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An cell-assembly derived physiological 3D model of the metabolic syndrome, based on adipose-derived stromal cells and a gelatin/alginate/fibrinogen matrix

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ABSTRACT

One of the major obstacles in drug discovery is the lack of *in vitro* three-dimensional (3D) models that can capture more complex features of a disease.Here we established a *in vitro* physiological model of the metabolic syndrome (MS) using cell-assembly technique (CAT), which can assemble cells into designated places to form complex 3D structures. Adipose-derived stromal (ADS) cells were assembled with gelatin/alginate/fibrinogen. Fibrin was employed as an effective material to regulate ADS cell differentiation and self-organization along with other methods. ADS cells differentiated into adipocytes and endothelial cells, meanwhile, the cells were induced to self-organize into an analogous tissue structure. Pancreatic islets were then deposited at designated locations and constituted the adipoinsular axis with adipocytes. Analysis of the factors involved in energy metabolism showed that this system could capture more pathological features of MS. Drugs known to have effects on MS showed accordant effects in this system, indicating that the model has potential in MS drug discovery. Overall, this study demonstrated that cell differentiation and self-organization can be regulated by techniques combined with CAT. The model presented could result in a better understanding of the pathogenesis of MS and the development of new technologies for drug discovery.

1. Introduction

Researchers have recently developed techniques to fabricate tissues and organs in which both cells and biomaterials have carefully defined architectures. A three-dimensional (3D) bio-assembly tool is capable of extruding cells and biomaterials into spatially organized 3D constructs [1]. Cell printing equipment can print cells as a stream of drops in 3D positions that mimic their respective positions in organs [2,3]. We have recently developed a 3D cell-assembly technique (CAT) based on Rapid Prototyping (RP) [4,5]. This technique can put different cells and materials into designated places to form complex 3D structures. The designed architecture facilitates cell growth, organization, and differentiation. Hepatocytes have been assembled with gelatin and alginate hydrogel to build 3D structures, in which the viabilities and functions of the cells could remain for more than 60 days [6].

Gelatin and alginate have been used as biomaterials in many simple scaffold structures such as sheets, fibers, and micro-capsules. The special property of gelatin in which it can be gelled at a low temperature allows the CAT extruded mixture to take shape at a low temperature. The combination of gelatin and alginate is advantageous because of chemical similarity to the extracellular matrix (ECM) [7]. However, the gelling processes of gelatin and alginate are not normal physiological process in living organisms and could not be controlled expediently. Consequently, it is difficult to induce cells in gel differentiation and facilitate self-organization into a functional structure [8]. Fibrin is normally used by the body as a temporary scaffold for tissue regeneration and healing [9]. Research shows that fibrin gel combines a number of important properties of an ideal scaffold to grow a variety of cells and tissue constructs [10]. The polymerization, constriction, and degradation of fibrin are controllable with the use of thrombin and aprotinin. Therefore, we conceived that a composite ECM with fibrin would help regulate cells' differentiation and self-organization into a functional tissue structure.

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Recently, tissue engineering has endeavored to expand into new horizons aside from the formation of tissues [11,12]. In the field of drug discovery, there is an increasing demand for *in vitro* 3D models that can capture more complex pathological features compared with what traditional two-dimensional (2-D) models have thus achieved [13,14]. Some *in vitro* 3D models have been used in physiological and pathological research [15,16], but their applications have been limited owing to their poorly controlled structure. For example, researchers have endeavored to develop drugs that can target the metabolic syndrome (MS) as a whole [17], but to date, there are still no approved drugs that can reliably reduce all metabolic risk factors. One of the major obstacles is the lack of *in vitro* 3D models that can capture most pathological features of MS [18–20]. MS is a cluster of growing epidemic diseases which present as energy metabolic disorders (obesity, diabetes) and cardiovascular diseases (hypertension, atherosclerosis) [21,22]. Adipocytes and β-cells constitute the adipo-insular axis that regulates energy metabolism [23], and endothelial cell dysfunction connects the pathogenesis of energy metabolic disorders with that of cardiovascular diseases [24]. With these mechanisms in mind, we attempt to organize the related cells to establish MS models using CAT.

In this work, we report on the feasibility of constructing an *in vitro* multicellular 3D model of the energy metabolic system for MS using CAT. Adipose-derived stromal (ADS) cells [25,26] and gelatin/alginate/fibrinogen were assembled in a well-designated 3D structure, and their differentiation into adipocytes and endothelial cells was controlled based on their respective positions within the structure; pancreatic islets were then deposited at the designated micro-holes. We also tested the factors involved in the energy metabolism and endothelial dysfunction of the multicellular model, whether chronic exposure to high glucose, a major inducement of MS, could lead to similar pathological changes in the multicellular model to MS, and whether drugs known to have effects on MS manifest accordant effects in the multicellular system.

2. Methods

2.1. Cell culture

ADS cells were isolated from rat subcutaneous adipose tissues [26]. The epididymal adipose tissues from Sprague-Dawley rats (100–150 g, Beijing university Medical Center of Laboratory Animals) were excised, washed and finely minced in 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 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 calf serum (FCS) at 37 °C in an atmosphere of 5% CO_2. Cells were grown to subconfluence and passaged by standard methods of trypsinization.

Pancreatic Islets were isolated from the rat pancreas [27]. Pancreas of SD rats was infused with 0.2% type V collagenase (Sigma), quickly excised, minced, and incubated at 37 °C for 30 min. Neutralized cells was washed in Hank's solution and centrifuged. Islets were separated using Ficoll gradient centrifugation (Ficoll 400 DL, Sigma). Islets were cultured in DMEM containing 100 mL/L FCS, 200 kU/L penicillin, 100 mg/L streptomycin, and 2 mmol/L L-glutamine (Gibco) at 37 °C in an atmosphere of 5% CO_2. Medium was changed every second day.

2.2. 3-D multicellular system construct and operation

The ADS Cells were trypsinized off the culture dishes upon subconfluence, washed and quantified. Then cells were mixed with gelatin (Tianjin green-island Company, 96 kDa, type B), alginate (SIGMA, 75–100 kDa, guluronic acid 39%) 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 (1mL, 0.45 × 16 RW LB). We used a software package (Microsoft, AT6400) to design the complex 3-D structure, which consisted of square grids and orderly channels about 400 um in diameter (Fig. 1a), this structure has been used in our previous work and has been proved effective. Following the designed structure, a refit nozzle controlled by computer was used to deposit the mixture on a glass chip at a temperature of 10 °C (Gelatin at gel state). The program was run 8 times consecutively at the same position to generation of a 10 × 10 × 2 mm^3 3-D configuration with the square pattern. When the process was finished, the 3-D structure was cross linked with 10% $CaCl_2$ for 1 min (crosslink the alginate), washed with DMEM three times. Then the 3-D structure was further stabilized by 50μU/ml Thrombin (polymerize fibrinogen) in a culture medium containing DMEM, 10% FCS, 1 μmol/L insulin, EGF and 50 U/mL aprotinin (Sigma), placed in a CO_2 incubator at 37 °C. After 3 days of culture, the medium was switched to another containing 10% FCS, 1 μM insulin, 1 μM dexamethasone, 0.5 mmol/L isobutylmethylxanthine (IBMX; Sigma), and 50 u/mL aprotinin for 3 d. At the 6th day, the pancreatic islets were aspirated and deposited at designated position of the 3-D structure. The medium was changed every other day. For 2-D control experiment, the cell culture plates were soaked with solution (gelatin: alginate: fibrinogen, 2:1:1, 0.5%) for 60 min, and then the solution was dumped and the plate was processed with 5% $CaCl_2$ for 1 min. ADS cells were plated at 5×10^5 cells/well plates and induced to adipocytes by IBMX or to endothelial cells by EGF.

