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약학석사학위논문

**Hanging drop 기술을 이용한 유전자  
변형체장소도 세포의 형성**

**Design of Optimal Pancreatic Islet Spheroids Using  
Hanging-Drop Method on the Functioned Surface**

2012年 8月

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**Functioned Surface**

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# ABSTRACT

For the treatment of type I insulin-dependent diabetes mellitus (IDDM), islet transplantation is considered as the promising strategy. However, because of detrimental conditions after transplantation, the function of the transplanted islets is not efficient enough to control the blood glucose level. Researchers developed islet spheroids to overcome this, and improve transplantation outcome as a result. Here, we investigated the islet spheroids formed using the functioned surface and increased the transfection efficiency of polymeric gene carrier.

Rat pancreatic islets were dissociated into single cells by treating 0.25% trypsin-EDTA. And  $5 \times 10^5$  islet single cells were seeded in a 12-well culture plate. Transfection complexes which consist of 5  $\mu$ g of DNA and corresponding amount of PEI were treated to each well and filled with extra culture medium to make 2 ml of total volume. Transfected single cells were reaggregated into spheroids using hanging drop method on the polystyrene petri dish. To improve the glucose sensitivity, polydopamine patterned superhydrophobic surface was adopted on the process. Single cells were resuspended in the culture medium (500 cells/30  $\mu$ l) to form 30  $\mu$ l drops onto the hydrophilic polydopamine coated part of the surface. The surface was then inverted and drops were incubated for 4-5 days. The lower dish was filled with sufficient PBS to prevent the drops from drying.

Intact islets and islet spheroids show no morphologic differences. Also, viability of islet spheroids was not damaged. Transfection efficiency of IL-10 using polyethyleneimine (PEI) increased to approximately 4.5-fold, compared to that of intact islets. This is due to the increase in total surface area that contacts to DNA-carrier complexes. If the incubation environments became more three dimension-like culture, glucose sensitivity tended to be increased. However, between islet spheroids formed with two different surfaces, there was no significant difference.

Formation of islet spheroids using hanging drop method would be advantageous in terms of reduced, controlled size and high transfection efficiency. Also, by using hanging drop method, glucose sensitivity of islet spheroids increased compared to those formed with two dimensional culture method which is the conventional cell aggregation methods using intermittent pipetting. With the surface which has greater contact angle, I could reduce the formation duration of islet spheroids.

Keywords:       Pancreatic islets  
                      Islet spheroids  
                      Hanging drop method  
                      Polydopamine patterned superhydrophobic surface  
                      Polyethyleneimine  
                      Interleukin-10



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# **1. INTRODUCTION**

## **1.1 Type 1 Diabetes mellitus**

Diabetes mellitus is one type of chronic diseases that occurs when the body cannot produce sufficient amount of insulin or the sensitivity of insulin is decreased. As a result, insulin it is not used efficiently to balance the blood glucose level. Diabetes mellitus can be divided into three types: Type 1 insulin-dependent Diabetes mellitus (IDDM), Type 2 non-insulin dependent Diabetes mellitus (NIDDM), and gestational Diabetes mellitus. According to World Health Organization (WHO), 345 million people worldwide are suffering from diabetes mellitus and diabetes deaths are projected to be doubled between 2005 and 2030. Also, it has been reported from Korean Diabetes Association that 2.4 million people in Korea which take up 5% of the whole nation, are considered as a diabetic patient, but only half of them noticed such health conditions. In case of Type 2 and gestational diabetes, oral medications with several different mechanisms are on the market and widely used these days. However, for the treatment of Type 1 diabetes only whole pancreas transplantation or conventional exogenous insulin injection has been in use and both of them have disadvantages of patient discomfort. Therefore, pancreatic islet transplantation is considered as potentially alternative treatment of type 1 diabetes mellitus, because it needs comparably minimal invasive surgical operations without repetitive insulin injections.

## 1.2 Pancreatic islet transplantation

For the treatment of Type 1 Diabetes mellitus, islet transplantation is used as one of the most promising strategies[1]. The procedure of islet isolation and transplantation were pioneered by Lacy et al. throughout late 1960s and early 1970s[2, 3]. However, because of detrimental conditions after transplantation, including instant blood-mediated inflammatory reaction (IBMIR)[4-6] and hypoxia[7], approximately 50% of transplanted islets cannot function properly right after transplantation[8]. Depending on transplantation methods such as throughintraportal, subcutaneous, kidney capsular, and so on, each of above mentioned conditions differ from each other, but still functionality loss of transplanted islets are a serious problem in every case. To overcome these challenging points, gene delivery into pancreatic islets[9-12] which can protect transplanted islets from harmful host biological responseshas been considered as one of the prominent strategies for successful transplantation outcomes[13, 14].

## 1.3 Rejection mechanisms of xeno-transplantation

The rejection mechanisms of xeno-transplanted islets consists of three sequential processes termed hyperacute, acute, and chronic rejection. First of all, hyperacute rejection occurs within 24 hours after transplantation. Host-derived antibodies, macrophages, and tissue factors play the major role in this step. Activated antibodies will trigger the complement activation and

macrophage will release various proinflammatory cytokines, like IL-1, TNF- $\alpha$ , IFN- $\gamma$ , and cytotoxic radicals. Tissue factors produced from the transplanted islets cause fibrin clot encapsulation of the islets with leukocytes infiltration. This inflammatory reaction is widely known as instant inflammatory reaction (IBMIR) and considered to have detrimental effects on the functionality of transplanted islets[15].

Secondly, acute rejection begins after first week of transplantation, because it needs time to activate T cells in lymph nodes. There are two recognition pathways which initiate the further immune responses: direct and indirect recognition. The former is mainly done by grafted dendritic cells from donors and known to be engaged in comparably fast acute rejection. On the other hand, indirect recognition is done by host dendritic cells. Therefore, it takes more time for immune cells to move to grafted islets and then load antigens.

Lastly, chronic rejection occurs months to years following transplantation. Main reason of this is the proliferation of smooth muscle cells and production of collagen by fibroblasts. Throughout this process, graft arterial occlusions occur and will finally cause ischemia and cell death. And this is the reason why recipients are requested to take immunosuppressive drugs for the rest of their lives.



## 1.4 Immunoregulatory effect of IL-10 on islet transplantation

Interleukin-10 is a kind of cytokine that is primarily secreted by antigen presenting cells (APCs) and show several different immunological effects including anti-apoptosis, anti-inflammation, and immunoregulation. Therefore, IL-10 is called pleiotropic cytokine which means one gene influences multiple phenotypic traits.

In the immunorejection process, no matter it is direct or indirect recognition, APCs activate helper T cells, as well as cytotoxic T cells. IL-10 plays an important role in the process of helper T cell activation. It blocks the secretion of pro-inflammatory mediators and activation of Th1 cells, which activates cytotoxic T cells. However, it also stimulates antibody mediated humoral immune system (Fig. 1). Since IL-10 has immunoregulatory effect, not immunosuppressive effect, so far its use for the improvement of transplantation outcome has been somewhat controversial[10, 16-22]. However, it is recently reported that by using different kind of immunosuppressive drug such as tacrolimus, or diluting the blood IL-10 concentration with anti-IL-10 antibody injection before antibody mediated immune response initiates, transplantation outcome can be improved[23]. Therefore, here I used a cationic polymer as an IL-10 gene carrier, because of its transient expression trait which might limit IL-10 expression to the certain period that antibody mediated immune response is not yet initiated.

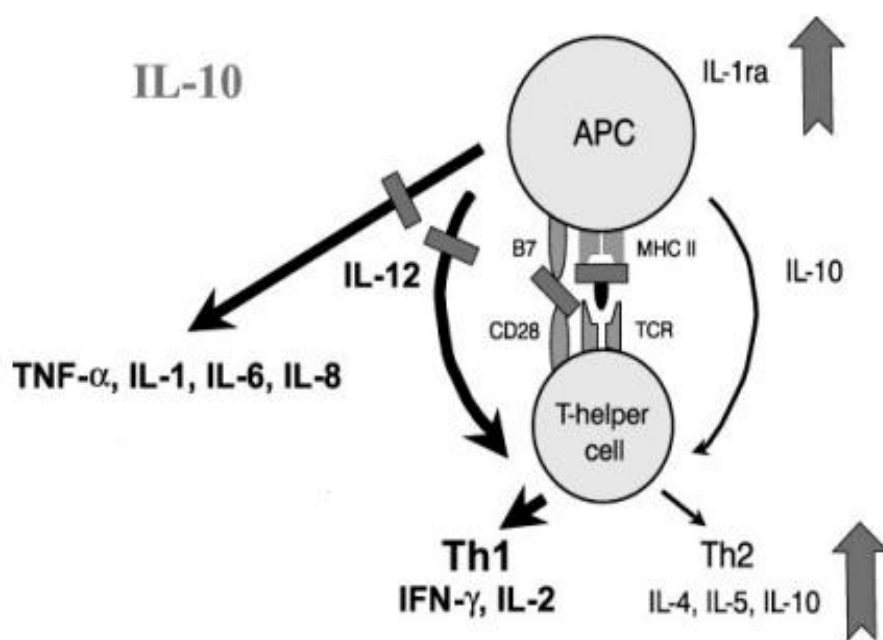


Figure 1. The immunoregulatory mechanism of IL-10 [23].

