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Controlled Adipose-derived Stromal Cells Differentiation into Adipose and Endothelial Cells in a 3D Structure Established by Cell-assembly Technique

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ABSTRACT: One of the major obstacles in engineering thick and complex tissues while vascularizing tissues *in vitro* is to maintain cell viability during tissue growth and structural organization. Adipose-derived stromal (ADS) cells were used to establish a multicellular system through a cell-assembly technique. Attempts were made to control ADS cells differentiation into different targeted cell types according to their positions within an orderly 3D structure. Oil red O staining confirmed that the ADS cells in the structure differentiated into adipocytes with a spherical shape while immunostaining tests confirmed that the endothelial growth factor induced ADS cells on the walls of channels differentiated into mature endothelial cells and then organized into tubular structures throughout the engineered 3D structure. The endothelin-1 and nitric oxide release rules of the endothelial cells were coincidental with those *in vivo*.

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This study provides a new approach to engineer orderly endothelial vessel networks *in vitro* and has potential applications in adipose-tissue engineering.

KEY WORDS: adipose-derived stromal (ADS) cells, cell-assembly technique, vascularize, endothelial vessel network, adipose-tissue engineering, tissue engineering.

INTRODUCTION

Cell-assembling technology is a remarkable new invention developed for tissue engineering. The three-dimensional (3D) bioassembly tool is capable of extruding cells and matrix into spatially organized 3D constructs [1–3]. Cell printing can place cells as a stream of drops in 3D positions that mimic their respective positions in a living organ [4,5]. We have recently developed a 3D cell-assembly technique (Figure 1(a)) based on rapid prototyping (RP) [6–11]. Using this technology, different cells and extracellular matrix (ECM) can be put into designated locations to form 3D constructs. The designed architecture facilitates cell growth, organization and differentiation. We have used hepatocytes with hydrogel to build a 3D structure, in which the hepatocytes retain their viability and functions for more than 60 days [7–9].

Engineered adipose tissue can be used in plastic and reconstructive surgery to augment soft tissue lost due to mastectomy or lumpectomy [12,13]. Adipose-tissue engineering with collagen scaffolds combined with human pre-adipocytes was recently reported [14,15]. Patrick et al. have succeeded in forming adipose tissue in the rat subcutis using a porous poly(lactic-co-glycolic acid) scaffold pre-seeded with autologously isolated pre-adipocytes [16,17]. One of the major obstacles in engineering thick and complex adipose tissue is vascularizing the tissue to maintain cell viability during tissue growth and inducing structural organization. Koike et al. co-seeded endothelial cells and perivascular cell precursors in an engineered construct that provided long-lasting and stable microvessels *in vivo* [18] while 3D vascularized skeletal muscle tissue by co-culturing myoblasts, endothelial cells and embryonic fibroblasts was engineered by Levenberg et al. [19]. However, the best solution is to vascularize the engineered tissue constructs before transplantation [20,21].

Herein, we report the production of engineered adipose tissue and vascularization of the constructs before transplantation.