2.3. Structural analyses by scanning electron microscopy

At the 6th day of culture, the multicellular 3-D structure were washed in phosphate buffer (pH 7.4) and fixed with 3% glutaraldehyde for 2 h. Then the samples were post-fixed with 0.5% OsO4 and rinsed with PB again. The samples were dried in vacuum freeze dryer for 12 h. After dehydrated, samples were sputter coated with gold–palladium. All micrographs were obtained in a scanning electron microscope (Hitachi S450, JAP).

2.4. Immunostaining

For immunofluorescence analyses, the assembled multicellular 3-D structure was fixed with 4% glutaraldehyde for 20 min at 20 °C, and washed 3 times with PBS. The structure was incubated with 50 μg/mL propidium iodide (PI, sigma USA) for 20 min (nuclear staining), then the structure were incubate with primary antibodies: rabbit anti-rat:CD31 (1:20 in PBS); rabbit anti-rat CD34; rabbit anti-rat insulin (1:50) (all from Santa Cruz, USA) for 30 min respectively. And then the structure was incubated with a secondary antibody (FITC-conjugated anti rabbit IgG, Santa Cruz, 1:20 in PBS) for 30 min. Finally, the samples were washed with PBS and observed by 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).

2.5. Dynamic insulin secretion experiment

We measured Insulin secretion kinetics by perfusion experiments. After the islets were deposited in the structure, the structures were precultured in normal glucose (5 mM) or high glucose (15 mM) medium. After 5 days, islets in or not in the 3-D structures (20 islets/per) were introduced to a 1-mL perfusion chamber and exposed to flowing perfusate (DMEM, 0.2 mL/min) with a basal glucose concentration (2mM) for 1 h for islet cell starvation. Basal insulin secretion was estimated at the same medium and then switched to a high glucose content (15 mM) perfusate and collected every 2 min for 20 min. Then the perfusate was returned to the basal solution and collected every 5 min for 15 min. The insulin content of collected samples was measured by using a rat insulin ELISA kit according to the manufacturers instructions (RB, USA) by a microplate reader (Bio-Rad 550). For drug experiment, rosiglitazone (3 μg/mL) and nateglinide (5 μg/mL) were added to the culture medium at the 11th day respectively.

2.6. Measurement of glucose consumption, FFA release, and adipogenesis

After seeding the islets, the multicellular 3-D system was further cultured in normal glucose (5 mM) or high glucose (15 mM) medium for more than 7 d. At the 13th day, we replaced the medium with DMEM containing 25 mM glucose. After 24 h, we collected the medium for the measurement of glucose consumption. Glucose concentration was measured by Glucose assay kit, (Biovision, USA). Glucose consumption rates were calculated subtracting residual glucose in the treated medium from glucose in nontreated medium. For determination of FFA release, we replace the medium with DMEM containing 2% fatty acid free BSA and 10 μM isoproterenol at the 15th day. Aliquots of the medium were collected and the FFA content was measured with FFA assay kits (Nanjing Jiancheng, PRC). For drug experiment, rosiglitazone was added to the culture medium at the 11th day.

We use oil red o staining to indicate the high glucose-induced adipogenesis of adipocyte. After seeding the islets, the 3-D multicellular system was cultured in normal glucose (5 mM) or high glucose (15 mM) medium for more than 8 d. At the 14th d, the 3-D multicellular system was fixed with 10% formalin, stained with 0.1 mg/mL oil red O solution for 2 h. Oil red O dye was extracted into isopropanol and absorbance was measured at 510 nm. For drug experiment, rosiglitazone (3 μg/mL) was added to the culture medium at the 7th day.

2.7. Measurement of adipocytokine by ELISA

After seeding the islets, the multicellular 3-D system was cultured in normal glucose (5 mM) or high glucose (15 mM) medium, three day-conditioned media were

