugated secondary antibody (1:5000). Antibody binding was detected by chemiluminescence radiography. Membranes were scanned, recorded digitally, and processed using Image J software.

**Characteristics of Pancreatic Functional Proteins for Differentiated BMSCs by Immunostaining**

The samples were fixed in 4% formaldehyde (ThermoFisher, Waltham, Mass), cryoprotected with 30% sucrose, and cut into 5-μm-thick sections. For immunostaining, the following rabbit primary antibodies were used: anti-α-Amy, 1:200; anti-CK7, 1:250; anti-C-peptide, 1:250; and anti-Flk-1, 1:200. Goat antirabbit Alexafluor 488 (1:500; Invitrogen) was used as a secondary antibody. For colabeling using antibodies from the same host species, sequential staining was conducted. After the first primary antibody staining, an additional blocking step was included before the addition of a secondary antibody. After actin staining, the slides were washed 3 times with 1× PBS (5–10 minutes) each before being mounted. Images were recorded on an Olympus microscope (Olympus, Tokyo, Japan).

**Quantification of Potential Metabolic Function of Differentiated BMSCs In Vitro**

For the insulin release assay in vitro, we treated cell clusters using 4 different glucose concentrations (5, 10, 15, and 25 mM). The samples were preincubated with the Krebs-Ringer bicarbonate (KRB) buffer for 1 hour. Subsequently, the clusters were removed and incubated for 1 hour in the KRB buffer containing different concentrations of glucose. The supernatant was collected and frozen at −80°C. We measured insulin levels using a rat insulin ELISA kit (Mercodia, Uppsala, Sweden) according to the manufacturer's protocol.

We measured Amy secretion according to 4 different cholecystokinin (CCK) concentrations: 0, 10⁻⁹, 10⁻⁸, and 10⁻⁷ mM. The Amy levels are expressed as ratios between the amount of Amy released into the extracellular medium and the total cellular...
Amy, as determined by permeabilizing cells with 0.1% SDS in 10 mM phosphate buffer (pH 7.8). Amylase levels were spectro-photometrically measured using the Phadebas amylase kit (Abcam, Guangzhou, China) according to the manufacturer’s protocol. We performed both processes at the end of the differentiation process.

In Vivo Studies of Differentiated BMSCs

To investigate the potential to use differentiated BMSCs to treat chronic pancreatitis (CP) in vivo, we randomly divided the rates into 6 groups (n = 10 rats per group): the control group, the model group, and 4 treatment groups (BMSCs cultured with TCF-GF(−), TCF-GF(+) and APB-GF(−), and APB-GF(+)). The CP rat model was induced by infusion of dibutyltin dichloride through the caudal vein. For the treatment group, 3 × 10⁶ differentiated BMSCs in 1 mL were injected through the caudal vein on days 20, 27, and 34 after model induction. For the model group, an equal volume of saline was injected into the caudal vein.

We collected pancreatic tissues 80 days after model induction for histopathological examination by the hematoxylin-eosin staining technique and detected the expression of alpha-smooth muscle actin (α-SMA), collagen types I and III, and interleukin-10 (IL-10) by ELISA.

Statistical Analysis

Data are expressed as mean (standard deviation). Significant differences among groups were determined by the Student t test.
for 2-group comparisons and analysis of variance of repeated measure followed by post hoc analysis for multiple-group comparisons. Probability values at $P < 0.05$ indicated statistical significance.

**RESULTS**

**Characterization of BMSCs Isolated From Bone Marrow**

Bone mesenchymal stromal cells in Passage 1 exhibited an irregular round shape and size (Fig. 1A), whereas in Passage 3, they changed to a regular form and had a spindle fibroblast-like or branched appearance (Fig. 1B). The third passage of BMSCs, 90.21% were positive for both CD90 and CD29, 98.53% were positive for CD90 and negative for CD45, and 93.38% were positive for CD29 and negative for CD45. At early-passage BMSCs, especially the third passage, BMSCs were positive for CD29 (integrin) and CD90 (cell surface antigen), but negative for CD45 (leucocyte antigen), the result was identified as rat BMSCs for morphology and surface antigens, indicating the purity of BMSCs.

**Repopulation of APB With BMSCs in Pancreatic Bioreactor Cultures**

The seeded BMSCs attached on the surface of the APB and exhibited different shapes. We observed the adhesion among neighboring cells by SEM (Fig. 2A). Also, laser-scanning confocal microscopy showed that BMSCs not only attached to the surface of the bioscaffold but also formed in clusters along the inner structure of the APB. The 3D scaffolds reseeded with the GFP-positive cells were counterstained with component collagen and DAPI (Fig. 2B). The APB, as the 3D scaffolding, could support the reseeding of BMSCs.

In addition, the DNA quantification of the BMSC-APB graft showed that 4 mL/min for the dynamic culture system was the optimal flow rate for BMSC proliferation on both day 4 and day 7, with a significant difference compared with other flow speeds ($P < 0.05$) (Fig. 3).

**The Ultrastructural Characteristics of BMSCs During Differentiation**

The SEM analysis revealed that undifferentiated BMSCs exhibited a spherical appearance with little microvilli at the cell surface (Fig. 4A). We did not observe any mature organelles by TEM (Figs. 4B, B1).