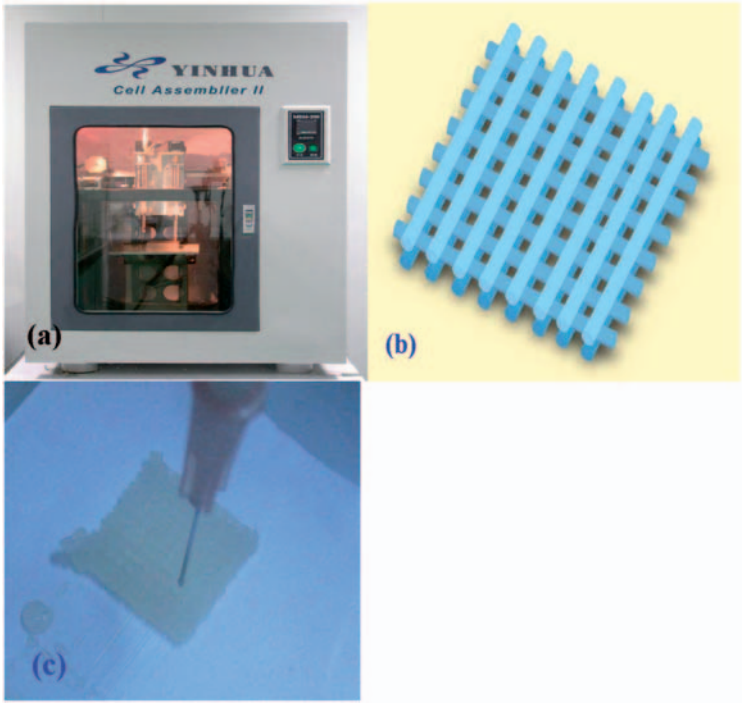

Figure 1. (a) The cell-assembly machine, with two nozzles controlled by computer respectively. (b) A 3D structure, which consisted of square grids and orderly channels, was designed by a software package. (c) Following the designed model, computer controlled a nozzle to deposit the mixture and generated a 3D structure on a glass chip.

Gelatin, alginate and fibrinogen as the ECM were used for assembling the cells and adipose-derived stromal (ADS) cells that were induced to differentiate into adipocytes and endothelial cells as reported [22]. We attempted to control the differentiation of the ADS cells into specific target cell types according to their positions within an orderly 3D structure. Oil red O staining showed that ADS cells in the structure differentiated into adipocytes with a spherical shape. Immunostaining test was used to determine that the endothelial growth factor (EGF) induced ADS cells on the walls of the channels differentiated into mature endothelial cells and then organized into tubular structures throughout the engineered 3D structure. The engineered adipose tissues secreted adipokines, typical biomarks of adipose tissue, such as leptin. The endothelin-1 (ET-1) and nitric oxide (NO) release of the endothelial cells were coincident with that of *in vivo*. Finally, the successful *in vitro* production of human adipose substitutes with orderly endothelial vessel

networks was accomplished; this reinforced the concept that customized autologous reconstructed adipose tissues could be produced to repair a wide range of soft-tissue defects.

METHODS

Cell Culture

ADS cells were isolated from rat subcutaneous adipose tissues [23]. The epididymal adipose tissues from Sprague-Dawley rats (100∼150 g, Beijing university medical Center of Laboratory Animals) were excised, washed sequentially in serial dilutions of betadine and finely minced in phosphate-buffered saline (PBS). Tissues were digested with 0.075% Type II collagenase (sigma) at 37°C for 30 min. Neutralized cells were centrifuged to separate mature adipocytes and the stromal-vascular fraction. Floating adipocytes were removed and pelleted stromal cells were filtered through a 100 μm cell strainer before plating. ADS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in an atmosphere of 5% CO_2. Cells were grown to subconfluence and passaged by standard trypsinization methods.

Construct of the 3D Multicellular System

The ADS cells were trypsinized off the culture dishes upon subconfluence, washed and quantified. Then the cells were mixed with gelatin (Tianjin green-island Company), alginate (SIGMA) and fibrinogen (SIGMA) gel (gelatin: alginate: fibrinogen, 2 : 1 : 1) at a density of 3×10^7 cells/mL. After mixing, 1 mL of the mixture was loaded into a sterilized syringe (1 mL, 0.45 × 16 RW LB). A software package (Microsoft, AT6400) was used to design the complex 3D structure, which consisted of square grids and orderly channels about 400 μm in diameter (Figure 1(b)). Following the designed structure, computer controlled a refit nozzle to deposit the mixture on a glass chip at 10°C (gelatin at gel state). The program was run 8 times consecutively at the same position to generation of a $10 \times 10 \times 2\,mm^3$ 3D configuration on the square pattern. When the process was complete, the 3D structure was treated with 10% $CaCl_2$ solution for 1 min (which crosslinked the alginate) then washed with DMEM three times. The 3D structure was then treated with 50 μU/mL of thrombin (which crosslinked the fibrinogen) in a DMEM culture medium containing 10% FBS, 1 μmol/L insulin, 10 ng/mL EGF and 50 U/mL aprotinin (Sigma), placed in a CO_2

incubator at 37°C. After 3 days of culture, the medium was changed to 10% FBS, 1 μM insulin, 1 μM dexamethasone (DXM; Sigma), 0.5 mmol/L isobutyl-methylxanthine (IBMX; Sigma), and 50 u/mL aprotinin for 3 d. Then the medium was changed to DMEM containing 10% FBS. As control experiments, the 3D structure was cultured in a medium without EGF for the first three days or without DXM and IBMX for the second three days.