## 1.5 Gene delivery using polyethyleneimine (PEI)

Polyethyleneimine (PEI) is one of the most frequently used polymeric gene carrier along with polyamidoamine (PANAM). It was first described for gene delivery purpose by Behr et al. in 1995[24]. And 25kDa branched PEI is reported to have the highest transfection efficiency with acceptable toxicity[25]. Similar to other cationic polymers, PEI condenses plasmid DNA into positively charged particles which interact with the anionic cell surface. After they enter into the cell through endocytosis, degradation of plasmid occurs and it is released into the cytosol, allowing the migration into the nucleus. During the endosomal stage of gene delivery, PEI is easily protonated and stimulates the influx of external protons by activating ATPase. While the influx of proton, Chloride ions come in together, making the endosome swell and the rupture of endosome. This is called “Proton sponge effect” and considered as a main reason explaining the reason why the transfection efficiency of PEI is higher than other cationic polymers[24].

Even though the transfection efficiency of polymeric vector is lower than viral vectors, there are more advantages in terms of safety concerns and technical features. For example, PEI has low immunogenicity, no oncogenesis, mass productivity, easy quality control, and the transfection process is simple enough for researchers to carry out. Therefore, I decided to use PEI as a carrier of IL-10 gene delivery.

## 1.6 Formation of pancreatic islet spheroids using hanging drop method

It has been known that gene transfection efficiency into islets is comparably low due to several following reasons. They are cell clusters of approximately 1,000 to 2,000 single cells including  $\alpha$ ,  $\beta$ , and  $\delta$  cells that result in aggregates which have a size of 80 to 400  $\mu\text{m}$  in diameter. Therefore, only few cells in 2-3 peripheral cell layers can be transfected with gene, although non-viral and/or viral gene delivery system are applied[26, 27]. Also, the fact that islets are detached and non-dividing cells leads to the low transfection efficiency[28]. Therefore, pancreatic islet spheroids are considered to improve the efficiency of gene delivery into islets. When pancreatic single cells are transfected and then reaggregated, the total surface area that cells and transfection complex contacts increases and transfected single cells can be located in the center of the spheroids. In addition, it is reported that reaggregated islet spheroids are small in size compared to the original one and this allows the spheroids to overcome the limitation of oxygen and nutrient transport[29], especially to the core part. In normal intact islet culture condition, researchers can easily observed the hypoxic damage of islets in the center part, as time goes by.

Several different techniques have been used for the cell aggregation such as hanging drop method, spinner flask method, plating upon non-tissue culture treated plates method, moulding method, and so on[30]. Among these

techniques, hanging drop method has advantages like size control, morphology control, and increased reaggregation efficiency. Hanging drop method was first used in animal tissue culture to generate primary cultures of fibroblasts from chick heart explant using solid substrata[31]. It is still in use for stem cell differentiation, spheroid preparation in tumor invasion model, and pancreatic islet spheroid formation[13].

## 1.7 Cell to cell communications between pancreatic islet cells

Pancreatic islet cells communicate with each other by exchanging intracellular  $\text{Ca}^{2+}$  ions through gap junctions which consist primarily of connexin-36 proteins. Through this communication, pancreatic islets can coordinate cellular activity and they can review the functional state of their neighbors. Connexin-36 proteins consist of one connexon and two connexons link adjacent cells. When peripheral pancreatic  $\beta$ -cells take up extracellular glucose through GLUT 2 transmembrane transporter, intracellular ATP concentration increases as a result of sequential glucokinase, glycolysis, citric acid cycle, and oxidative phosphorylation process. Increased ATP closes ATP-gated  $\text{K}^+$  channel followed by opening of voltage-gated  $\text{Ca}^{2+}$  channel and thus increase the intracellular  $\text{Ca}^{2+}$  concentration which finally acts as a signaling molecule and stimulates insulin secretion. The role of gap junction is known to be very significant in terms of total islet functionality. It is reported that

when connexin-36 gene is knocked out, insulin gene expression and stimulation index are decreased. However, after restoration of this gene, insulin biosynthesis and insulin secretion are increased[5].

## 1.8 Rationale

Pancreatic islet spheroids are reported to have advantages such as small in size and higher transfection efficiency. Therefore, growing number of researchers are showing interests in forming pancreatic islet spheroids. Here, we formed pancreatic islet spheroids using hanging drop method, because application of this technique in islet research has not been actively done and there is a need to characterize the spheroids using this method in detail. First, I tried to optimize the seeding concentration and incubation time to get ideal pancreatic islet spheroids. The pCMV-SP-IL-10 expression plasmid is delivered into islet single cells by using polymeric gene carrier, 25kDa branched PEI. Then I investigated the yield, functionality, viability, and transfection efficiency of formed pancreatic islet spheroids and compared these with those of intact islet.

I investigated the effects of surface hydrophobicity and droplet contact angle on the formation of islet spheroids. Depending on different contact angles, I hypothesized that the aggregation driving force within droplet changes and this may results in formation of more compactly packed spheroids. To confirm the hypothesis, I compared the viability and

functionality of spheroids formed by two dimensional culture method and hanging drop method with two different surfaces with different hydrophobicity: polystyrene and polydopamine patterned superhydrophobic surface.

## 2. MATERIALS AND METHODS

### 2.1 Materials

#### 2.1.1 Animals

Male outbred Sprague-Dawley rats aged 7 weeks (weighing between 200 -300 g) were used as donors of pancreatic islets. They were purchased from Orient-Bio (Kyunggi, South Korea), and were kept under pathogen-free conditions. All animal procedures were approved by our Institute of Laboratory Animals Resources of Seoul National University.

#### 2.1.2 pCMV-SP-IL-10 plasmid

The IL-10 cDNA was amplified by polymerase chain reaction (PCR) using pCAGGS-IL-10 as a template[32]. The PCR primer sequences are as follows; forward primer, 5'-CCCGCTCGAGATGCCTGGCTCAGCACTGCTA-3', backward primer, 5'-CGACGCGTGGTGTTTGTAGCTTTTCATTTGAT-3'. For cloning convenience, the XhoI and MluI site were introduced to the forward and backward primers, respectively (the enzyme sites are underlined). The amplified IL-10 cDNA was digested with XhoI and MluI and purified by agarose gel electrophoresis and elution. The IL-10 cDNA was inserted into pCI (Promega, Madison, WI, USA) at the XhoI and MluI sites, resulting in construction of pCMV-IL-10. The DNA fragment encoding the

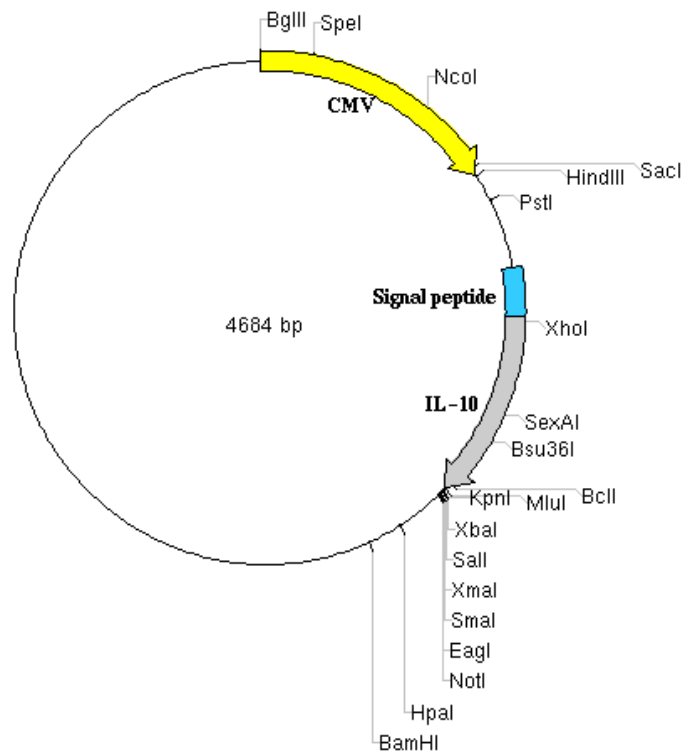


secretion signal peptide was synthesized chemically and inserted into pCMV-IL-10 at the *NheI* sites. ATGCTGCTGCTGCTGCTGCTGCTGGGGCCTGC GCCTGCAGCT is the signal peptide sequence. GAGCCTGGGCCCA. The furin recognition site was located at the 3'-end of the SP cDNA. Therefore, after expression of SP-IL-10 fusion protein, SP may be removed from the protein. The construction of pCMV-IL-10 was confirmed by direct sequencing.

The SP cDNA was inserted in the upstream of the IL-10 cDNA to produce SP linked IL-10. It was previously reported that production of an SP-linked peptide promotes secretion of therapeutic peptide[33]. The SP-linked IL-10 expression plasmid was constructed by inserting DNA encoding the IL-10 secretion SP into pCMV-IL-10 at the *NheI* sites (Fig. 2). After the expression of the SP and IL-10 fusion protein, SP-IL-10 would be processed by digestion by furin in Golgi, producing IL-10.