Scanning electron microscopy analysis revealed that differentiated BMSCs at day 21 were spindle fibroblast-like or branched in shape with denser microvilli at the cell surface (Fig. 5A). There was a major increase in the number of epithelial-like cell clusters with a complex intercalation of the extracellular matrix (ECM) (Figs. 5B, C). A significant amount of ECM fibers accumulated around each newly differentiated cell as well as cell

![FIGURE 6](image6.png)

**FIGURE 6.** Quantification of morphological changes for cell aspect ratio. The aspect ratio was higher in GF(+) groups compared with GF(−) groups. The aspect ratio was higher in BMSCs cultured on the APB than in BMSCs in the TCF group.

![FIGURE 7](image7.png)

**FIGURE 7.** Quantitative analysis of cell morphology counted per condition. The proportion of round cells decreased, whereas the branched cells increased with culture time. All of the 4 culture systems had a prominent branched shape after 21 days.
clusters that had developed from the BMSCs differentiating into endocrine and exocrine cells (Figs. 5D, D1, D2).

Transmission electron microscopy revealed that acinar-like cells were organized around ductal structures (Fig. 5E). The epithelial cells formed small clusters separate from the ducts (Fig. 5F). Acinar cells increased in number and formed amylase/Amy-positive cell clusters (Fig. 5F). The cell clusters for insulin also increased in number and formed islet-like structures (Fig. 5F) that contained capillaries (Fig. 5F).

In addition, TEM revealed glycogen positivity in ductal cells, nuclei, secretory granules, mitochondria (white arrow), and smooth and rough endoplasmic reticulum in differentiated BMSCs (Fig. 5G).

Quantification of the Morphological Changes of BMSCs During Differentiation

We quantified the morphological changes by measuring the aspect ratio and found that the aspect ratio increased from day 3 to day 21 in all groups (Fig. 6). Also, in both culture systems, the aspect ratio was higher in GF(+) groups compared with GF(−) groups. In addition, with or without GF, the aspect ratio was higher in BMSCs cultured on the APB than in BMSCs in the TCF group. The aspect ratio was higher in APB-GF(−) cells than in TCF-GF(−) cells, with a significant difference from day 14 (P < 0.05), and it was higher in APB-GF(+) cells than in TCF-GF(+) cells, with a significant difference from day 5 (P < 0.05).

As shown in Figure 7, cell morphology in round, branched, and spindle cell shapes were identified during the differentiation process in all 4 groups. Overall, the initial state of differentiation was predominantly characterized by round morphology. The proportion of round cells decreased, whereas that of branched cells increased with culture time. The spindle cells increased from day 0 in all 4 groups, but decreased after 14 days in the TCF-GF(−) group, 7 days in APB-GF(−) and TCF-GF(+) cells, and 5 days in the APB-GF(+) group. All 4 culture systems had a prominent branched shape after 21 days. The round cells decreased significantly (P < 0.05) from day 14 in the TCF-GF(−) system, from day 7 in the APB-GF(−) and TCF-GF(+) group, and from day 3 in the APB-GF(+) group. The spindle shape cells increased significantly (P < 0.05) from day 14 in the TCF-GF(−) system, from day 7 in APB-GF(−) and TCF-GF(+) cells, and from day 3 in the APB-GF(+) group. The branched cells increased significantly (P < 0.05) from day 14 in the TCF-GF(−) system, from day 7 in APB-GF(−) and TCF-GF(+) group, and from day 5 in the APB-GF(+) group.

![FIGURE 8. Number of focal adhesion points culture time on different culture system. The number of focal adhesion points was determined per μm² on different culture system. Focal point density was higher in GF(+) groups compared with GF(−) groups. The focal point density was higher in BMSCs cultured on the APB than in BMSCs in the TCF group.](image1)

![FIGURE 9. The proliferation of BMSCs grown on culture system.](image2)
The focal points as a function of cell adhesion and spreading were also determined (Fig. 8). The focal point density increased with culture time, indicating cells spreading and adhesion in the culture system. The focal point density increased from day 3 to day 21 in all groups. Also, in both culture systems, the focal point density was higher in GF(+) groups compared with GF(−) groups. In addition, with or without GF, the focal point density was higher in BMSCs cultured on the APB than in BMSCs in the TCF group. The focal point density was higher in APB-GF(−) cells than in TCF-GF(−) cells, with a significant difference from day 14 (P < 0.05), and it was higher in APB-GF(+) cells than in TCF-GF(+) cells, with a significant difference from day 5 (P < 0.05).