Structural Analyses Using Scanning Electron Microscopy

At the 6th day of culture, the multicellular 3D structures were washed with PBS (pH 7.4) and fixed with 3% glutaraldehyde for 2 h; the samples were then post-fixed with 0.5% OsO_4 and rinsed with PBS. The samples were dried under a vacuum freeze dryer for 12 h. The dehydrated samples were sputter coated with gold–palladium. All micrographs were obtained in a scanning electron microscope (Hitachi S450, JAP).

Immunostaining

For immunofluorescence analyses, the multicellular 3D structure was fixed with 4% glutaraldehyde for 20 min at 20°C and then washed 3 times with PBS. The structure was incubated with 50 μg/mL propidium iodide (PI, sigma USA) for 20 min (nuclear staining) and then with primary antibodies (rabbit anti-rat : CD31, 1 : 20 in PBS from Santa Cruz, USA) for 30 min and with a secondary antibody (FITC-conjugated anti rabbit IgG, 1 : 20 in PBS, Santa Cruz,) for 30 min. Finally, samples were washed with PBS and observed with a fluorescence microscope (OLYMPUS BX51, JAP) or confocal microscopy (Leica TCS SP2, Germany). Image acquisition and analysis were performed using the Applied DP-Controller system (OLYMPUS, JAP) and Image-pro Plus 5.0 (Media Cybernetics, USA).

Assessment of Adipogenesis

On day 6, the multicellular 3-D structures were cultured in normal glucose (5 mM) or high glucose (15 mM) medium. After a total of 14 d, oil red O staining was used to indicate the ADS cells differentiation into adipocytes. The adipocyte cells were stained with Oil red O as described [24]. The 3-D structure cultured in the plate was washed with PBS and fixed with 10% formalin for 1 h. After staining with 0.1 mg/mL oil red O solution for 2 h, the cells were washed 3 times with water and observed with a microscope. The oil red O dye was extracted into isopropanol and

the absorbance measured at 510 nm using a microplate reader (Bio-Rad 550, Hercules, CA, USA). The cells of the control group were processed in the same way.

Measurement of Leptin by ELISA

After 6 days of culture, the multicellular 3D structure was cultured in a normal glucose (5 mM) or high glucose (15 mM) medium. The three day-conditioned media were harvested on the day 21. The leptin in cell culture media was measured using rat leptin ELISA kits (RB, USA). The assays were conducted in 96-well microplates according to the manufacturer's recommendation, with a microplate reader. The cells of the control group were dealt with the same process.

Measurement of ET-1 and NO Release

The multicellular 3D structure was cultured with 5 mM glucose, on the 13th day, the medium with DMEM containing 25 mM glucose was replaced. To study the ET-1 secretion of the cells, the culture medium was collected at 3, 6, 9, 12, and 24 h. ET-l released into the culture media was measured using a ELISA Kit (RB, USA) according to the manufacturer's instructions. To study the NO secretion of the cells, the media were harvested at the 15th day. The NO concentrations in the culture media were detected using a NO Detection kit (JinMei Biotech Co. Ltd.) based on a nitrate reductase method. Cells from the control groups were dealt with the same process.

Statistical Analysis

Statistical analysis was performed with Student's *t*-test using both confidence interval estimate analysis and t-score probability hypothesis testing method for two independent sample groups. However, there is considerable probability of statistical error due to the limited number of samples in this study.