### 2.1.3 Modification of polydopamine patterned superhydrophobic surface

Following the process previously reported[34], F-silane compound-functionalized anodic aluminum oxide surface was fabricated. First of all, 99.999% pure aluminum surface with the thickness of 0.25 mm was washed



**Figure 2.** The circular genetic map of pCMV-SP-IL-10.

in the acetone by sonication for 5 min followed by ethanol and deionized water rinse. Then it was electropolished with the perchloric acid ( $\text{HClO}_4$ , 70%) and ethanol mixture (1:5 in volume) under 7°C and 15 V for 5 min. Here, aluminum surface worked as an anode and graphite as a cathode. This surface was anodized in 0.9 M phosphoric acid ( $\text{H}_3\text{PO}_4$ , 85%) using 120 V cell voltage and graphite as a cathode for 6 h at 3°C. Porous aluminum oxide layer was removed by immersing the surface in 1.8 wt% chromic acid ( $\text{CrO}_3$ , 99.9%) and 8.6 wt% phosphoric acid mixture for 3 h at 65°C. This textured aluminum surface was then anodized again with the same conditions for 30 min. The pores of aluminum oxide were widened by immersing the surface in 5 wt% phosphoric acid for 30 min at 45°C. This anodic aluminum oxide surface was dried at 50°C overnight followed by 7 min of oxygen plasma treatment. The fluorine compound was evaporated under low pressure and deposited on anodic aluminum surface.

Anodic aluminum oxide (AAO) surface is fabricated by photolithography followed by polydopamine coating as previously reported[35]. After spin-coating of dot-patterned photoresist onto the surface with 3,500 rpm for 35 min, soft banking process was done for 2 min at 110 °C. The dot-patterned photomask was then aligned onto the softbanked surface followed by UV exposure centered at 350 nm for 30 sec. By immersing the surface in the developer (MIF200) for 60 sec, photomask was removed. Then partially UV-exposed surface was immersed in dopamine solution and incubated overnight

for the coating. Following acetone washing and deionized water rinse, the polydopamine patterned superhydrophobic surface was dried under stream of nitrogen. Depending on the patterning, contact angle of drops on the surface can be changed (Fig 3). The general modification process of polydopamine patterned superhydrophobic surface is shown in Figure 4.

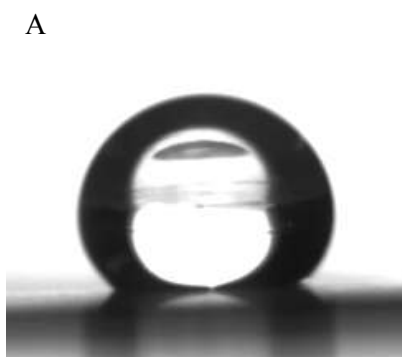
## 2.2 Methods

### 2.2.1 Pancreatic islet isolation

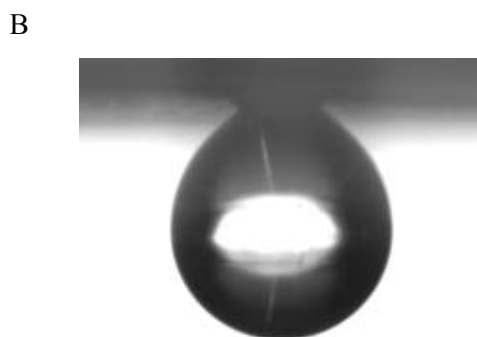
Pancreatic islets were isolated from male Sprague-Dawley rats by collagenase digestion and subsequent Ficoll (Sigma, St. Louis, MO, USA) density gradient purification followed by washing twice with RPMI-1640 culture medium (Sigma) which contains 10% fetal bovine serum (FBS; Sigma), 2mM sodium pyruvate (Gibco-BRL, Grand Island, NY, USA), 23.8 mM sodium bicarbonate (Sigma), 6mM HEPES (Gibco-BRL), 11mM glucose (Sigma) and 1% penicillin/streptomycin (Gibco-BRL). The islets were handpicked on the following day for the further purification and cultured in the culture medium under humidified air containing 5% CO<sub>2</sub> at 37°C.

### 2.2.2 Preparation of PEI/IL-10 gene complex

Transfection complexes were prepared by mixing pDNA encoding either enhanced green fluorescent (eGFP) reporter gene or sp-IL-10 and 25kDa branched PEI in culture medium without 10% FBS. The mixture was then



138.15°



131.8°

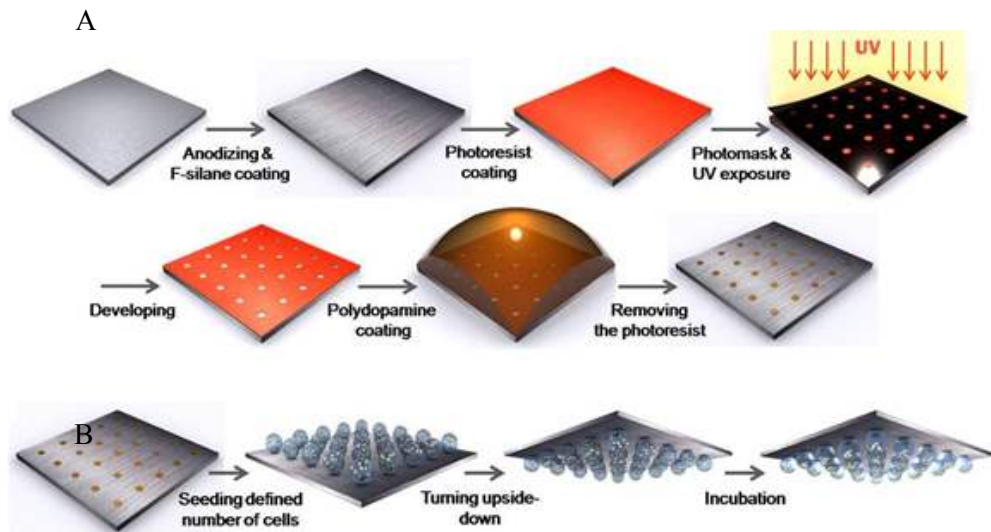


55.3°



59.3°

**Figure 3. Contact angle of droplets placed on the polydopamine patterned surface and normal polystyrene petri dish, respectively. (A,C). The droplets hanging on the same surfaces (B, D).**



**Figure 4. Schematic procedure to prepare the spheroform. The spheroform is fabricated by photolithography followed by polydopamine coating (A). Procedures for spheroid formations on the spheroform surfaces (B).**

incubated for 30 min at room temperature (RT) to form the complex. N (nitrogen of PEI)/P (phosphate of DNA) ratio used in the following experiments was fixed to 5:1.

### 2.2.3 Dispersion of islet single cells

Isolated pancreatic islets (1,000 islets) were dissociated into single cells by treating 0.25% trypsin-EDTA digestive enzyme (Gibco-BRL; 1 ml) and intermittent mild vortexing during approximately 10 min of the process in water bath at 37°C. After trypsinization for 10 min, dissociated islet single cells were washed at least twice with culture media containing 10% of FBS.

### 2.2.4 Gene transfection into islet single cells

Gene delivery into islet cells was carried out after complex of pCMV-SP-IL-10 expression plasmid with branched polyethylenimine (PEI; 25kDa) in culture medium without 10% FBS for 30 min at room temperature. For the transfection, the dispersed islet single cells were seeded in a 12-well culture plate ( $5 \times 10^5$  cells/2 ml/well), and then the pCMV-SP-IL-10/PEI complex (5 µg DNA) was treated into them and incubated in the culture medium without FBS for 6 h under humidified air supplemented by 5% CO<sub>2</sub> at 37°C. After transfection, the single cells were washed twice with culture media in order to eliminate redundant transfection complexes.

Transfection efficiency of PEI non-viral carrier was calculated with

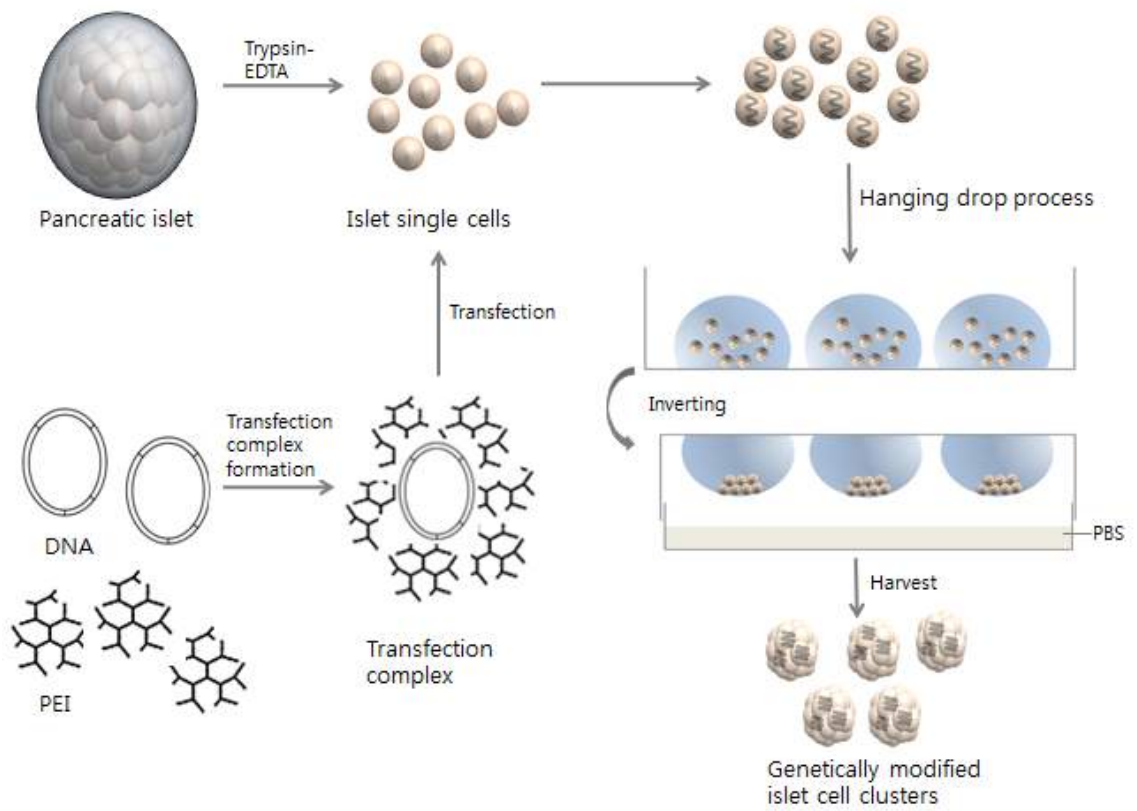
fluorescence- activated cell sorter (BD FACSCalibur™, Franklin Lakes, NJ, USA). To do that, pCMV-eGFP (enhanced green fluorescent protein) expression plasmid was complexed with branched PEI polymer (N/P ratio 5:1; 5µg DNA) and the complex was treated into islet single cells. Simple confirmation of GFP expression was observed with confocal laser scanning microscopy (CLSM; Carl Zeiss-LSM510, Göttingen, Germany).