BMSC Proliferation During Differentiation

To determine whether the APB allowed BMSCs to grow and to compare its efficacy with that of other culture systems (Fig. 9), we performed a BrdU incorporation assay to analyze the time-dependent growth of BMSCs from days 3 to 21. From day 3, the cell proliferation rate was significantly higher in the APB dynamic system than in the TCF culture, with or without GF (P < 0.05). Also, in the presence of GF, the proliferation rate was higher in both the APB system and the TCF system, with a significant difference from day 3 (P < 0.05). These results indicate that the APB efficiently facilitated pancreatic-like cell growth and that GF promoted BMSC proliferation.
Evaluation of the Gene Expression of Pancreatic Markers

To assess the ability of the APB to support BMSC differentiation, we assessed the expression of pancreatic genes at different time points (Fig. 10). Pancreatic duodenal homeodomain containing transcription factor (PDX-1) and pancreatic exocrine transcription factor (PTF-1) gene expression increased, whereas Oct4 expression decreased from day 3 to day 21 in all groups. Furthermore, in both culture systems, the expression of PDX-1 and PTF-1 was

FIGURE 11. The expression of pancreatic functional proteins. A, The expression of pancreatic functional protein α-Amy by Western blot. B, The expression of pancreatic functional protein CK7 by Western blot. C, The expression of pancreatic functional protein Flk-1 by Western blot. D, The expression of pancreatic functional protein C-peptide by Western blot.
higher, whereas Oct4 expression was lower in the GF(+) groups compared with the GF(−) groups. In addition, with or without GF, the expression of PDX-1 and PTF-1 was higher, whereas the expression of Oct4 was lower in BMSC cells cultured on the APB. The expression of PDX-1 and PTF-1 was higher in APB-GF(−) cells than in TCF-GF(−) cells, with a significant difference from day 7 (P < 0.05), and it was higher in APB-GF(+) cells than in TCF-GF(+) cells, with a significant difference from day 3 (P < 0.05). Conversely, Oct4 expression was lower in APB-GF(−) cells than in TCF-GF(−) cells, with a significant difference from day 5 (P < 0.05), but it was lower in APB-GF(+) cells than in TCF-GF(+) cells, with a significant difference from day 3 (P < 0.05).

Evaluation of the Expression of Pancreatic Functional Proteins

To verify these findings, we performed Western blotting of pancreatic functional proteins, (Fig. 11). The protein expression of α-Amy, CK7, C-peptide, and Flk-1 increased from day 3 to day 21 in all groups. Also, in both culture systems, the expression of all 4 proteins was higher in GF(+) groups compared with GF(−) groups. In addition, with or without GF, the expression of the 4 proteins was higher in BMSCs cultured on the APB than in BMSCs in the TCF group. The expression of C-peptide was higher in APB-GF(−) cells than in TCF-GF(−) cells, with a significant difference from day 5 (P < 0.05), and it was higher in APB-GF(+) cells than in TCF-GF(+) cells, with a significant difference from day 3 (P < 0.05). The expression of the other 3 proteins was higher in APB-GF(−) cells than in TCF-GF(−) cells, with a significant difference from day 14 (P < 0.05), but it was higher in APB-GF(+) cells than in TCF-GF(+) cells, with a significant difference from day 5 (P < 0.05). The gene and proteins results indicate that pancreatic cell differentiation was enhanced in the presence of APB and that GF promoted BMSC differentiation.

The Expression of Pancreatic Functional Proteins for Differentiated BMSCs by Immunostaining

The immunostaining analysis showed that BMSCs could differentiate into endocrine β-cells and exocrine acinar cells attached and localized to different vitro systems (Fig. 12). Unlike on TCF (Figs. 12A, C), the immunostaining image showed the positive expression of C-peptide (an analog of insulin marker identifying endocrine β cells) and amylase (a digestive enzyme identifying exocrine acinar cells) in their specific topographical locations on the APB. In other words, similar to native pancreatic tissue, most C-peptide–positive cells were localized along island regions, whereas α-Amy–positive cells were lined with tubular ductal spaces and lobulus acinar structures on the APB because of their seeding route (Figs. 12B, D).

In addition, immunostaining assessment revealed the localization of CK7 (a marker contributing to the formation of the duct structures) and Flk-1 (a factor associated with the endothelial layer of the vasculature) (Fig. 13). Distinct from TCF system, where CK7 and Flk-1 were interdispersed (Fig. 13A, C), the immunostaining image demonstrated that the intact vasculature and ductal structure could be detected on the APB, which indicated the preservation of architecture of vasculature and ductal structure after BMSC differentiation (Figs. 13B, D).

These results indicate that the APB is capable of supporting pancreatic lineage and pancreatic-like phenotypes. It may allow the reconstitution of major cellular constituents as in the native pancreas.
Assessment of Differentiated BMSCs

We detected insulin secretion levels (pg) of the cell clusters in the presence of 5, 10, 15, and 25 mM glucose (Fig. 14A). The insulin levels were enhanced when concentrations of glucose increased in all 4 groups. At the lowest concentration (5 mM), there was no significant difference among the 4 groups ($P > 0.05$). At a concentration of 10 mM, in the presence of GF, there was a significant difference between the dynamic and the static system ($P < 0.05$). In both systems, however, there was no significant difference between the presence or absence of GF ($P > 0.05$). At glucose concentrations of 15 and 25 mM, with or without GF, insulin secretion levels were higher in the APB groups than in the TCF groups ($P < 0.05$), and the levels of insulin were significantly higher in the presence of GF in both APB and TCF culture systems ($P < 0.05$).

We detected $\alpha$-Amy secretion levels in the culture systems in the presence of $10^{-11}, 10^{-10}, 10^{-9}$, and $10^{-8}$ mM CCK (Fig. 14B). The Amy levels were enhanced with increasing CCK concentrations in all 4 groups. At a concentration of $10^{-11}$ or $10^{-10}$ mM, there was no significant difference among the 4 groups ($P > 0.05$). At a concentration of $10^{-9}$ or $10^{-8}$ mM, we observed significant differences in $\alpha$-Amy secretion levels between the APB system and the TCF system, with or without GF ($P < 0.05$), and between the presence and absence of GF in both culture systems ($P < 0.05$).