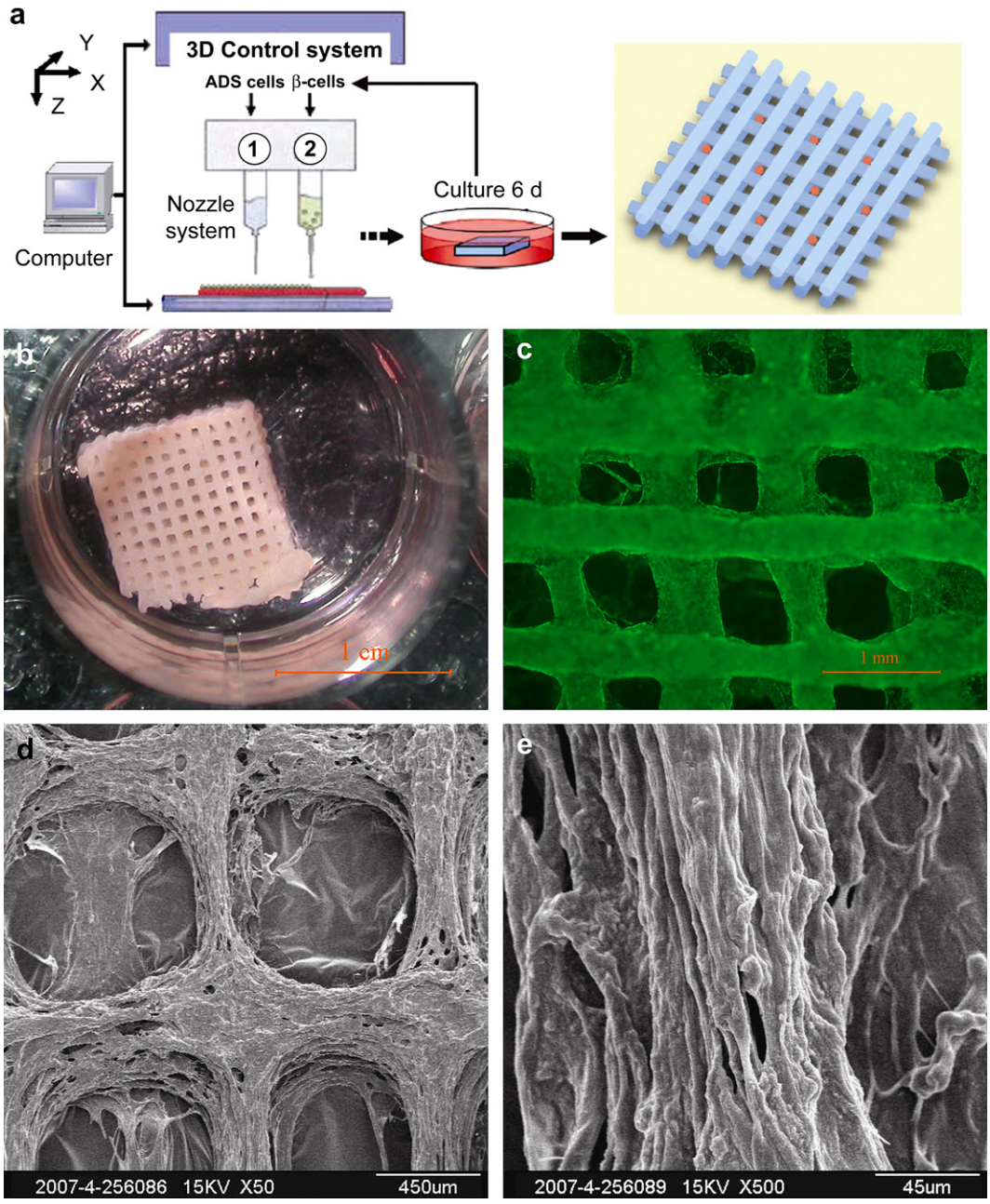

Fig. 1. Construction and analyses of the 3D multicellular system. (a) 3D multicellular system construct and operation. (b) The 3D structure cultured in a plate. (c) Immunostaining of the 3D structure with mAb against CD34+ (in green). (d)(e) Scanning electron micrographs (SEM) showed the development of extensive ECM and cell networks in the structure after 6 days of culture. (f)(g) SEM of the 3D structure just after preparation. (h) Immunostaining of the pancreatic islets in the 3D structure using anti-insulin in green. pancreatic islets keep integrity globoid shapes for more than three weeks in the structure, (i) some envelopes of islets were broken.

harvested at the 21th day. Leptin, resistin and adiponectin were measured in cell culture media using rat leptin ELISA kits (RB, USA), rat resistin ELISA kits (TPI, USA) and rat adiponectin ELISA kits (USCNLIFE, USA) respectively. The assays were conducted in 96-well microplates according to the manufacture's recommendations, with a microplate reader. Results were expressed as a rate of adipocytokine level to DNA content of the cells. Adipocytes in 2-D culture and in 3-D culture without islets were deal with the same process. For drug experiment, rosiglitazone (3 μg/mL) was added to the culture medium at the 12th day.

2.8. DNA assays

Following the measurement of adipocytokine, samples and cell-free structure were carefully collected and homogenized, and then digested (0.14 mg/mL papainase in 100 mM phosphate buffer with 10 mM ethylenediaminetetraacetate, EDTA, and 10 mM cysteine) for 20 h at 60 °C, using 2 mL of the enzyme solution per sample [42]. DNA content was determined fluorimetrically following the binding of Hoechst 33 258 dye (Sigma) using calf thymus type I DNA as a standard. The fluorescence of negative, cell-free structure was subtracted from the fluorescence values of experimental groups to account for fluorescence of the material alone.

2.9. Measurement of endothelin-1(ET-1) and nitric oxide (NO)

After seeding the islets, the multicellular 3-D system was culture with 5 mM glucose. At the 13th day, the medium were replaced with DMEM containing 25 mM glucose. Two day-conditioned media were harvested at the 15th day. ET-l released into the culture media was measured by ELISA kit (RB, USA) according to the manufacturer's instructions. NO concentrations in the culture media were detected by using NO Detection kit (JinMei Biotech Co. Ltd.) based on nitrate reductase

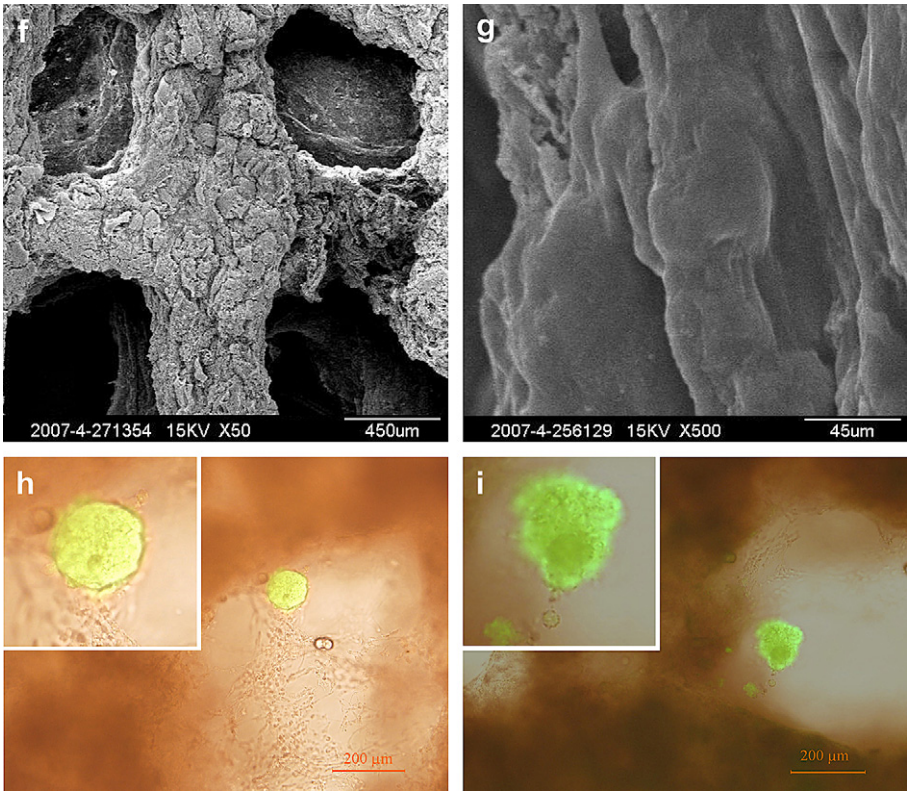

Fig. 1. (*continued*).

method. Endothelial cells in 2-D culture and in 3-D culture without islets were dealt with the same process. For drug experiment, rosiglitazone (3 μg/mL) was added to the culture medium at the 10th day.

2.10. Statistical analysis

Statistical analysis was performed by 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 a considerable probability of statistical error due to the limited numbers of subjects in this study.