RESULTS

In this study, rat ADS cells, that differentiate into adipocytes and endothelial cells, were implanted in a gelatin, alginate and fibrinogen ECM (Figure 1(c)). Gelatin has a special property as it can be gelled at low temperatures and the alginate can be crosslinked with $CaCl_2$ and the fibrinogen with thrombin. Once the mixture was crosslinked,

the whole construct could be handled easily without losing its integrity. The gelatin/alginate/fibrinogen hydrogel was much more stable than gelatin/alginate or gelatin/fibrinogen hydrogels and remained for at least 8 weeks (Figure 2(a)). Due to the loss of Ca^{2+} ions from the crosslinked alginate molecules, hydrogel consisting of gelatin and alginate became very brittle within 1 week after culture (Figure 2(b)). While the hydrogel consisting of gelatin and fibrinogen constricted (or shrunk) and degraded in 2 weeks due to the plasmin secreted by living cells (Figure 2(c)). The complete fabrication process required only 20 min. Scanning electron micrographs have showed extensive ECM and cell networks in the structure after 6 days of culture (Figure 3(b)).

To test whether the ADS cells in the 3D structure were acturally controlled differentiation into endothelial cells and adiposities, we

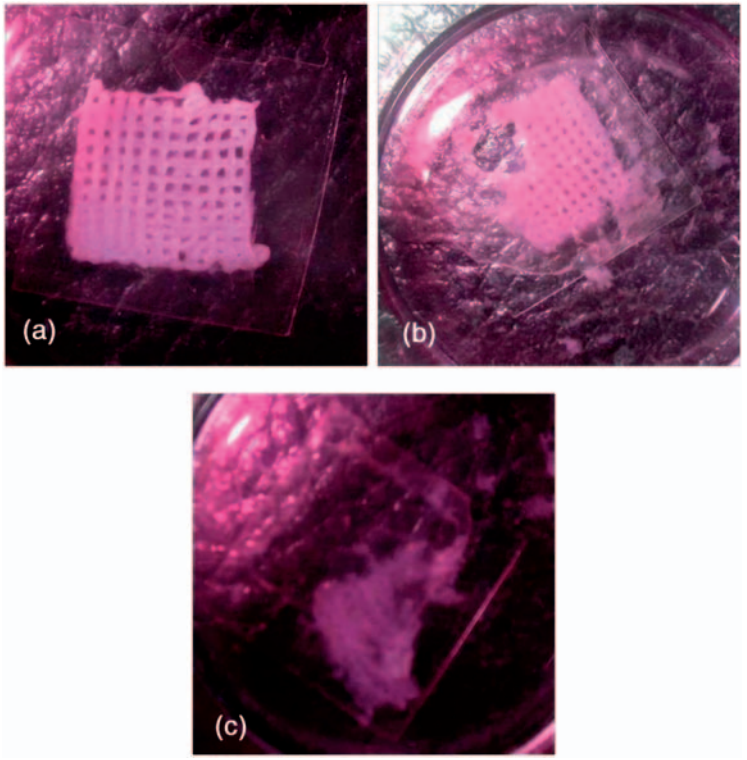

Figure 2. Comparative experiments about the biocompatible materials. ADS cells were mixed with (a) gelatin, alginate, and fibrinogen (crosslinked with $CaCl_2$ and thrombin); (b) gelatin and alginate (crosslinked with $CaCl_2$); (c) gelatin and fibrinogen (polymerized through thrombin).