CP Treatment With Differentiated BMSC In Vivo

Consistent with the pathological change indicated in Figure 15, both the pathological score and the pancreatic fibrosis score (Table 1) were lower in each treatment group compared with the model group ($P < 0.05$). In the 4 treatment groups, with or without GF, the pathological score and the pancreatic fibrosis score were lower in the APB group than in the TCF group ($P < 0.05$). In addition, both culture systems, the scores were higher in the GF (−) groups than in GF(+) groups ($P < 0.05$).

The tissue expression levels of $\alpha$-SMA and collagen types I and III (Table 2) were lower in each treatment group than in the model group ($P < 0.05$), whereas the levels of IL-10 in pancreatic tissue were higher in the treatment groups than in the model group ($P < 0.05$). In the 4 treatment groups, with or without GF, the expression levels of $\alpha$-SMA and collagen types I and III were lower, but the levels of IL-10 were higher in the APB group than in the TCF group ($P < 0.05$). In addition, in both culture systems, the expression levels of $\alpha$-SMA and collagen types I and III were lower, but the levels of IL-10 were higher in the GF(+) groups than in the GF(−) groups ($P < 0.05$).

DISCUSSION

For both in vivo and in vitro tissue regeneration, it is essential for the donor cell to have the following characteristics: (1) the ability to differentiate into pancreatic cell types, (2) high proliferative potential (ability to expand to high numbers before seeding onto the APB), (3) easy accessibility (an autologous cell source, either differentiated or stem), and (4) a lack of immunogenicity. These characteristics point to the possibility of using BMSCs as a source for reseeding on the APB or as in vivo cell therapy.

The growth of cells is regulated by their microenvironment, including the attachment among cells and the presence of signaling molecules. It is important for cells to proliferate and...
differentiate in a culture system mimicking the microenvironment of their innate tissue, which cannot be accomplished by traditional 2-dimensional culture systems. The ACM, with a preserved complex multifaceted ECM composition, 3D spatial orientation, and microstructure, has a tissue-specific biological nature, as well as biocompatible properties, that can promote cell adhesion, viability, proliferation, and differentiation. Also, the ACM has physiological levels of GFs, such as insulin-like GF, bone morphogenetic protein 4, and cytokines, for regeneration and implantation. This may explain why APBs have the ability to promote BMSC differentiation.

Furthermore, a dynamic culture with an appropriate flow rate is better for BMSC proliferation and differentiation than a static culture in the APB. Dynamic culture benefits the delivery of O2 and nutrients, and metabolic waste can be easily removed in flow culture systems. In addition, the flow speed in the dynamic culture system can generate a liquid shearing force, which can appropriately modulate BMSC proliferation and differentiation. Lowering or enhancing the flow rate affects the proportion of apoptotic BMSCs. At a flow rate of 4 mL/min, BMSCs can survive and proliferate with high viability in the dynamic culture system. Therefore, we used a flow rate of 4 mL/min in our study.

In our study, BMSCs could be reseeded to repopulate the surface and the center of APB, as indicated by morphological assessment. With respect to the ultrastructural characteristics of BMSCs during differentiation, the shape of cells changed to spindle or branched, and cells exhibited denser microvilli. Both endocrine and exocrine cells increased in number and formed epithelial cell-like clusters, with significant amounts of accumulated ECM fibers. In addition, our study clearly showed glycogen positivity, nuclei, secretory granules, and mature organelles such as mitochondria and smooth and rough endoplasmic reticulum. This indicates that BMSCs differentiate into pancreatic-like cells. A previous study indicated that cell aggregation is a necessary condition for BMSC differentiation. This finding agrees with that of our current research.

In addition, we quantified the morphological changes by measuring the aspect ratio of cells, which is an indicator for the elongated cell shapes, as well as the morphology of cells in different shapes. Our research showed that aspect ratio and cell shape changed as a time course effect during differentiation. Although differentiation might cause changes in the cell shape, cell morphology could also alter the differentiation of mesenchymal lineage. The branched shape might increase the contractility of the cytoskeleton and lead to preferential differentiation, whereas the...
round shape might induce low contractibility, resulting in low differentiation ability of MSCs.\(^3\)\(^8\)\(^–\)\(^4\)\(^0\) Our results indicate that BMSC differentiation can be enhanced in the presence of the APB and that GF promotes BMSC differentiation because of the morphological changes of cells.

To identify the state of cell adhesion and spreading in 4 culture systems, the focal point density was also determined. Cell adhesion and spreading by means of integrin to the ECM proteins is a potent promoter of cell growth, differentiation, and gene expression.\(^4\)\(^1\) Our study findings indicate that the APB system seems more suitable for cell adhesion and spreading, and GF can promote BMSC adhesion and spreading.

For the purpose of BMSC differentiation, we added GF in both culture systems. During the differentiation process, BMSCs proliferated in all 4 groups. The cell proliferation rate was higher in the APB (dynamic) system than in the TCF (static) culture system. The proliferation rate was higher in the APB-GF(+) system than in the APB-GF(−) system. These results indicate that the APB efficiently facilitates pancreatic-like cell growth and GF promotes BMSC proliferation during differentiation.