3. Results

3.1. Assembly of the 3D multicellular system

Considering the important roles of adipocytes, β-cells, and endothelial cells in energy metabolism and MS pathogenesis, we assumed that these cell types would be required for the construct of the model for MS. We selected ADS cells isolated from rat, which can differentiate into adipocytes and endothelial cells. We mixed ADS cells with gelatin, alginate, and fibrinogen solutions sequentially. Following the designed model, a nozzle controlled by a computer was used to deposit the mixture on a glass chip and generate 3D structures (Fig. 1b). These 3D structures were then cross linked and polymerized (fibrinogen to fibrin) with CaCL2 and thrombin, respectively. The complete process required about 20 min. Once the mixture was cross linked, the whole construct could be handled easily without losing its integrity. The cells were homogeneously embedded in the matrices (Fig. 1c and d), and the hydrogel state could remain for at least 8 weeks. Due to the loss of Ca^{2+} ions from the cross linked alginate molecules, hydrogel, consisting of gelatin and alginate, became very brittle within one week after culture. The hydrogel consisting of gelatin and fibrin constricted and degraded in two weeks due to fibrin constriction and plasmin secretion of living cells. The gelatin/alginate/fibrin hydrogel was much more stable than the gelatin/alginate or gelatin/fibrin hydrogels. Fibrin constriction caused the tensile force on the surface of the channels in the 3D structure, the high tensile force induced the channels to become round (Fig. 1c–e), and this constriction could be weakened by adding aprotinin.

Scanning electron micrographs showed the development of an extensive cell networks in the structure after six days of culture (Fig. 1d–g). The pancreatic islets (containing β-cells) from rat were deposited into the designated micro holes in the 3D structure at the 7th day. Anti-insulin immunostaining showed that 90% of the pancreatic islets could keep their integrity of globoid shape more than three weeks in the structure (Fig. 1h). Some envelopes of islet were broken, but the β-cells still congregated and kept secreting insulin (Fig. 1i).

To test whether the ADS cells in the 3D structure can be controlled in terms of their differentiation into endothelial cells and adipocytes, we 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 endothelial growth factor (EGF), CD34 (ADS cells and endothelial cells indicator) and CD31 (mature endothelial cells indicator) staining revealed that over 90% of the ADS cells on the walls of the channels were differentiated into mature endothelial cells (CD31+/CD34+) and connected with one another, forming a vessel-like structure (Fig. 2a, c and f). This differentiation of ADS cells was based on EGF induction (Fig. 2b and d) and cell position in the 3D structure (Fig. 2f). The structure without fibrin almost fell apart and few ADS cells in the hydrogel differentiated into mature endothelial cells (Fig. 2e). From the fourth day, the structures were treated with insulin, IBMX, and dexamethasone for three days. On the 12th day, Oil red O staining revealed that the ADS cells in the structure differentiated into adipocytes with a spherical shape (Fig. 2g). The cells under the

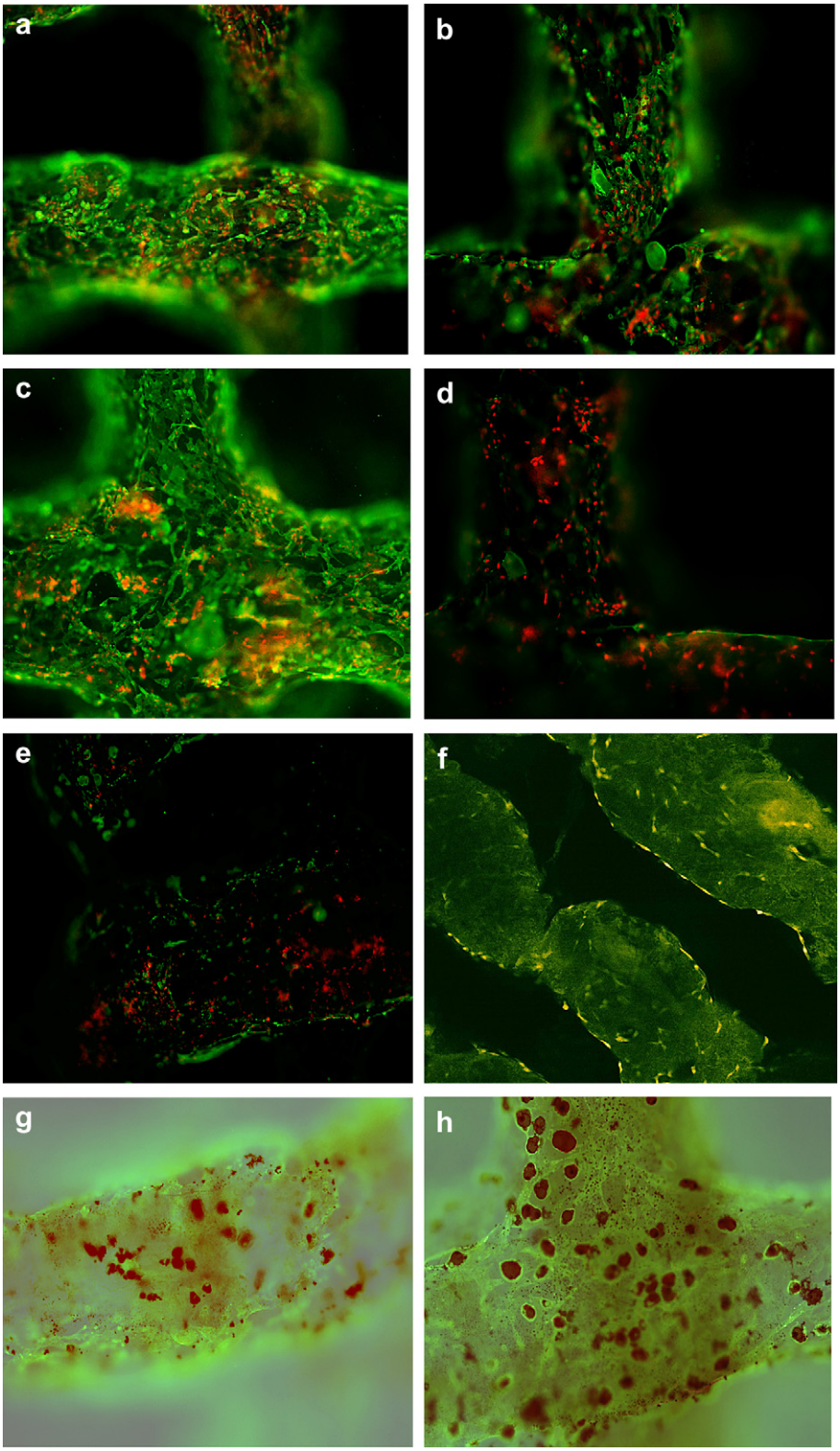

Fig. 2. ADS cells in the 3-D structure controlled to differentiate into endothelial cells and adipocytes. (a, b) Immunostaining of the 3-D structure using mAbs for CD34 + cells (ADS cells and endothelial cells) in green and PI for nuclear in red. (c–f) Immunostaining of the 3-D structure using mAbs for CD31 + cells (mature endothelial cells) in green and PI for nuclear in red. ADS cells in the 3-D structure were cultured with EGF (a, c, e, f) or without EGF (b, d) (negative control). (e) ADS cells with gelatin and alginate, without fibrin. Image of (f) was observed by confocal microscopy. (g, h) Immunostaining of the 3-D structure using mAbs for CD31 + cells in green and Oil red O staining of the 3-D structure for adipocytes in red. ADS cells in 3-D structure were culture with EGF (g) or without EGF (h) for three days, and then treated with insulin, IBMX and dexamethasone.

walls of the channels were more sensitive to differentiation into adipocytes than those on the surface of the walls. However, if not induced with EGF, more than 90% of the cells on the walls of the channels would differentiate into adipocytes (Fig. 2h).