### 2.2.5 Aggregation of genetically modified pancreatic islet spheroids

Briefly, the different numbers of genetically modified islet single cells were re-suspended in the culture medium (125, 250 or 500 cells/30 µl). The 30 µl droplets were put onto square shaped petri dish (Greiner, Bio IneGmbH, Frickenhausen, Germany). The petri dish was then inverted and incubated in humidified air containing 5% CO<sub>2</sub> at 37°C to form pancreatic islet spheroids. The lower part of each dish was filled with sufficient amount of PBS buffer to prevent the droplet drying during 4-5 days of incubation. The aggregated spheroids in the droplets were harvested and further cultured for following experiments. The general scheme of the experiment is shown in Figure 5.

In case of hanging drop method using polydopamine patterned superhydrophobic surface, seeding concentration was fixed to 500 cells/30 µl which showed high formation yield and regular shape. And the volume of each droplet was 12 µl, because it was the maximum volume that the surface





**Figure 5. Experimental scheme for generating ICCs from intact islets using hanging drop method.**

can hold droplets stably. Further process was same as I described above in spheroids formation using square shaped petri dish.

To form the pancreatic islet spheroids with two dimensional culture method,  $6 \times 10^5$  dissociated islet single cells were culture again in a 12-well plate (Corning, New York, NY, USA) in the incubator containing 5% CO<sub>2</sub> at 37 °C. After 6 days of incubation with intermittent pipetteing, single cells reaggregate spontaneously.

### 2.2.6 In vitro viability of genetically modified pancreatic islet spheroids

The viability of the genetically modified spheroids was investigated with both Live/Dead® cell viability assay (Molecular Probe, Invitrogen Co., Carlsbad, CA, USA) and Cell Counting Kit-8 assay (CCK-8; Dojindo Molecular Technologies Inc., Rockville, Maryland, USA). For Live/Dead® cell viability assay, calcein was stained only in viable cells (green color) and ethidium homodimer-1 was stained only in damaged cells (red color). The stained spheroids were visualized by the CLSM (Carl Zeiss). For CCK-8 assay, 100 µl of cell suspension was (30 spheroids/well) dispensed in 96-well plate and incubated for 24 h in the incubator with 5% CO<sub>2</sub> at 37°C to give time for dehydrogenase secretion. Then 10 µl of CCK-8 solution was added to each well and the plate was further incubated for 4 h. The amount of dehydrogenase secreted from live cell was measured by microplate reader

(Bio-Tek Instrument, Inc., Highland Park, Winooski, VT, USA) with excitation at 450 nm and emission at 590-630 nm. The CCK-8 data were revised by DNA quantification using PicoGreen Quantitative DNA assay kit (Molecular Probe, Invitrogen Co., CA) for compensating size variation of the spheroids.

### 2.2.7 Insulin secretion of genetically modified pancreatic islet spheroids

To evaluate the functionality of the spheroids, glucose-stimulated insulin secretion (GSIS) was carried out. Briefly, the spheroids were seeded on each Millicell® cell culture insert (Millipore, MA, USA) (30 spheroids/insert) and they were pre-incubated in low glucose solution (2.8 mM) in humidified air containing 5% CO<sub>2</sub> at 37°C for 1 h. Then they were further incubated in fresh low glucose solution for 2 h. After incubation, the culture solution was collected and stored at -20°C until further experiment. And then the spheroids were transferred to high glucose solution (28 mM) and continuously incubated for 2 h. Finally, we measured the secreted amount of insulin protein in the low and high glucose solution by rat insulin ELISA kit (EZRMI-BK; Millipore, MA, USA). In addition, stimulation index (SI) values of different concentration of glucose to the cultured spheroids were calculated with the following equation:

$$\text{Stimulation index (SI)} = \frac{\text{Secreted amount of insulin at high glucose solution}}{\text{Secreted amount of insulin at low glucose solution}}$$

## 2.2.8 Transmission electron microscopy (TEM)

To confirm the cell-to-cell interaction within the spheroids, transmission electron microscopy (TEM; JEOL, Tokyo, Japan) was used. Generally, the cell-to-cell interaction in islet is very important for rapid secretion and sensitivity of insulin[5]. Briefly, the pancreatic islet spheroids or intact islets (control group) were fixed with modified Karnovsky's fixative containing 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate buffer with pH 7.2 at 4°C for 2-4 h followed by washing three times with the same buffer. In post-fixation step at 4°C for 2 h, 1% osmium tetroxide in 0.05 M sodium cacodylate buffer with pH 7.2 was used. The samples were washed again with distilled water at room temperature for two times briefly. Then 0.5 % uranyl acetate was used at 4°C for 30 min for en bloc staining. After dehydration and transition with 100 % propylene oxide at room temperature for 15 min twice, the samples were infiltrated with the propylene oxide and Spurr's resin mixture. For the polymerization of the block, samples were kept at 70°C for 24 h. The blocks were sectioned with ultramicrotome (MT-X, RMC, Tuscon, AZ, USA) and stained with 2 % uranyl acetate for 7 min and Reynolds' lead citrate for 7 min again. After the whole process, samples were observed with TEM.

### 2.2.9 Connexin-36 immunostaining

1% Agarose, cell culture grade, in isosmotic PBS for the fixation of spheroids and intact islets. Embedded cells were kept in 10% paraformaldehyde for at least 24 h. The agar blocks were embedded again in paraffin and then sectioned in the microtome, Probe-On-Plus Slides (Fisher Scientific) at thickness of 4  $\mu\text{m}$ . The sections were deparaffinated in xylene and dehydrated in 100%, 95%, 85%, 70%, and 0% alcohol. Then peroxidase blocking was done with 0.3%  $\text{H}_2\text{O}_2$  followed by microwave antigen retrieval in 0.01 M citrate buffer at pH 6.0. After non-specific binding blocking with 4% Bovine serum albumin and PBST, rabbit anti-rat connexin-36 antibody (Biorbyt, orb15452) was treated for 60 min. (1:200). Then goat anti-rabbit Alexa Fluor488 (A11008, Invitrogen) was treated for 30 min. Stained samples were observed using confocal laser scanning microscopy (CLSM; Carl Zeiss-LSM510).

### 2.2.10 IL-10 secretion from genetically modified spheroids

To confirm the IL-10 expression of genetically modified spheroids, we measured the secreted amount of IL-10 in culture medium during culture period. Briefly, genetically modified spheroids were put into each Millicell® cell culture insert (Millipore) and incubated. Culture medium was collected at day 2,4,6, and 8 during cultivation period. For media exchange, the inserts

were transferred every two-day to other wells filled with fresh culture medium. The secreted amount of IL-10 in culture medium was measured by using IL-10 ELISA kit (E90056Mu, Usen Life Science Inc., Wuhan, China).

### 2.2.11 Statistical analysis

All results were expressed as mean  $\pm$  standard deviation (SD). Statistical analysis by Student's t-test or one-way analysis of variance (ANOVA) with Tukey's HSD (Honestly Significantly Different) test (SigmaStat 3.5, Systat Software, San Jose, CA, USA) was evaluated with  $P < 0.05$  significance.

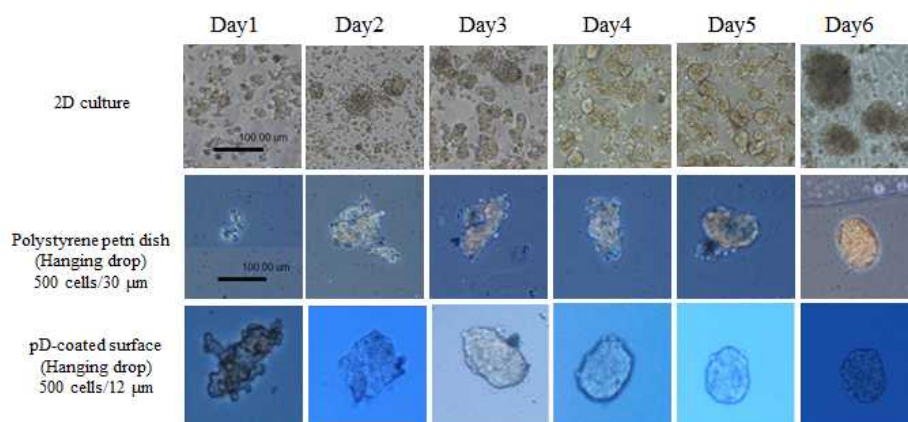
### 3. RESULTS

#### 3.1 Preparation of islet spheroids from pancreatic islets

After isolation and purification by handpicking on the following day, rat pancreatic islets were dissociated by treating 0.25% trypsin-EDTA and the viability of single cells was above 90% which was confirmed by Trypan blue (Sigma) exclusion test.