To assess pancreatic differentiation, we evaluated the gene expression of PDX-1 and PTF-1, which are known to be important to pancreatic function. Pancreatic duodenal homeodomain containing transcription factor plays a vital role in pancreatic development and differentiation,\(^4\)\(^2\) whereas PTF-1, as a transcriptional regulator of exocrine-specific genes and an exocrine transcription factor, is responsible for pancreatic exocrine function and exocrine gene expression.\(^4\)\(^3\) In our research, without GF, PDX-1, and PTF-1 expression was higher on the APB system than on the TCF system, with a significant difference from day 3 with GF. The gene expression of PDX-1, PTF-1, and Oct4 showed that APB promoted BMSC differentiation into pancreatic-like cells, reducing their pluripotency capacities, especially with the help of GF.

To identify the pancreatic-like cells, we assessed the expression of proteins such as α-Amy, CK7, C-peptide, and Flk-1. The α-Amy is a well-established marker of pancreatic acinar cells. Both CK7 and Flk-1 are localized normally in the pancreatic duct structures in the adult pancreas and contribute to the formation of the large intralobular, interlobular, and main ducts.\(^4\)\(^5\) Coupled with CK7, Flk-1 functions as the receptor of vascular endothelial growth factor, which is associated with the endothelial layer of the vasculature.\(^4\)\(^6\),\(^4\)\(^7\) These 2 markers are known to be expressed during pancreatic morphogenesis in the fetus.\(^4\)\(^8\) C-peptide is expressed in pancreatic endocrine cells, which indicates β-cell function.\(^4\)\(^9\)

Our study showed that the expression of α-Amy, CK7, and Flk-1 was significantly higher in BMSC cells cultured on the

### Table 1. The Pancreatic Fibrosis and Pathological Score

| Groups                | n | Pathological Score | Pancreatic Fibrosis Score |
|-----------------------|---|--------------------|--------------------------|
| Control group         | 10| 0                  | 0                        |
| Model group           | 10| 9.89 (3.31)        | 2.51 (0.41)              |
| Treatment group       |   |                    |                          |
| TCF-GF(−)             | 10| 7.79 (2.57)        | 0.96 (0.18)              |
| TCF-GF(+)             | 10| 5.84 (2.11)        | 0.78 (0.17)              |
| APB-GF(−)             | 10| 4.91 (1.33)        | 0.71 (0.24)              |
| APB-GF(+)             | 10| 2.11 (0.86)        | 0.37 (0.09)              |
| F                     |   | 3.182              | 4.055                    |
| P                     |   | 0.038              | 0.024                    |

Data are expressed as mean (standard deviation).
TABLE 2. The Expression of α-SMA, Collagen Type I and III, IL-10 in Pancreatic Tissue (mg/ml)

|                | n  | α-SMA         | Collagen Type I | Collagen Type III | IL-10   |
|----------------|----|---------------|-----------------|-------------------|---------|
| Control group  | 10 | 70.22 (24.41) | 42.59 (19.56)   | 230.79 (85.55)    | 2808.11 (927.58) |
| Model group    | 10 | 168.56 (71.25)| 94.37 (37.51)   | 789.68 (293.47)   | 3137.68 (986.89)  |
| Treatment group|    |               |                 |                   |         |
| TCF-GF(−)      | 10 | 79.65 (21.28) | 45.13 (15.21)   | 263.14 (81.52)    | 3573.57 (721.13)  |
| TCF-GF(+)      | 10 | 56.45 (18.21) | 29.21 (10.12)   | 136.14 (45.23)    | 4864.27 (2071.41) |
| APB-GF(−)      | 10 | 46.52 (14.13) | 24.32 (8.17)    | 131.31 (41.34)    | 4618.98 (1861.12) |
| APB-GF(+)      | 10 | 22.15 (7.28)  | 10.15 (3.25)    | 73.14 (18.25)     | 7614.08 (2071.41) |

F  4.003  5.184  3.523  5.216
P  0.033  0.013  0.035  0.009

Data are expressed as mean (standard deviation).

APB than in the TCF group after day 14 without GF, but it was higher from day 5 with GF. Also, C-peptide levels were higher in cells cultured in the APB system than in TCF cells after day 5 without GF and from day 3 with GF. The pancreatic functional protein results indicate that BMSCs differentiate into 2 major pancreatic lineage cell types: (1) endocrine cells, which are arranged mainly in groups as islets of Langerhans and secrete different polypeptides delivered to other parts of the body via the vasculature; and (2) acinar exocrine cells, whose secretions are carried away through the ductal system. Thus, GF strengthens the stimulatory effects of APB on BMSCs.

Research has indicated that the mechanical characteristics of the ACM, such as the stiffness and biomechanical strength, are related to BMSC differentiation through TGF-β. Also, the changing ECM density inhibits capillary morphogenesis and neovascularization in vivo in a manner consistent with that observed in vitro. This may be another mechanism mediating the inductive effects of APB on BMSCs.