3.2. Dynamic insulin secretion

To determine whether β-cells, which were assembled in the system as a unit of pancreatic islets, can mimic in vivo function, we used a perfusion system for a time-dependent estimation of insulin response to glucose stimulation. Fig. 3 shows the dynamic insulin release patterns of free or assembled β-cells stimulated by glucose. In response to 15 mM glucose stimulation, assembled β-cells showed a significant increase in insulin secretion compared to free β-cells. To determine whether chronic exposure to high glucose could cause pathological changes of the β-cells, we culture free or assembled β-cells with high-glucose (15 mM). After 5 days of culture, both free β-cells and assembled β-cells showed dramatic decreases in glucose-induced insulin release, and the decrease rate of the assembled β-cells was higher than that of the free β-cells. Nateglinide could revise the insulin secretion decrease of the assembled and free β-cells; whereas rosiglitazone only had the same effects on the assembled β-cells.

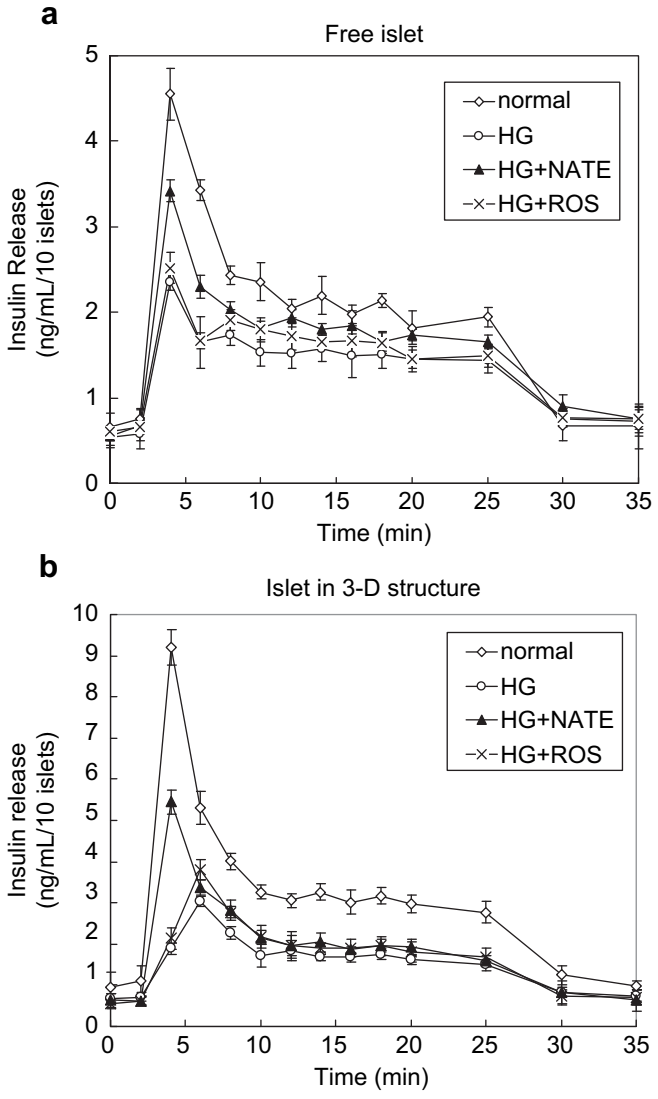

Fig. 3. Insulin secretion kinetics of (a) the free islet, (b) the islet assembled in the 3-D structure were measured by perfusion experiments. Cells were incubated in normal glucose (NG, 5 mM) or high glucose (HG, 15 mM) for 5 d. For drug experiment, rosiglitazone (ROS, 3 μg/mL) or nateglinide (NATE, 5 μg/mL) were added to the HG culture medium at the 4th day. Next the cells were introduced to a perfusion chamber and exposed to flowing perfusate with a basal glucose concentration (2 mM) for 1h and then switched to a high glucose content (15 mM) perfusate for 20 min. Then the perfusate was returned to basal glucose concentration. The insulin content in the extracts was determined by ELISA insulin kit. Data are mean ± s.d., $n = 3$.

Notably, after pre-cultured with high glucose, the glucose-induced insulin secretion peak of the assembled β-cells was delayed compared to normal control, this delay could be compensated by nateglinide, however, this phenomenon was not observed in the free β-cells. Derangements in the kinetics of insulin release, including the decrease and delay of the secretion peak value, were one of main characteristics of diabetes [28,29]. These results are consistent with the other *in vivo* experiments [30,31], suggesting that the adipocytes in the 3-D structure could help the β-cells to capture more pathologic feature of MS. Further investigation of adipocytokine secretion would subsequently reveal the mechanism of this phenomenon.

3.3. Glucose consumption, FFA release, and adipogenesis

Glucose consumption and free fatty acids (FFA) mobilization of adipocytes are two basic metabolic processes in energy metabolism. To determine the glucose consumption of cells in the 2-D, 3-D (without β-cells) and multicellular 3-D (with β-cells) culture systems, we culture the cells with 25 mM glucose, and thereafter the media were collected for glucose consumption assays (Fig. 4a). Cells in the 3-D structure showed higher glucose consumption than in the 2-D structure, seeding β-cells in the 3-D structure significantly enhanced the glucose consumption. Similarly, after stimulated with isoproterenol, cells in the 3-D structure showed higher FFA release than in the 2-D structure, and the β-cells significantly increase the FFA release of the adipocytes (Fig. 4b).

To determine whether long-term exposure to high glucose could cause similar pathological changes of glucose and lipid metabolism of MS, we cultured cells with high-glucose (15 mM) for 7 d. Pre-culture with high glucose inhibited the glucose consumption of all culture systems, the decrease rates of the multicellular 3-D system was the highest. Rosiglitazone revised the glucose consumption inhibited by high glucose, the increase rates of the multicellular 3-D system was the highest. Contrarily, pre-culture with high glucose increased the isoproterenol-induced FFA release of all culture systems. Rosiglitazone just inhibited the FFA release which increase in the multicellular 3-D system. These results suggest that β-cells assembled in the structure facilitate the system to mimic glucose and FFA metabolism dysfunction of MS and to show more obvious effects of rosiglitazone.