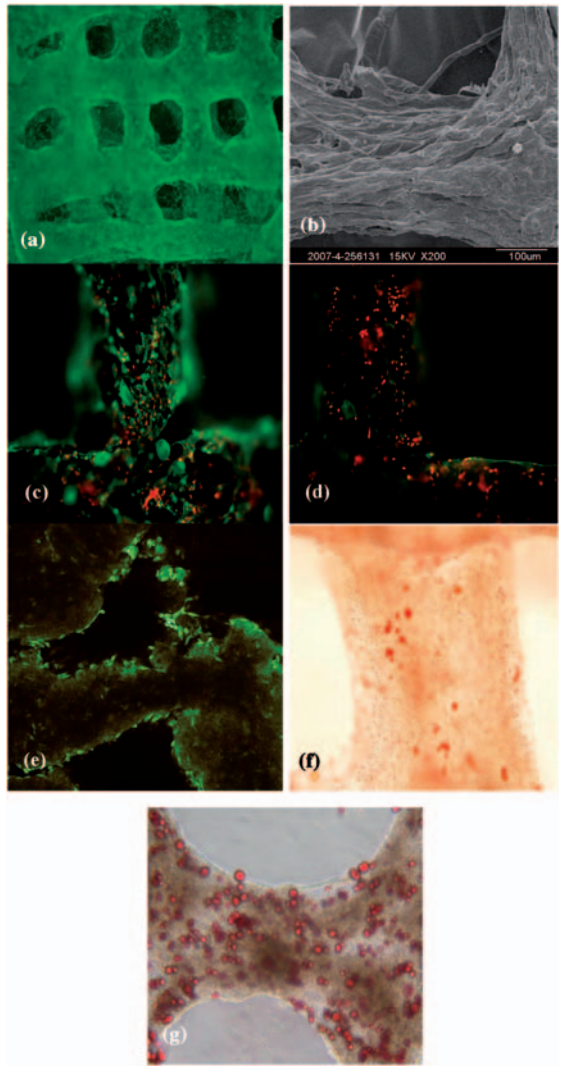

Figure 3. Structural analyses. (a) The 3D structure cultured in a plate. Immunostaining of the 3D structure using mAbs for CD31+ cells in green. (b) Scanning electron micrographs show the extensive ECM and cell networks in the structure after 6 days of culture. ADS cells in the 3D structure differentiated into endothelial cells and adipocytes through control. Immunostaining of the 3D structure using mAbs for CD31+ cells (ADS cells and endothelial cells) in green and PI for nuclear in red. ADS cells in the 3D structure were cultured with EGF (c, e) or without EGF (d). (e) Image of the structure observed with confocal microscopy, Immunostaining of the 3D structure using mAbs for CD31+ cells in green. (f, g) Oil red O staining of the 3D structure for adipocytes in red. ADS cells in 3D structure were cultured with EGF (f) or without EGF (g) for 3 days, and then treated with IBMX and dexamethasone.

examined the composition and distribution of the cells in the 3D structure by immunostaining and oil Red O staining. After 3 days of culture with EGF, CD31 (mature endothelial cells indicator) staining revealed that over 90% of the ADS cells on the walls of channels were differentiated into mature endothelial cells (CD31+) and connected with each other to form vessel-like structures (Figure 3(a), (c), and (e)). This differentiation of ADS cells was based on EGF inducing (Figure 3(c) and (d)) and cell position in the 3D structure (Figure 3(e)). From the 4th day, the structures were treated with insulin, IBMX and dexamethasone for 3 days. On the 12th day, oil red O staining revealed that ADS cells in the structure differentiated into adipocytes with a spherical shape (Figure 3(f) and (g)). The cells away from the walls of the channels were more sensitive to differentiation into adipocytes than those on the surface of walls. This was confirmed by the fact that over 90% of the cells on the walls of channels would also differentiate into adipocytes if not induced with EGF (Figure 3(g)).

The synthesis and storage of fatty acids with nutrients is the main function of adipocytes *in vivo* and also the pathogenesis of obesity. To test whether high glucose promotes adipogenesis of cells in different structures, we cultured cells with normal and high glucose. After total of 12 days of culture, the adipogenesis of cells in the DXM and IBMX-induced 3-D structure was 1.59-folds higher than that in the DXM and IBMX-not-induced structure (Figure 4(a)). After culture with high glucose for 12 days, the adipogenesis of cells in the DXM and IBMX-induced structure was 3.29-folds higher than that not dealt with DXM and IBMX. The adipogenesis rate in the high glucose, DXM and IBMX 3-D structures was higher than that in the non DXM and IBMX structures. These results demonstrate that cells in the structure can be induced to differentiate into adipocytes and mimic adipose functions *in vivo*.