We also assessed the metabolic function of differentiated BMSCs. To demonstrate the endocrine function, we compared the insulin secretion levels at different glucose concentrations. The insulin levels were enhanced when glucose concentrations increased. Insulin secretion levels were significantly higher in the APB groups than in TCF groups at glucose concentrations of 15 and 25 mM. The GF could significantly increase insulin secretion. Such a trend was not significant at low glucose concentrations.

Similarly, α-Amy levels were enhanced when concentrations of CCK increased. At CCK concentrations of 10⁻³ and 10⁻⁸ mM, α-Amy levels were significantly higher in the APB system than in the TCF system. α-Amy secretion significantly increased in the presence of GF. Low levels of CCK did not follow such a trend. Previous studies indicated that CCK at less than 10⁻⁸ mC could stimulate Amy secretion in a concentration-dependent manner, whereas CCK at higher concentrations might inhibit Amy secretion. We used less than 10⁻⁸ mC CCK in our study.

The assay of metabolic function indicated that the APB with GF promoted the pancreatic organogenesis of BMSCs and supported BMSC differentiation into primary functional units, which maintained their respective phenotypic expression (endocrine ß-cells, insulin; exocrine acinar cells, Amy) in proximity to their respective native niches.

Because whole-organ ACM physiologically resembles the original tissue, including intact 3D architecture, preserved native ECM components, vascular networks, and biomechanical properties, it can guide tissue regrowth and encourage cell differentiation when combined with biological agents. Our study findings are in agreement with that of this previous study.

Another previous study indicated that BMSCs are less immunogenic, as they do not express MHC class II markers or elicit a strong immune response, as evidenced by a lack of activation of T cells. In addition, BMSCs are easily acquired and easily accessible. Based on these merits of BMSCs, our findings can support in vivo cell therapy efforts as well as in vitro treatments in whole-pancreas regeneration.

A pathological hallmark of CP is progressive fibrosis, which is mediated by pancreatic stellate cells (PSCs). One of the earliest cellular events at the initiation of fibrosis is activation of PSCs, which can be mediated concomitantly by a variety of factors, such as oxidative stress, cytokines, and GFs. The process of PSC activation involves significant expression of α-SMA, which can promote the secretion of collagen types I and III. Interleukin-10 has been indicated to be a protective cytokine and has been shown to decrease the severity of CP and to reduce the likelihood of systemic complications. Also, it can downregulate collagen synthesis in both unstimulated and activated PSCs.

Our study showed that the expression of α-SMA and collagen types I and III could be downregulated, whereas the expression of IL-10 could be upregulated in differentiated BMSCs in vivo. This indicates that differentiated BMSCs inhibit the process of fibrosis after transplantation in vivo through the causal vein and exert protective effects on pancreatic tissue.

Note that this study had several unavoidable limitations. First, after BMSC differentiation, the cells did not have the intact phenotypic properties and functions of native pancreatic cells. We plan to improve our differentiation strategy in further research. Second, this study was based on an animal model. In future studies, we will aim to provide insights regarding the feasibility and usefulness of bioscaffolds and cells in humans. Third, our study focused on the APB and BMSCs in vitro. Efforts to improve pancreatic tissue engineering in vivo will be made in future research. Despite these limitations, we believe that our data on the effects of APB on BMSCs are valuable and reliable.

CONCLUSIONS

Acellular pancreatic bioscaffold supported the proliferation and viability of BMSCs in a dynamic culture system with an optimal flow rate of 4 mL/min. During the differentiation process, APB could (1) induce BMSC differentiation into pancreatic-like cells which express gene markers and pancreatic functional proteins and (2) promote secretion of metabolic enzymes. Growth factor could significantly improve proliferation, differentiation, and cell engraftment in APB. Our study sheds light on the
possibilities of developing pancreatic cell therapies and improving pancreatic tissue engineering.

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REFERENCES

1. Ibadov RA, Arifjanov AS, Ibragimov SK, et al. Acute respiratory distress-syndrome in the general complications of severe acute pancreatitis. Ann Hepatobiliary Pancreat Surg. 2019;23:359–364.

2. Zhou X, Wang W, Wang C, et al. DPP4 inhibitor attenuates severe acute pancreatitis-associated intestinal inflammation via Nrf2 signaling. Oxidative Med Cell Longev. 2019;2019:6181754.

3. Wang X, Yan Y, Tan Q, et al. MEX3A knockdown inhibits the development of pancreatic ductal adenocarcinoma. Cancer Cell Int. 2020;20:63.

4. Luo W, Yang G, Luo W, et al. Novel therapeutic strategies and perspectives for metastatic pancreatic cancer: vaccine therapy is more than just a theory. Cancer Cell Int. 2020;20:66.

5. Rijkse E, Ijzermans JN, Minnee RC. Machine perfusion in abdominal organ transplantation: current use in the Netherlands. World J Transplant. 2020;10:15–28.

6. Porzionato A, Stocco E, Barbon S, et al. Tissue engineered grafts from human decellularized extracellular matrices: a systematic review and future perspectives. Int J Mol Sci. 2018;19:4117.

7. Mendez JJ, Ghaedi M, Steinbacher D, et al. Epithelial cell differentiation of human mesenchymal stromal cells in decellularized lung scaffolds. Tissue Eng Part A. 2014;20:1735–1746.