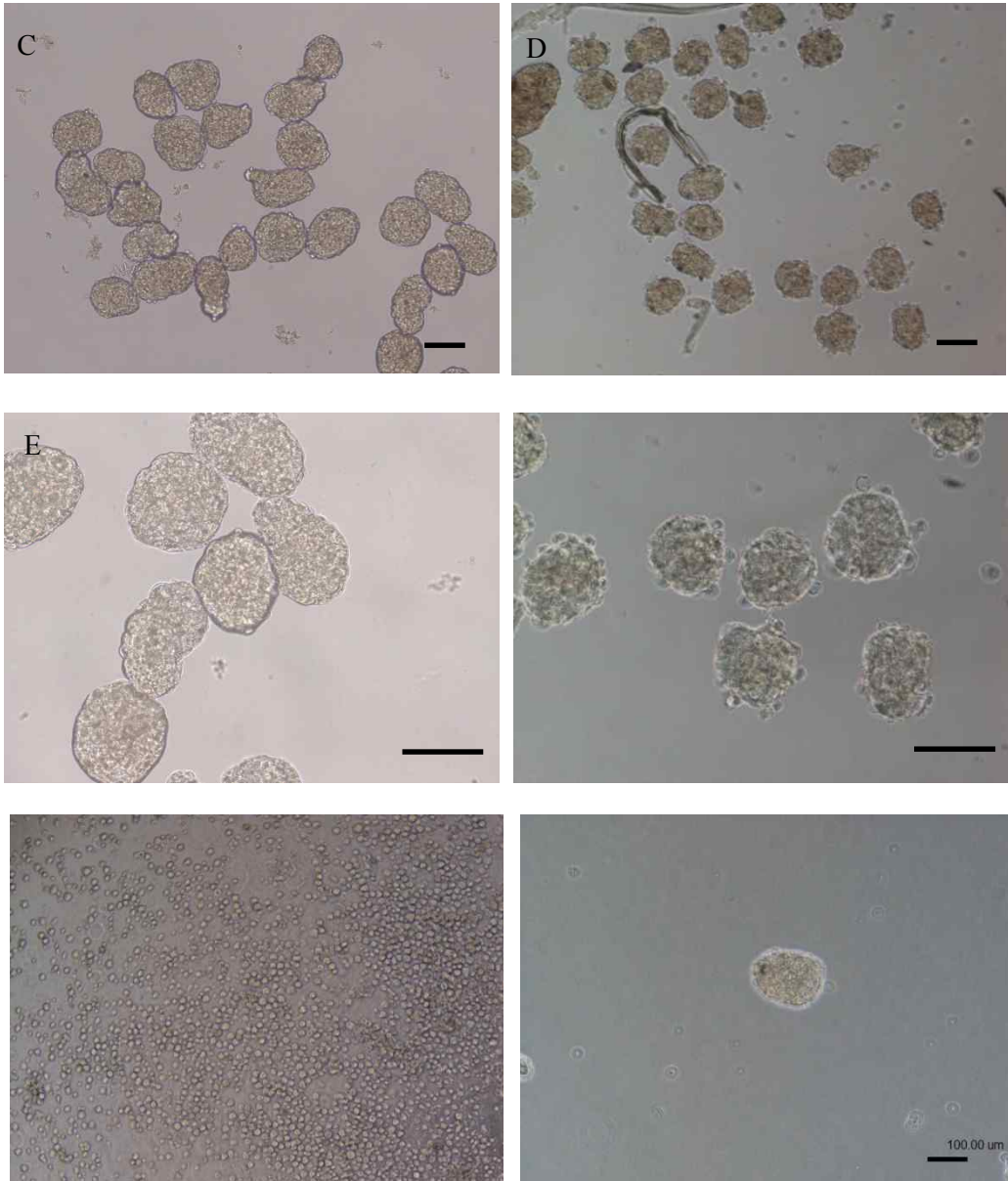
The hanging drop process took 7 days in case of polystyrene surface and 4 days in polydopamine patterned superhydrophobic surface (Fig.6). In both cases, compactly reaggregated pancreatic islet spheroids with standardized morphology were obtained (Fig.7). Rat pancreatic islets and the spheroids show no remarkable differences regarding the general morphologic aspect. Cell borders were clearly distinguished and peripheral cells formed continuous and smooth boundaries. And, composite cells located inside were closely packed.

The size distribution was calculated for three different seeding densities; 125, 250, and 500 cells per each drop (30  $\mu$ l). The average diameter was calculated as an average of the longest and the shortest diameter of the spheroids. And the results of obtained spheroids with each density were 53.75, 60.29, and 72.07  $\mu$ m, respectively. The average diameters of the spheroids with 125 and 250 cells per drop condition were similar to each other, whereas the spheroids with 500 cells showed statistical difference compared to other



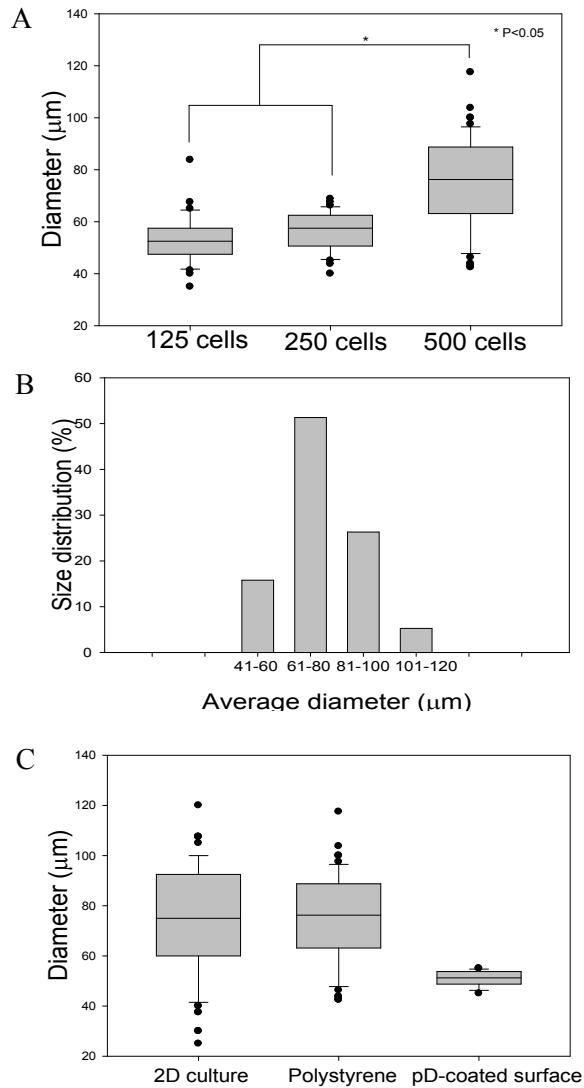
**Figure 6. Morphology of the spheroids formed with three different formation process; two dimensional culture method, hanging drop method on polystyrene petri dish and on polydopamine patterned superhydrophobic surface depending on incubation time.**





**Figure 7. Morphology of pancreatic islet spheroids. After cell clustering with 500 cells/30  $\mu$ l using hanging drop method on polystyrene surface (A, C). 200 cells/12  $\mu$ l using hanging drop method on polydopamine patterned superhydrophobic surface (B, D). Photos of the spheroids with magnification of  $\times 100$ , and  $\times 200$ . (E) Pancreatic islet single cells and (F) intact islet with magnification of  $\times 100$ . Scale bar = 100  $\mu$ m**

two groups (Fig.8A). We concluded from this result that density of 500 cells per drop is necessary for forming more compact and stable spheroids. Therefore, all experiments below were done with the condition of 500 cells per drop. In this optimal condition, diameters of about 90% of formed spheroids were in the narrow range from 81 to 120  $\mu\text{m}$  (Fig.8B). Compared to the size distribution results of the spheroids formed using two dimensional culture method, the spheroids formed with hanging drop technique showed narrow size range. And depending on type of surface that is used for hanging drop method, more precisely depending on the contact angle of droplets, size range changed. It is proven that as contact angle of hung droplets increases, the size distribution of formed spheroids tend to be decreased (Fig.8C).