Synthesize and storage of fatty acid with overnutrition is the main function of adipocytes and also the pathogenesis of obesity. To test whether high glucose promotes adipogenesis of adipocytes in different culture systems, we cultured cells with high glucose. After total of 12 days of culture, the adipogenesis of the 2-D, 3-D and multicellular 3-D system were increased by 2.1, 5, and 7.3 folds compared to normal level, respectively (Fig. 4c). Rosiglitazone could increase the adipogenesis of all the groups; the increase rate of the multicellular 3-D culture system was the lowest. These results suggest that, in the multicellular 3-D system, the assembled β-cells promote adipogenesis of adipocytes, but rosiglitazone has only moderate effect on adipogenesis. These results accord with the other *in vitro* researches [32,33].

3.4. Adipocytokine

In addition to the storage of lipid, adipocytes perform an endocrine function by secreting adipocytokines to regulate energy

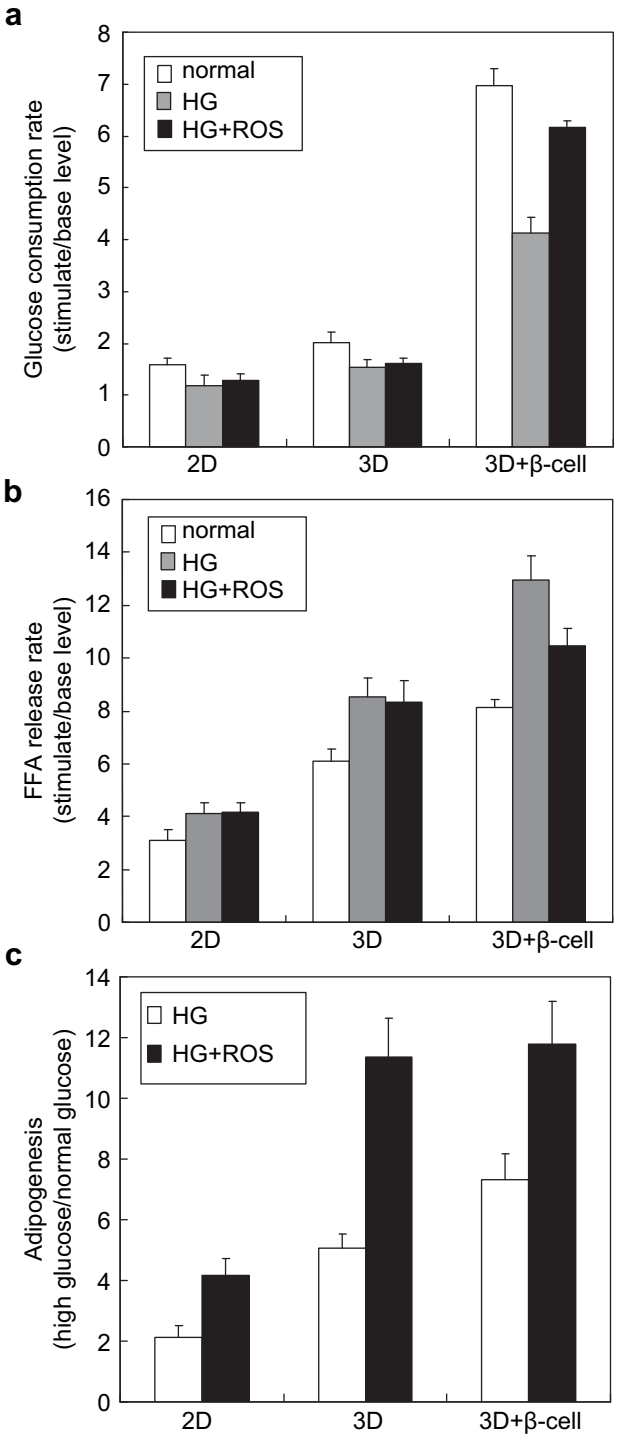

Fig. 4. Measurement of Glucose metabolism and lipid metabolism. Long-term exposure to high glucose (HG 15 mM) was used to induce the pathological changes of the cells in different culture system. For drug experiment, rosiglitazone (ROS, 3 μg/mL) was added to the HG culture medium. (a) Glucose consumption of cells was stimulated with 25 mM glucose and the glucose concentration of the medium was measured with Glucose assay kit. (b) FFA release of cells was stimulated with isoproterenol (10 μM) and the FFA content was measured with FFA assay kits. Results were expressed as the ratios of the stimulate level to base level. (c) Adipogenesis.was stimulated by long exposure to HG and the lipid accumulation was measured with Oil red O assay. Absorbance is designated as 1.0 for cell cultured in normal glucose. Data are mean ± s.d., $n = 6$.

metabolism [34]. To study the adipocytokine secreted by adipocytes, we analyzed the secretion of leptin, resistin, and adiponectin of different culture systems. Leptin can inhibit both energy intake and insulin secretion, and is antagonistic to insulin action. Leptin secretion of adipocytes in the 3-D structure was higher than that in the 2-D structure; β-cells inhibit leptin secretion of adipocytes in the 3-D structure (Fig. 5a). Resistin is capable of being antagonistic to insulin action and, thus, may serve as a link between obesity and type 2 diabetes. Adipocytes in the 3-D structure showed higher resistin secretion than in the 2-D structure (Fig. 5b). Adiponectin can increase insulin sensitivity and protect endothelial cells. Adipocytes in 3-D structure showed higher adiponectin secretion than in 2-D culture (Fig. 5c). These data suggest that adipocytes in the 3-D culture systems have higher adipocytokine secretion than in the 2-D culture system. However β-cells have no obvious effect on normal secretion level of adiponectin and resistin, except leptin.

To determine whether chronic exposure to high glucose can induce pathological changes of adipocytokine secretion of adipocytes, we cultured the different systems with high glucose. Compared with the normal secretion level, pre-culture with high glucose: (i) increased the leptin secretion of all groups; (ii) increased the resistin secretion of all groups; (iii) inhibited the adiponectin secretion of all groups; (iv) adipocytokine secretion changes in the multicellular 3-D group were more moderate than those in the other groups. Rosiglitazone could restore the leptin, resistin, and adiponectin secretion derangement; these effects were more obvious in the multicellular 3-D group. These results are consistent with the other *in vivo* experiments [35,36].