8. Kim IG, Ko J, Lee HR, et al. Mesenchymal cells condensation-inducible mesh scaffolds for cartilage tissue engineering. Biomaterials. 2016;85:18–29.

9. Junyert S, Garcin CL, Szczekowski JLA, et al. Specialized cytoines induce self-organization of stem cells. Proc Natl Acad Sci U S A. 2020;117:7236–7244.

10. Lanzoni G, Oikawa T, Wang Y, et al. Concise review: clinical programs of pancreatic tissue engineering. Pancreas. 2020;51:107:4872–1746.

11. Mizuno H, Tobita M, Uysal AC. Concise review: adipose-derived stem cells as a novel tool for future regenerative medicine. Stem Cells. 2012;30:804–810.

12. Lin HT, Otsu M, Nakauchi H. Stem cell therapy: an exercise in patience and prudence. Philos Trans R Soc Lond Ser B Biol Sci. 2013;368:2010334.

13. Ra JC, Shin IS, Kim SH, et al. Safety of intravenous infusion of human adipose tissue-derived mesenchymal stem cells in animals and humans. Stem Cells Dev. 2011;20:1297–1308.

14. Rastegar F, Shenaj D, Huang J, et al. Mesenchymal stem cells: molecular characteristics and clinical applications. World J Stem Cells. 2010;267–80.

15. Wang Y, Chen X, Cao W, et al. Plasticity of mesenchymal stem cells in immunomodulation: pathological and therapeutic implications. Nat Immunol. 2014;15:1009–1016.

16. Zhou L, Liu W, Wu Y, et al. Oral mesenchymal stem/progenitor cells: the immunomodulatory masters. Stem Cells Int. 2020;2020:1327405.

17. Fawzy El-Sayed KM, Elahmady M, Adawi Z, et al. The periodontal stem progenitor cell inflammatory-regenerative cross talk: a new perspective. J Periodontal Res. 2019;54:81–94.

18. Brown C, McKee C, Bakshi S, et al. Mesenchymal stem cells: cell therapy and regeneration potential. J Tissue Eng Regen Med. 2019;13:1738–1755.

19. Ma Z, Song G, Liu D, et al. N-acetylcysteine enhances the therapeutic efficacy of bone marrow-derived mesenchymal stem cell transplantation in rats with severe acute pancreatitis. Pancreatology. 2019;19:258–265.

20. Leung PS, Ng KY. Current progress in stem cell research and its potential for islet cell transplantation. Curr Mol Med. 2013;13:109–125.

21. Li M, Zhang T, Jiang J, et al. ECM coating modification generated by optimized decellularization process improves functional behavior of BMSCs. Mater Sci Eng C Mater Biol Appl. 2019;105:110039.

22. Yin H, Wang Y, Sun Z, et al. Induction of mesenchymal stem cell chondrogenic differentiation and functional cartilage microtissue formation for in vivo cartilage regeneration by cartilage extracellular matrix-derived particles. Acta Biomater. 2016;33:96–109.

23. Salvatori M, Katari R, Patel T, et al. Extracellular matrix scaffold technology for bioartificial pancreas engineering: state of the art and future challenges. J Diabetes Sci Technol. 2014;8:159–169.

24. Tsuchiya T, Balestrini J, Mendez J, et al. Influence of pH on extracellular matrix preservation during lung decellularization. Tissue Eng Part C Methods. 2014;20:1028–1036.

25. Orlando G, Farney AC, Iskandar SS, et al. Production and implantation of renal extracellular matrix scaffolds from porcine kidneys as a platform for renal bioengineering investigations. Ann Surg. 2012;256:363–370.

26. Zhao W, Li JJ, Cao DY, et al. Intravenous injection of mesenchymal stem cells is effective in treating liver fibrosis. World J Gastroenterol. 2012;18:1048–1058.

27. Kakabadze A, Mardaleishvili K, Loladze G, et al. Reconstruction of mandibular defects with autogenous bone and decellularized bovine bone grafts with freeze-dried bone marrow stem cell paracrine factors. Oncol Lett. 2017;13:1811–1818.

28. Santhakumar R, Vidyasekar P, Verma RS. Cardiogel: a nano-matrix scaffold with potential application in cardiac regeneration using mesenchymal stem cells. PLoS One. 2014;9:e114697.

29. Wang X, Li Y, Du Y, et al. The research of acellular pancreatic bioscaffold as a natural 3-dimensional platform in vitro. Pancreas. 2018;47:1040–1049.

30. Goh S, Bertera S, Olsen P, et al. Perfusion-decellularized pancreas as a natural 3D scaffold for pancreatic tissue and whole organ engineering. Biomaterials. 2013;34:6760–6772.

31. Gaetani R, Aouad S, Demaddalena LL, et al. Evaluation of different decellularization protocols on the generation of pancreas-derived hydrogels. Tissue Eng Part C Methods. 2018;24:697–708.

32. Ungerleider JL, Johnson TD, Hernandez MJ, et al. Extracellular matrix hydrogel promotes tissue remodeling, arteriogenesis, and perfusion in a rat hindlimb ischemia model. JACC Basic Trans Sci. 2016;1:32–44.

33. Crapo PM, Gilbert TW, Badyalik SF. An overview of tissue and whole organ decellularization processes. Biomaterials. 2011;32:3233–3243.