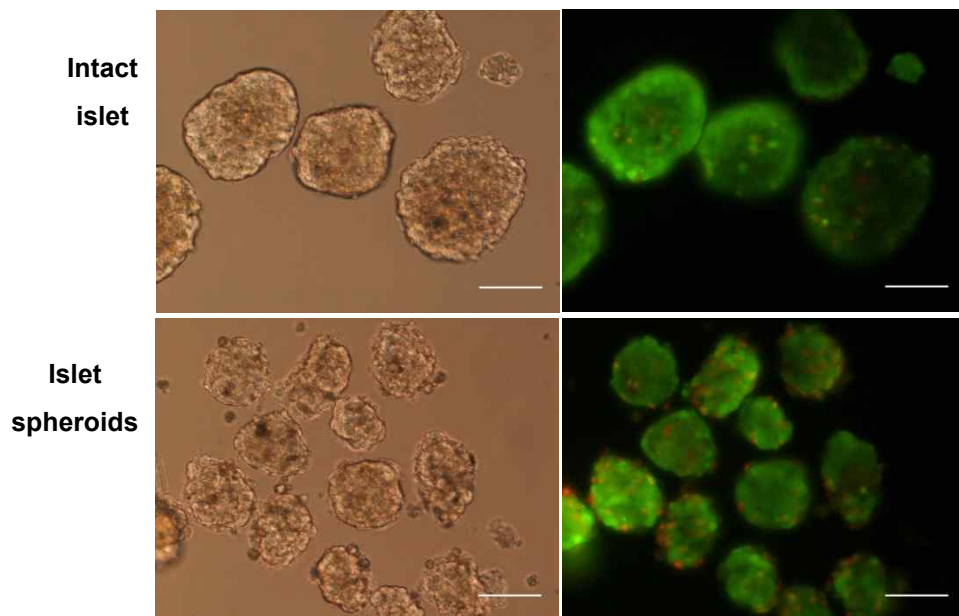


**Figure 8. Size distribution of pancreatic islet spheroids. (A) Size comparison of the spheroids formed with hanging drop method on polystyrene dish. Cell seeding densities were 125, 250, and 500 cells/30  $\mu$ l, respectively. (B) Size distribution of the spheroids formed with 500 cells/30  $\mu$ l condition with hanging drop technique on polystyrene surface. (C) Size distribution of the spheroids formed with 3 different conditions; two dimensional culture method, polystyrene surface, pD-coated surface, respectively.**

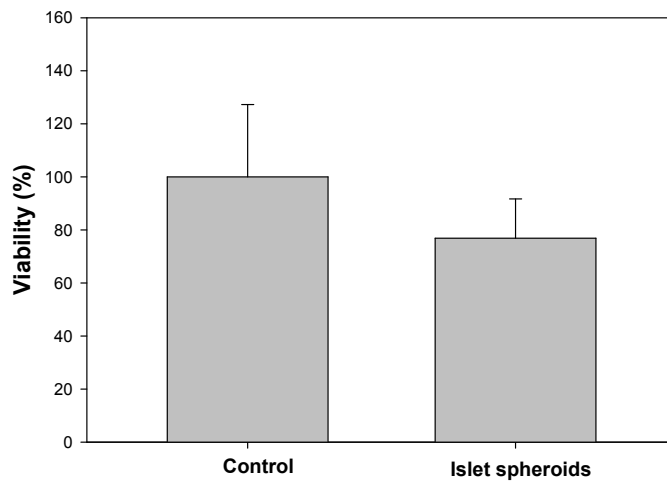
### 3.2 Characterization of genetically modified pancreatic islet spheroids

During the hanging drop process, cells need to be cultivated for 6-7 days in drops without media change in case of the process with polystyrene surface. It is possible that this long cultivation in the same culture media might damage the viability of the spheroids. Therefore, cell viability of both spheroids and control islets incubated for 7 days were evaluated. In Live/Dead® cell viability assay, some dead cells were appeared among the spheroids. Also, control islets were partly damaged during 7 days of incubation and showed some dead cells (Fig.9). To quantitatively analyze the cell viability, we carried out CCK-8 analysis which was revised by DNA quantification. Compared to control group, the relative cell viability of the spheroids were  $71.90 \pm 7.39\%$  (Fig.10). From this result, we concluded that cultivation environment of hanging drop method with no media change for 6-7 days and suspended position did not greatly affect the viability of the spheroids. Also, according to the Live/dead® cell viability assay, spheroids formed with hanging drop method using pD-coated surface were not damaged during 4 days of incubation (data not shown).

To check if the hanging drop process influences the functionality of the spheroids, we investigated the insulin secretion ability. Measurement of insulin secretion by in vitro revealed that spheroids formed using two dimensional culture method and hanging drop method exhibited a decreased

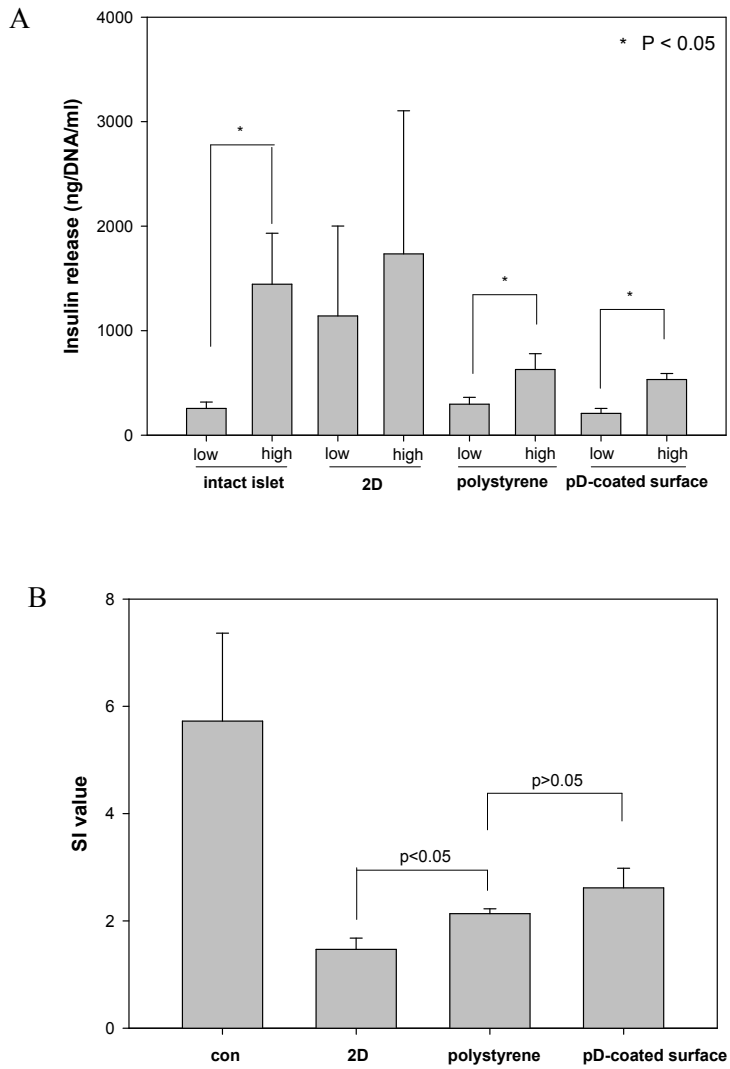


**Figure 9. Viability of intact islets incubated for 7 days and the spheroids formed with 500 cells/30 $\mu$ l. Intact islets and the spheroids treated with calcein AM (green) and ethidium homodimer-1 (red) for the determination of live and dead cells. Photographs were obtained under fluorescence microscope. Magnification =  $\times 200$ . Scale bar = 100  $\mu$ m.**



**Figure 10. CCK-8 assay of intact islets incubated for 7 days and the spheroids with 500 cells/30  $\mu$ l condition for quantitative viability analysis. The difference between two groups is not statistically significant. ( $P=0.428$ ).**

secretion activity in response to high glucose stimulation and increased secretion activity in response to low glucose stimulation (Fig.11A). In case of islet spheroids formed using hanging drop method with polystyrene surface or polydopamine patterned superhydrophobic surface, the insulin secretion response depending on glucose stimulation was statistically different, similar to the result of intact islets. However, the spheroids formed with two dimensional culture method didn't show the statistical difference between secreted insulin amount in low and high glucose solution. This is considered to be due to insufficient formation of gap junctions between single cells and not efficient cellular communication between them as a result. Although it is comparably lower than responsiveness of intact islets, data suggested that the spheroids formed by hanging drop method appear to recover the functionality which was greatly damaged in two dimensional culture process. To evaluate the responsiveness quantitatively, I calculated SI index of each samples (Fig.11B). Compare to SI index of two dimensional cultured spheroids, that of the spheroids formed with hanging drop method showed higher value. However, different from my hypothesis, SI index between polystyrene and polydopamine patterned superhydrophobic surface group didn't show statistically meaningful difference.