In addition to the storage of lipid, adipocytes provide an endocrine function by secreting adipocytokines to regulate metabolic energy [25]. Leptin can inhibit energy intake, insulin secretion and antagonistic to insulin action. To study the adipocytokine secreted by adipocytes, we analyzed the secretion of leptin from different structures. After being cultured with normal glucose for 6 days, leptin secretion of cells in the DXM and IBMX-induced 3D structure was higher than that in the non DXM and IBMX structure (Figure 4(b)). To determine whether chronic exposure to high glucose can induce pathological changes of adipocytokine secretion of adipocytes, we cultured the different structures with high glucose for 12 days, the increase rates of leptin secretion of cells in the DXM and IBMX-induced structures (3.54-fold of the normal) were

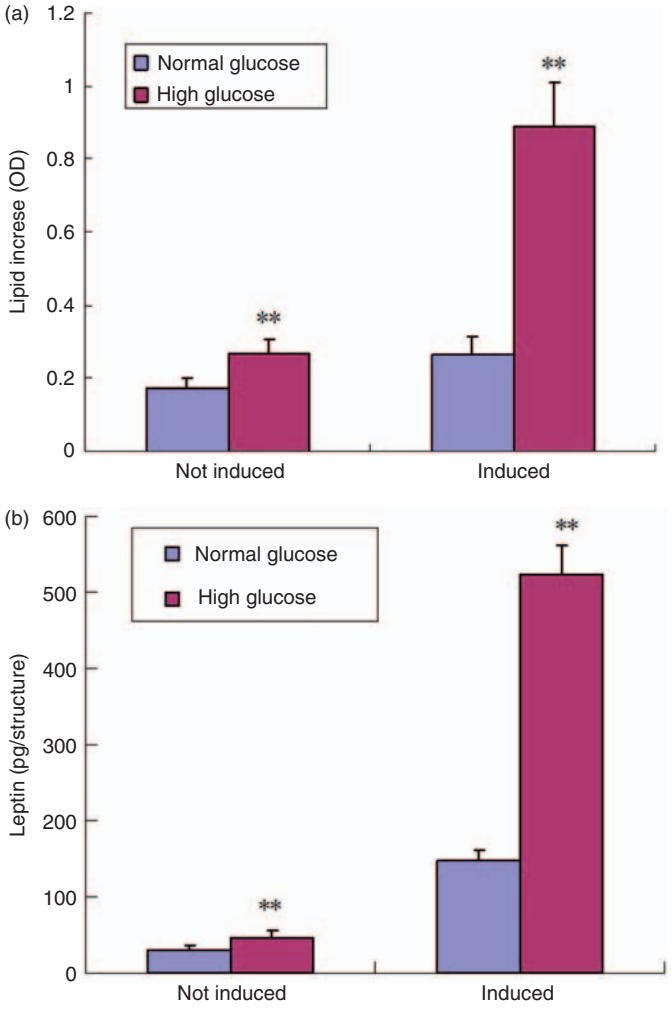

Figure 4. Measurement of adipogenesis of the cells in different structures. Long-term exposure to high glucose (HG 15 mM) was used to induce the pathological changes of the cells in different culture systems. (a) Adipogenesis was stimulated by long-term exposure to HG and the lipid accumulation was measured with oil red O assay. (b) Leptin secretion of the adipocytes in different culture systems, Leptin content in the medium was determined by ELISA kit. Data are mean ± s.d., $n = 6$. $^{**}p<0.01$, versus normal glucose-treated group.

much higher than those in the non DXM and IBMX 3-D structures (1.51-fold of the normal). These results further demonstrate that cells in the structure can be induced to differentiate into adipocyte and simulate adipose endocrine function *in vivo* [26,27].

Endothelin-1 and NO are vasoactive factors secreted by endothelial cells. ET-1 that can induce constriction of the structure, while NO can induce vasodilatation. ET-1 and NO secretion derangements have been identified as the major contributors to endothelial dysfunction associated with many diseases [28]. To study the ET-1 secretion of the cells, we cultured different structures with high glucose for 24 h. Time-dependent spontaneous ET-1 accumulation in the culture media of the structures was observed for 24 h (Figure 5(a)). Our data clearly showed that the base ET-1 secretion of the cells in the EGF-induced structure was higher than that in the non induced structure. The addition of high glucose (25 mM) significantly enhanced the ET-1 accumulation rates of cells in all the structures without any kinetic changes. While the ET-1 accumulation rate in the EGF-induced structure was higher than that in the not induced structure.