3.5. Endothelial dysfunction

ET-1 and NO secretion derangements have been identified as major contributors to endothelial dysfunction associated with MS [24]. To study the ET-1 and NO secretion of endothelial, we cultured different systems with high glucose for two days. After stimulated with high glucose, endothelial cells in the 3-D structure showed higher ET-1 secretion rate than in the 2-D culture, and the assembled β-cells increased the ET-1 secretion rate of endothelial in the 3-D structure; Rosiglitazone decreased the ET-1 secretion stimulated by high glucose; the decrease rate of the multicellular 3-D system was the highest (Fig. 6a). However, we did not find any obvious modification of NO production in the 2-D and 3-D systems after stimulating with high glucose, except for the increase of the multicellular 3-D system. Rosiglitazone could significantly increase the NO production of the 3-D and multicellular 3-D systems, but has no effect on the 2-D system (Fig. 6b).

Our results suggest that the secretion of β-cells could stimulate the secretions of ET-1 and NO. Insulin plays an important role in inducing ET-1 and NO release; Anti-insulin could decrease the ET-1 and NO secretion of the multicellular system, but has no significant effects on other systems without β-cells. These results suggest that β-cells increase ET-1 and NO secretion of culture system largely through secretion insulin, comparing with other culture systems, endothelial cells in the multicellular 3-D system could capture more pathological features of MS and rosiglitazone had more accordant effects on these systems [37,38].

4. Discussion

We give the initial description on the establishment of the energy metabolic system model using CAT. This approach integrates recent advancements in biomaterials, tissue engineering, stem cell, and MS research into a more efficient and useful methodology. We used gelatin, alginate, and fibrinogen as the composite ECM. While gelatin gel took shape at a temperature of 10 °C,

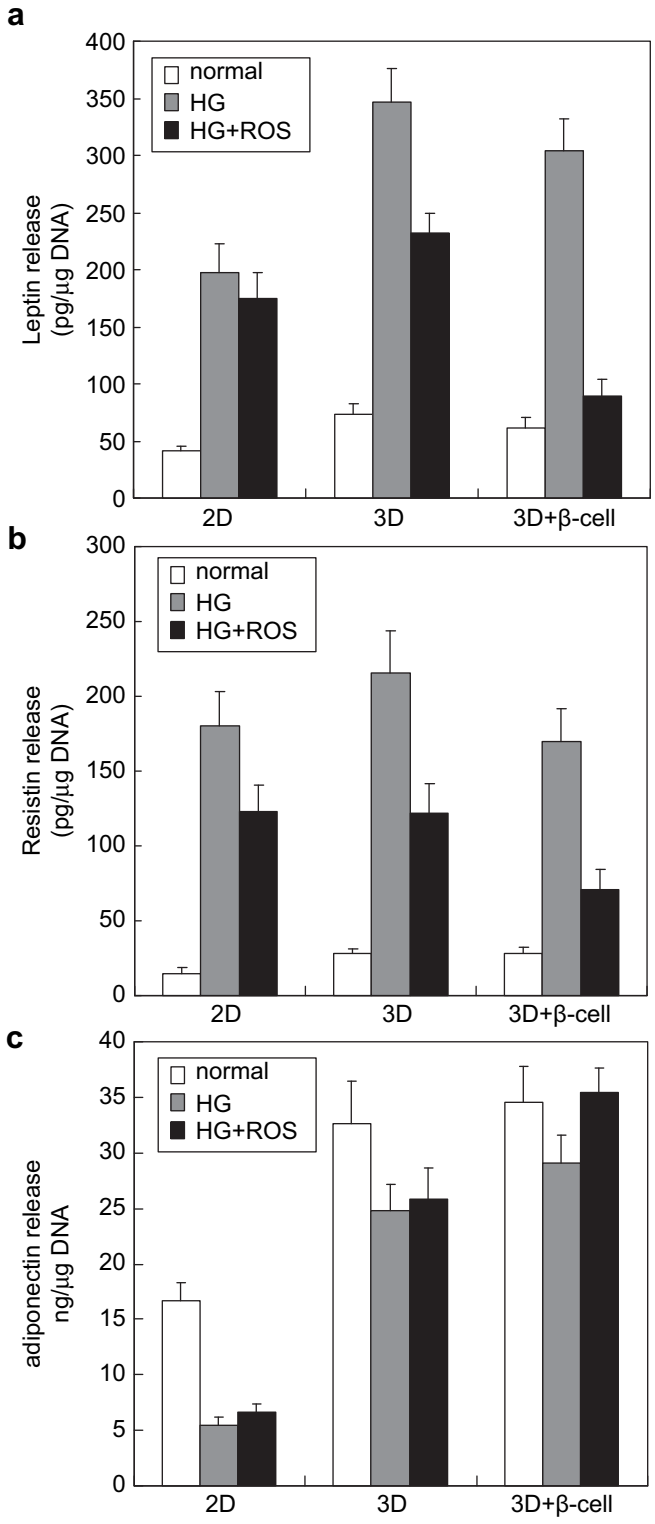

Fig. 5. Adipocytokine secretion of adipocytes in different culture systems. Long-term exposure to high glucose (HG 15 mM) was used to induce the pathological changes of the cells in different culture system. For drug experiment, rosiglitazone (ROS, 3 μg/mL) was added to the HG culture medium. Adipocytokine secretion of adipocytes in different culture system: (a) leptin secretion (b) resistin secretion (c) adiponectin secretion. Adipocytokine content in the medium was determined by ELISA kit. Results were expressed as the ratios of the adipocytokine secretion level to the DNA content of the cells. Data are mean ± s.d., $n = 6$.

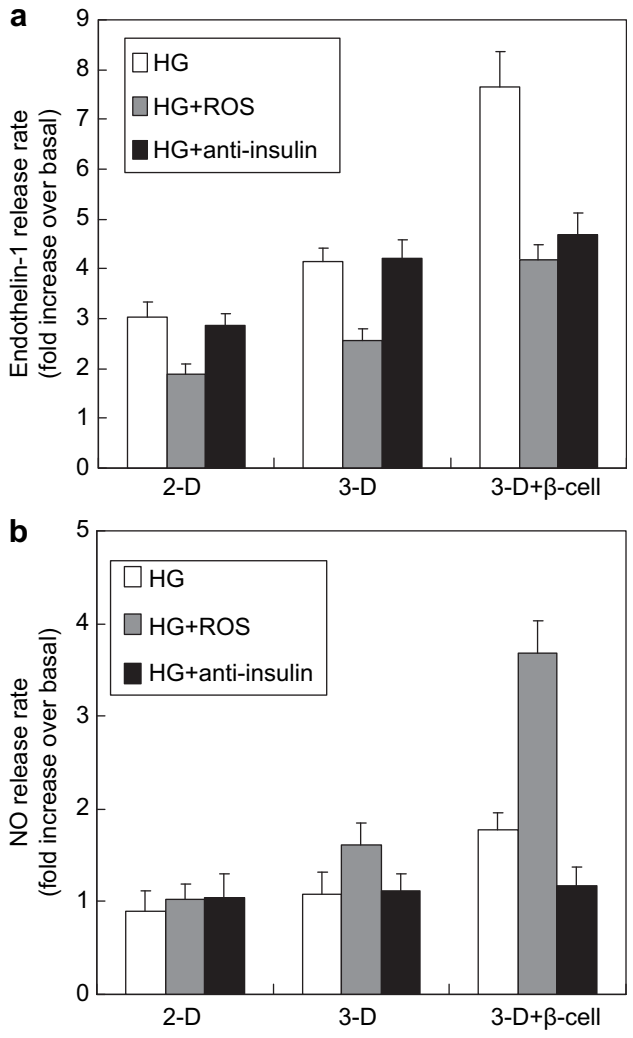

Fig. 6. Endothelin-1 and NO secretion of endothelial in different culture systems. Cells were cultured with high glucose (HG 25 mM) for two days. (a) Endothelin-1 concentrations in the culture medium were measured by ELISA kit. (b) NO concentrations in the culture medium were detected by NO Detection kit. For drug experiment, rosiglitazone (3 μg/mL) was added to the HG culture medium. Results were expressed as fold increase to base level. Data are mean ± s.d., $n = 6$.