34. Kniazeva E, Kachgal S, Putnam A. Effects of extracellular matrix density and mesenchymal stem cells on neovascularization in vivo. Tissue Eng Part A. 2011;17:905–914.

35. Hwang NS, Vorghese S, Elisseef J. Controlled differentiation of stem cells. Adv Drug Deliv Rev. 2008;60:199–214.

36. Cartmell SH, Porter BD, Garcia AJ, et al. Effects of medium perfusion rate on cell-seeded three-dimensional bone constructs in vitro. J Tissue Eng Regen Med. 2014;8:1016.

37. Kilian KA, Buganja B, Lahn BT, et al. Geometric cues for directing the differentiation of mesenchymal stem cells. Proc Natl Acad Sci U S A. 2010;107:4872–4877.

38. Lavenos S, Racquier JC, Louna G, et al. Cell interaction with nanopatterned surface of implants. Nanomedicine (London). 2010;5:937–947.
40. Lavenus S, Berreur M, Trichet V, et al. Adhesion and osteogenic differentiation of human mesenchymal stem cells on titanium nanopores. *Eur Cell Mater*. 2011;22:84–96; discussion 96.

41. Manohar M, Verma AK, Venkateshaiah SU, et al. Pathogenic mechanisms of pancreatitis. *World J Gastrointest Pharmacol Ther*. 2017;8:10–25.

42. Park CH, Lee JY, Kim MY, et al. Oligonol, a low-molecular-weight polyphenol derived from lychee fruit, protects the pancreas from apoptosis and proliferation via oxidative stress in streptozotocin-induced diabetic rats. *Food Funct*. 2016;7:3056–3063.

43. Barlass U, Dutta R, Cheema H, et al. Morphine worsens the severity and prevents pancreatic regeneration in mouse models of acute pancreatitis. *Gut*. 2018;67:600–602.

44. Fafián-Labora J, Morente-López M, Sánchez-Dopico M, et al. Influence of mesenchymal stem cell-derived extracellular vesicles in vitro and their role in ageing. *Stem Cell Res Ther*. 2020;11:13.

45. Rooman I, Heremans Y, Heimberg H, et al. Modulation of rat pancreatic acinoductal transdifferentiation and expression of PDX-1 in vitro. *Diabetologia*. 2000;43:907–914.

46. Di Rocco G, Tritarelli A, Toietta G, et al. Spontaneous myogenic differentiation of Flk-1-positive cells from adult pancreas and other nonmuscle tissues. *Am J Phys Cell Phys*. 2008;294:C604–C612.

47. Dygai AM, Skurikhin EG, Pershina OV, et al. Role of hematopoietic stem cells in inflammation of the pancreas during diabetes mellitus. *Bull Exp Biol Med*. 2016;160:474–479.

48. Xue A, Julovi SM, Hugh TJ, et al. A patient-derived subrenal capsule xenograft model can predict response to adjuvant therapy for cancers in the head of the pancreas. *Pancreatology*. 2015;15:397–404.

49. Boughton C, Allen JM, Tauschmann M, et al. Assessing the effect of closed-loop insulin delivery from onset of type 1 diabetes in youth on residual beta-cell function compared to standard insulin therapy (CLOuD study): a randomised parallel study protocol. *BMJ Open*. 2020;10:e033500.

50. Park JS, Chu JS, Tsou AD, et al. The effect of matrix stiffness on the differentiation of mesenchymal stem cells in response to TGF-β. *Biomaterials*. 2011;32:3921–3930.

51. Subramanian K, Owens DJ, OBrien TD, et al. Enhanced differentiation of adult bone marrow-derived stem cells to liver lineage in aggregate culture. *Tissue Eng Part A*. 2011;17:2331–2341.

52. Gukovskaya AS, Gukovsky I, Jung Y, et al. Cholecystokinin induces caspase activation and mitochondrial dysfunction in pancreatic acinar cells. Roles in cell injury processes of pancreatitis. *J Biol Chem*. 2002;277:22595–22604.

53. Gong T, Heng BC, Lo ECM, et al. Current advance and future prospects of tissue engineering approach to dentin/pulp regenerative therapy. *Stem Cells Int*. 2016;2016:9204574.

54. Rasmussen I, Ringdén O, Sundberg B, et al. Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. *Transplantation*. 2003;76:1208–1213.

55. Bynigeri RR, Jakkampudi A, Jangala R, et al. Pancreatic stellate cell: Pandora’s box for pancreatic disease biology. *World J Gastroenterol*. 2017;23:382–405.

56. Komar HM, Serpa G, Kerscher C, et al. Inhibition of Jak/STAT signaling reduces the activation of pancreatic stellate cells in vitro and limits caerulein-induced chronic pancreatitis in vivo. *Sci Rep*. 2017;7:1787.

57. Yu FX, Su LF, Dai CL, et al. Inhibition of pancreatic stellate cell activity by adipose-derived stem cells. *Hepatobiliary Pancreat Dis Int*. 2015;14:215–221.

58. Li Y, Li Z, Du Y, et al. Bone mesenchymal stem cells transplantation in the treatment of chronic pancreatitis of rats model. *Chin J Hepatobiliary Surg*. 2020;6:455–458.