To study the NO secretion, we cultured different structures with 1, 5, and 10 μg of insulin, respectively, for 24 h. The treatment of the EGF-induced structures with insulin resulted in a concentration-dependent increase in NO production (Figure 5(b)). The insulin stimulated the production of NO from endothelial cells; the NO provides vasodilation to enhance glucose disposal. There was a statistically significant increase in the NO production at 5 μg (1.48-fold of the 1 μg group, $P<0.05$) and a maximum NO level at 10 μg (1.72-fold of the 1 μg group and 1.18-fold of the 5 μg group, $P<0.01$). Treatment of the non induced structures with insulin also resulted in an increase in NO production, but the NO increased rates were significantly lower than those of the EGF-induced structures. There were no significant differences among the different dosage insulin-stimulate groups.

DISCUSSION

We developed a new approach to induce endothelial vessel networks in engineered adipose tissue. For tissue engineering to surpass the tissue thickness limit of 100–200 μm, it must overcome the challenge of creating functional blood vessels to supply cells with oxygen and nutrients and to remove waste products [29–35]. We used a software package to fabricate the complex structure model with orderly channels; the diameter of the channels was less than 100 μm. We selected ADS cells isolated from rat as the seed cells. ADS cells are multipotent cells found in adipose tissue [36]. Scientists recently have demonstrated that ADS cells can differentiate into not only adipocytes but also endothelial cells, neurons or osteoblasts in certain conditions. The main benefit of