alginate and fibrinogen were then cross linked or polymerized with $CaCl^{2+}$ or thrombin, respectively. Consequently, we developed a cell/ECM construct in which the mechanical properties of gelatin hydrogel were enhanced by combining it with alginate and fibrin. The main reason for the enhanced mechanical properties and stability of the hydrogels consisting of gelatin/alginate/fibrin was that the stiffer component (alginate) prevented the softer one (fibrin) from yielding and degrading, while the softer component protected the stiffer one from brittle fractures. Additionally, the polymerization, constriction, and degradation processes of fibrin in hydrogel were controlled by thrombin and aprotinin in this study. Results showed that the mechanical properties induced by fibrin constriction in the 3D structure helped in ADS cell differentiation and self-organization. These results are consistent with previous research that fibrin is normally used by the body as a temporary scaffold for tissue regeneration, and the polymerization, constriction, and degradation of fibrin play a role in tissue regeneration in vivo [9,10,39]. Our work indicated that fibrin provides new opportunities for CAT.

Previous researches demonstrated that, in comparison with conventional 2-D cultures, cells in 3-D cultures more closely

resemble to the *in vivo* situation with regards to cellular environment, cell shape, gene expression and biological behavior [40,41]. Here we used the cell-assembly technique to design and assemble a complex architecture with internal channels, which based on the requirements of growth, function, differentiation, signaling of different types of cells, as well as different detecting technologies. Results showed that cells in the 3-D structure have higher bioactive viabilities than in the 2-D culture system, including insulin secretion, glucose and lipid metabolism, adipocytokine secretion, NO and ET-1 secretion. These results demonstrated that the well-designed 3-D architecture could provide the different types of cells with suitable environment that facilitates cells keeping their respective bioactivities and functions. For example, adipocytes in our 3-D structures could capture distribution, morphology and bio-behavior features which ordinarily appear *in vivo* environment. These results are consistent with the recent researches of 3-D cultivation of preadipocytes on Fibrous Polymer Scaffolds [42].

ADS cells are multipotent cells found in adipose tissue and have received more attentions recently [43]. This is the first attempt that ADS cells were controlled differentiation into different targeted cell types according to their positions within an orderly 3-D structure. Immunostaining tests have demonstrated that EGF could easily induce ADS cells on the walls of the channels to differentiate into mature endothelial cells and form tubular structures throughout the engineered 3-D structures. Contrarily, after pre-culture with EGF, ADS cells under the walls of the channels were more sensitive to differentiation into adipocytes than the cells on the walls. The reasons could be explained as: (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, just like what the morphogen gradient do [44]; (ii) the mechanical properties in the surface of channels could induce the ADS cells to differentiate into endothelial cells more easily [45]; (iii) once differentiated into mature endothelial cells, the ADS cells will lost other differentiation potentials. Further investigations were needed to confirm these hypotheses. At present, one of the major obstacles in engineering thick and complex tissues is the need to vascularize the tissue *in vitro* for maintaining cell viability during tissue growth and inducing structural organization [46]. Our technique has therefore provided a new approach to engineer orderly endothelial vessel networks *in vitro*.

We organized ADS cells, endothelial cells, adipocytes, and β-cells in an orderly 3D structure and mimicked their respective *in vivo* positions. We hypothesized that these cells could mimic the energy metabolic system through paracrine and intercellular interactions as that *in vivo*. The results demonstrate that the adipocytes in the system could influence the glucose-induced insulin secretion kinetics of β-cells. After long-term exposure to high glucose, the adipocytes became obese and secreted more FFA, leptin and resistin, which are actively involved in crosstalk with β-cells, decrease and delay the insulin secretion peak value of β-cells [47]. Moreover, the secretions of β-cells also affected the functions of the adipocytes in that they increased glucose consumption, FFA release, and adipogenesis; decreased leptin release; and increased the homeostasis of adipocytokine secretion to high glucose stimulation. The endothelial cells formed a vessel wall-like structure on the walls of the channels and then formed an endothelial barrier between adipocytes and β-cells as that *in vivo*. In summary, the adipocytes and β-cells of the multicellular system constitute an adipoinsular axis to regulate energy metabolism, secretions from adipocytes and β-cells affect ET-1 and NO release of endothelial cells, and all these intercellular interactions are coincident with those of the *in vivo* experiments [48,49]. Further investigations were needed to confirm these conclusion. Notably, the major inducement of MS, chronic exposure to high glucose, causes more similar pathological changes of MS in the multi-cellular system, including obesity, insulin, and adipocytokine secretion derangement, insulin resistance, and endothelial cell dysfunction. Moreover, rosiglitazone showed positive effects in the 3D model. Rosiglitazone belongs to the novel thiazolidinedione ((TZD) class of anti-diabetic agents, which has therapeutic potential to improve MS besides diabetes [50]. Overall, these results demonstrated that the 3D model has great potential in MS drug discovery.

5. Conclusion

The interest of establishing *in vitro* models that capture the more complex features of diseases for drug discovery motivated us to assemble a multicellular MS model using CAT. Compounding of new biomaterials was used. Fibrin was employed as an effective material to regulate ADS cell differentiation and self-organization along with other methods. The results demonstrated that adipocytes and islets in the 3D structure constituted the adipoinsular axis. The model could capture most physiological and pathological features of MS *in vitro*. Consequently, this model displayed great potential in MS drug discovery. Establishment of such a multicellular 3D model of the energy metabolic system has practical and scientific implications. The model provides the essential step in pathogenesis study and drug discovery of MS *in vitro*. If we assemble cells that are isolated from human beings, this 3D model can capture species-specific aspects in drug discovery. Construct and study of such a multicellular 3D system can provide not only a research model for examining cell–cell interaction, stem cell differentiation, and cell self-organization mechanism but also provide new approaches for tissue engineering.

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Appendix

Figures with essential colour discrimination. Certain figures in this article, in particular Figs. 1 and 2, have parts that are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.01.111.

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