**Figure 11. Insulin secretion of intact islet and pancreatic islet spheroids.**

**(A) Glucose stimulated insulin release of intact islets incubated for 7 days, spheroids formed with 2D method, spheroids formed with hanging drop method on polystyrene surface, and on polydopamine patterned superhydrophobic surface. \*  $P < 0.05$ .**

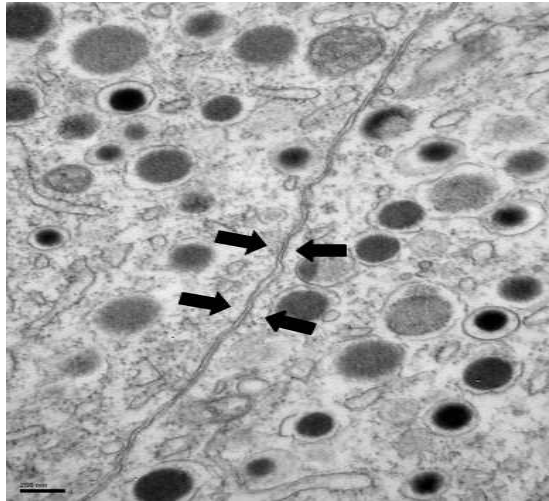


### 3.3 Cell to cell interaction of genetically modified spheroids

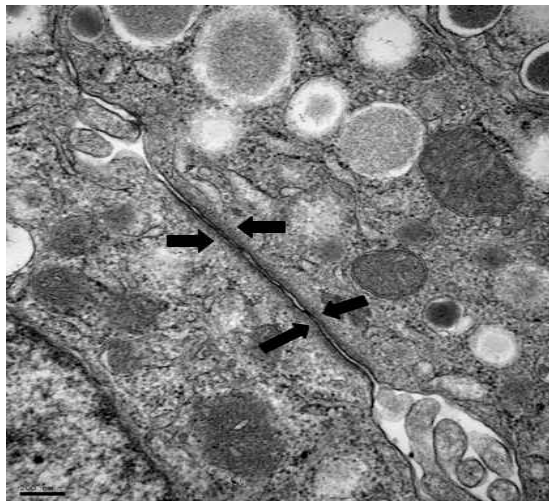
Newly formed gap junctions in pancreatic islet spheroids were confirmed by both TEM analysis and connexin-36 staining. Through the observation of narrowed down and 'lip-like' regions between single cells which are marked with black arrow heads, we can confirm the existence of gap junctions in both cases of the spheroids and intact islets (Fig.12). Cellular communications between islet single cells are known to be done by exchanging calcium ions through these gap junctions. And each of these gap junctions consists of 6 connexin proteins that form the transmembrane channel. Previous studies have shown that the presence of gap junctions between pancreatic  $\beta$ -cells is required for proper secretion profile of insulin [26, 36].

To confirm the existence of gap junction prominently, connexin-36 immunostaining was done. Green fluorescence indicates connexin-36 and blue one shows nucleus (Fig 13). All samples seemed to have connexin-36 proteins, but compared to spheroids prepared with two dimensional culture method, those with hanging drop method showed brighter green fluorescence. From this result, I concluded that hanging drop method, especially using polydopamine coated surface which can provide more three dimension-like culture, is ideal for islet single cells to form more compact spheroids and this enables to make more gap junctions among each other.

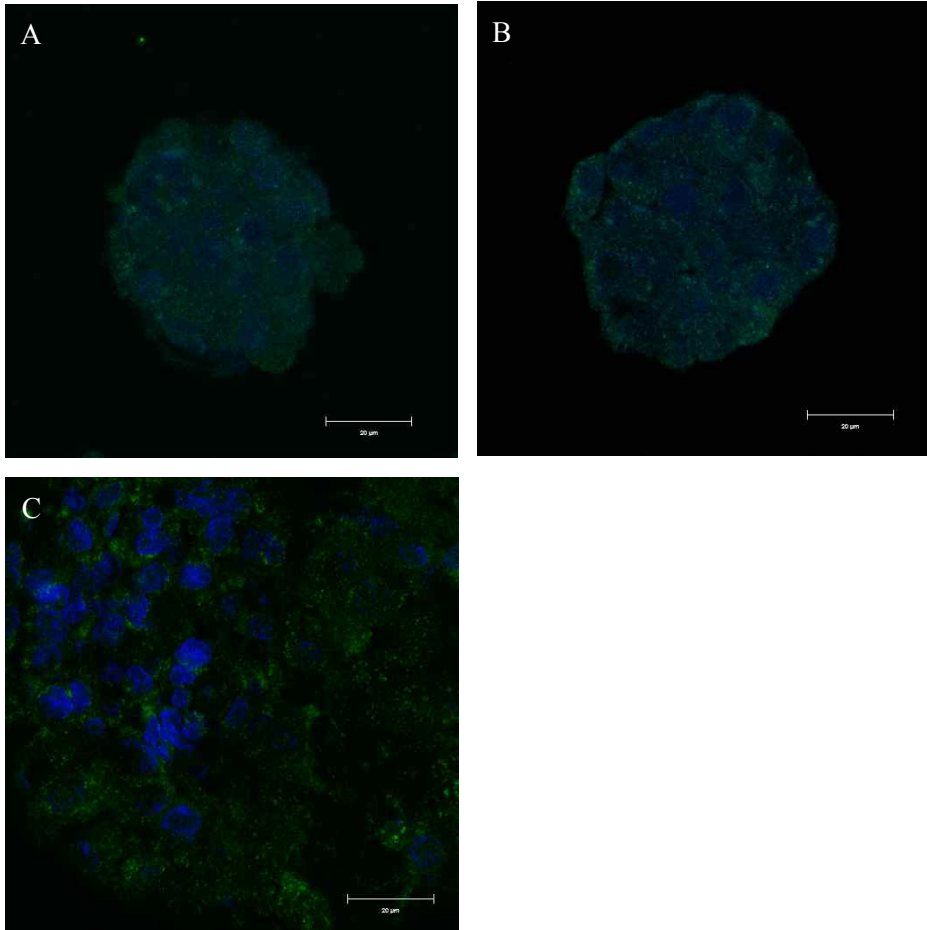
A



B



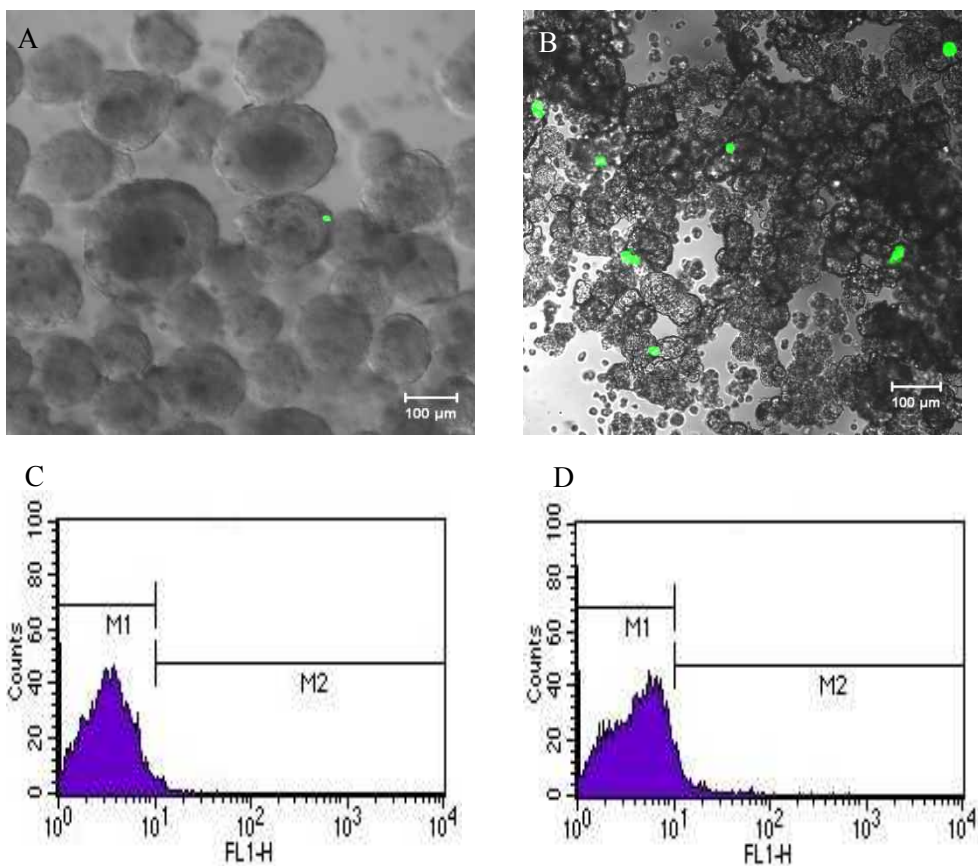
**Figure 12. Transmission electron microscope (TEM) image of gap junctions. Magnification  $\times 60,000$ . Scale bar = 200 nm. (A) intact islet. (B) pancreatic islet spheroids formed by hanging drop method**



**Figure 13. Immunostaining of intact islet and pancreatic islet spheroids formed with two dimensional culture, hanging drop on polystyrene or polydopamine patterned superhydrophobic surface. Each sample is stained with FITC conjugated connexin-36 antibody and DAPI.**

### 3.4 Efficiency of PEI-mediated gene delivery

The transfection efficiency of pancreatic islets is comparably low, because they grow in suspension culture and they are non-dividing primary cells. It is well known that surface area of suspension cells that contacts with carrier particles is smaller than adherent cells. Also, since they are non-dividing cells, they don't progress through the cell cycle which normally allows increased insertion of exogenous DNA when nuclear membrane disappears in mitosis. Therefore, whether using viral or non-viral strategy, transfection of islets is broadly known as a very difficult work. To investigate the transfection of intact islets and islet spheroids formed after single cell transfection, we delivered eGFP plasmid by PEI with same condition. According to confocal microscopy, we could hardly recognize the expression from intact group (Fig 14A). On the other hand, expression of islet spheroids group could be easily confirmed (Fig 14B). To quantitatively analyze the transfection efficiency, we did FACS analysis. In case of intact islet, the transfection efficiency was  $1.54 \pm 0.04\%$  (Fig. 14C). The efficiency of islet spheroids which were reaggregated after transfection in single cell state was  $6.75 \pm 0.21\%$  (duplicated) (Fig. 14D). We speculated that this great improvement was due to the increase of total surface area that contacts to DNA-carrier complex. This played an important role in enabling approximately 4.5-fold higher efficiency of islet spheroids compare to that of intact islets. Therefore, hanging drop method is considered as an