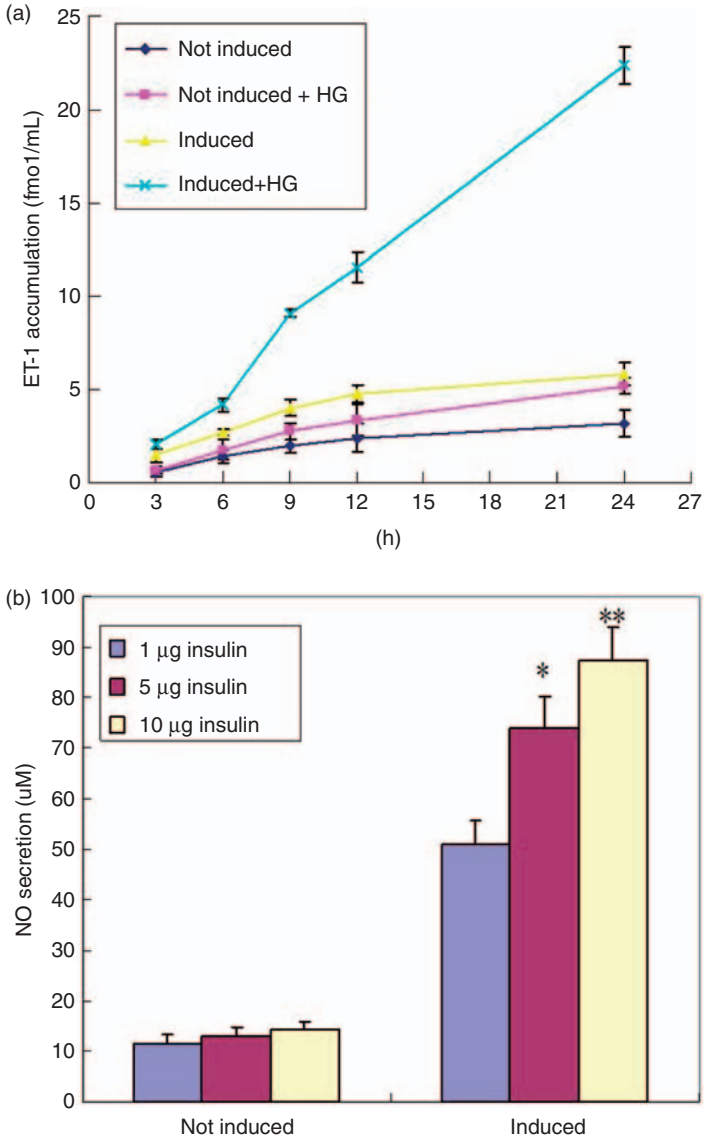

Figure 5. Endothelin-1 and NO secretion of the endothelial cells in the 3D structures. At the 13th day, the structure was cultured with DMEM containing 25 mM glucose. (a) ET-1 secretion kinetics of the endothelial cells were measured for 24 h, ET-1 concentrations in the culture media were measured by ELISA kit. Data are mean ± s.d., $n = 3$. (b) The media were harvested at the 15th day. NO concentrations in the culture media were detected using NO Detection kit. Data are mean ± s.d., $n = 6$. *$p<0.01$, versus 1 μg insulin-treated group; **$p<0.01$, versus 5 μg insulin-treated group.

ADS cells are that they can be easily harvested from patients by a simple, minimally invasive method and easily cultured.

We used gelatin, alginate and fibrinogen as the ECM; gelatin has a special property of gelling at low temperatures while alginate and fibrinogen can be crosslinked or polymerized with $CaCl_2$ or thrombin, respectively. Fibrin has been used as an effective scaffolding material to grow a variety of cells and tissue constructs [37]. Fibrin has numerous advantages as a scaffold material as it is normally used by the body as temporary scaffold for tissue regeneration and healing and can be autologously sourced [37,38]. In this study, we developed a scaffold process that enhanced the mechanical properties of the gelatin hydrogel by combining with alginate and fibrin. The main reason for the enhanced mechanical properties and stability of the hydrogels consisting of gelatin/alginate/fibrinogen was that the different structures and crosslinking of the molecules restrained degradation and strengthened the structure formed.

This is the first attempt to show that ADS cells could be controlled to differentiate into different targeted cell types according to their positions within an orderly 3D structure. Immunostaining tests demonstrated that EGF easily induced ADS cells on the walls of the channels to differentiate into mature endothelial cells and form tubular structures throughout the engineered 3D structures. In contrast, after pre-culture with EGF, the ADS cells under the channel walls were more sensitive to differentiate into adipocytes than the cells on the walls. The reasons could be: (i) the EGF concentration under the walls of the channels was lower than that on the surface of the channels due to the diffusion gradient; (ii) the mechanical properties of the surface of the channels induced the ADS cells to differentiate into endothelial cells more easily; and (iii) once differentiated into mature endothelial cells, the ADS cells lost other differentiation potentials. Further investigations were needed to confirm these hypotheses. Our technique has provided a new approach to engineer orderly endothelial vessel networks *in vitro*.

In this study, the adipocytes and endothelial cells, that were arranged in the 3D structures, simulated the respective *in vivo* positions. We hypothesized that these cells mimic the respective *in vivo* functions. After long-term exposure to high glucose, the adipocytes became obese and secreted more leptin, while the cells in the structures with no DXM and IBMX had lower adipogenesis, leptin secretion responses. The results also indicated that the ET-1 release ability of the endothelial cells in the structures increased under high glucose stimulation, while the treatment of the endothelial cells in the structures with insulin

resulted in a concentration-dependent increase in NO production. The cells in the structure, with no DXM or EFG, had lower ET-1 and NO secretion under high-glucose or insulin stimulation. These results clearly show that the adipocytes and endothelial cells in the structure, induced from ADS cells, mimic their respective functions *in vivo*.

CONCLUSIONS

The approach that we have developed enables the formation of *in vitro* endothelial vessel networks in adipose engineered tissue. The results show that ADS cells controlled differentiation into adipocytes and endothelial cells according to their positions within an orderly 3D structure. Furthermore, the differentiated cell types, induced from ADS cells, mimic, respective *in vivo* functions. These studies confirm the potential of cell-assembly technique and stem cell technique in the engineering of 3D vascularized tissues with the formation of endothelial networks. This research provides a new approach to engineering orderly endothelial vessel networks *in vitro* and has potential applications in adipose tissue engineering, as well as a tool for the *in vitro* studies of tissue vascularization mechanisms.

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