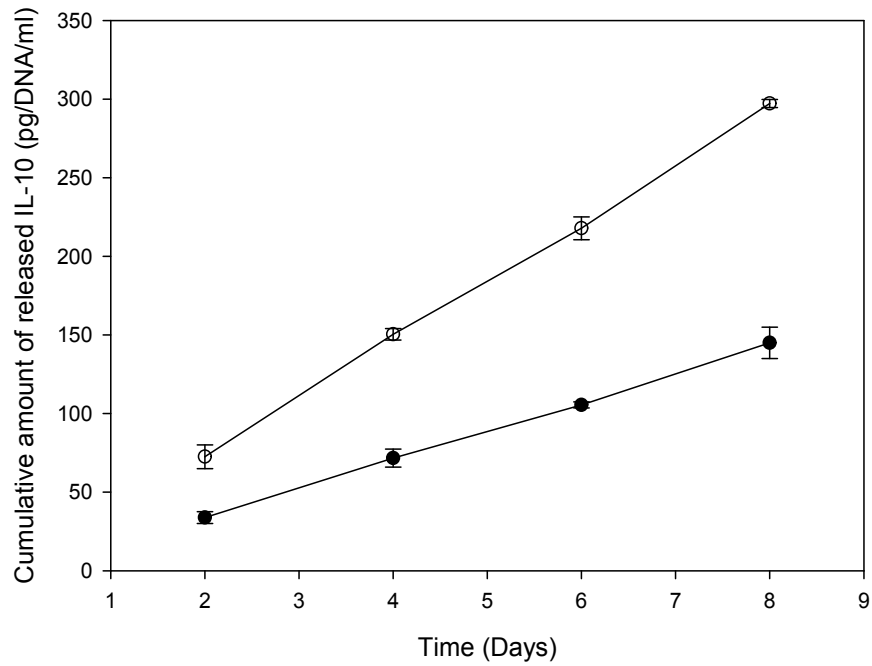


**Figure 14. Transfection efficiency of PEI-mediated gene delivery into intact islets and islet spheroids which are formed after single cell transfection. Confocal laser scanning microscope (CLSM) image of intact islets (A) and islet spheroids (B) formed after PEI mediated gene delivery. FACS analysis of the same groups, respectively; intact islet (C), islet spheroids (D)**

advantageous technique, because it is possible to enhance transfection efficiency.

### 3.5. IL-10 secretion profile from genetically modified ICCs

As a representative immunosuppressive cytokine, delivery of IL-10 gene to improve transplantation outcome is under active investigation[10]. Therefore, we tried to deliver IL-10 gene with polymeric carrier, PEI and released amount of IL-10 from transfected intact islet and islet spheroids formed by hanging drop method after single cell state transfection was compared. The release profile of each group was observed up to 8 days with media change every other day and revised by DNA quantification. Accumulation of IL-10 increased gradually throughout the 8 days of experiment in both groups (Fig. 15). The total amount of released IL-10 in case of islet spheroids group was about twice than that of intact group. This is because of the different transfection efficiency between the two groups which was about 4-fold difference.



**Figure 15. Cumulative IL-10 secretion profiles of transfected intact islets and ICCs which were revised by DNA quantification. Black circle indicateds released amount of IL-10 from transfected**

## 4. DISCUSSION

The pancreatic islet cell transplantation for the treatment of type 1 diabetes mellitus has been widely adapted around the world, since Dr. James Shapiro and colleagues reported what is now known as the Edmonton protocol. With the use of sirolimus, tacrolimus, and daclizumab, 7 consecutiverecipients achieved euglycemia. It has been recently reported that 50-68% of recipients did not need additional insulin after transplantation, but only 10% of recipients could live independent from insulin, 5 years after transplantation. Therefore, there is still long way to improve the islet transplantation procedure.

Islet spheroids are considered to have some advantage over intact islets, in terms of improved viability, because of its small size. Among several different techniques to form islet spheroids, hanging drop method has not been deeply investigated. In this study, I tried to figure out the optimal conditions of hanging drop methods for making islet spheroids and investigate them at first. And then by changing the strength of driving force that enables single cells to aggregate, I tried to investigate the influence of physical strength on the performance of islet spheroids.

Regardless of the type of surface, the size and morphology was controlled in case of islet spheroids formed using hanging drop method. And this is



meaningful in terms of forming islet spheroids with expected size with narrow size distribution and further application for various purposes such as microencapsulation[29]. I decided the seeding concentration of 500 cells/30  $\mu$ l as the optimal one, because size difference of islet spheroids formed in lower concentrations was not statistically significant. This is probably due to the certain amount of force that is required between composite islet single cells. In the lower concentration conditions, it is likely that driving force is not enough for all single cells to form the aggregate. From this, I hypothesized that by increasing the driving force within the droplets, single cells may form more tightly packed spheroids which allow them to function better. To provide stronger driving force to cells, I used polydopamine patterned superhydrophobic surface which can hold water droplets with bigger contact angles and this results in stronger force.

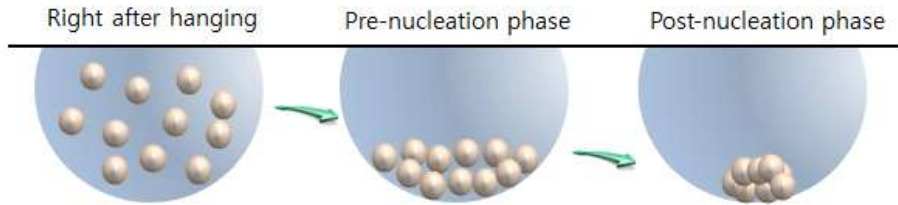
The normal insulin secretion pattern inside the body is pulsatile, as a result of communication of islet single cells by exchanging  $\text{Ca}^{2+}$  ions through gap junctions which consist mostly of connexin-36 proteins. So far, many researchers mentioned connexin-36 as a very important protein for the biosynthesis and release of insulin[5]. A lot of experiments have been carried out to improve cell to cell communications either by chemically or biologically enhancing the expression of connexin-36 gene. However, there are very a few research papers dealing with this issue in physical point of view.

It was reported that proteins undergo three phases in crystallization

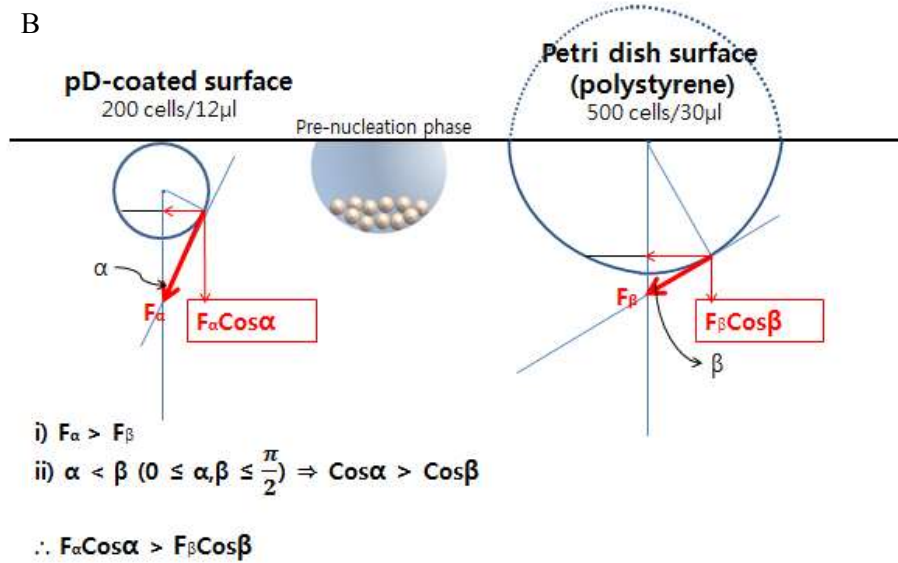
process; dispersed phase, pre-nucleation phase, and post nucleation phase[37]. The driving force for cell aggregation plays the most important role in pre-nucleation phase. Depending on different contact angles, the force that each single cells within droplets changes. In case of droplets hanging on polydopamine coated superhydrophobic surface, cells aggregate thicker and as a result, more number of cells locate higher position. The force that cells get within the droplets is same as those on the slope with a certain contact angle of each location. If the weight of a single cell is  $m$  and the contact angle is  $\alpha$ , the force that single cell gets is  $mg\sin\alpha$ . If  $0 \leq \alpha, \beta \leq \frac{\pi}{2}$ ,  $\sin \alpha$  is bigger than  $\sin \beta$ . Since more number of cells in case of polydopamine coated surface are located with bigger contact angle than normal polystyrene petri dish, total driving force within droplets hanging on the polydopamine coated surface is stronger (Fig. 16).

Even though the final functionalities of islet spheroids formed on two different surfaces with different contact angles were similar to each other, this research was still meaningful as a first step toward the further investigation.

A



B



**Figure 16. Three phases of islet spheroids formation (A). Influence of microgravity on pre-nucleation phase (B).**

## 5. CONCLUSION

In this study, I investigated optimal conditions of forming islet spheroids using hanging drop method such as seeding concentration, incubation days, and etc. Since hanging drop method has not been actively investigated in the formation of islet spheroids, I also investigated the influence of the method on morphology, size distribution, viability, functionality, and gap junction formation. I confirmed that transfection efficiency of PEI increased up to approximately 4 times by transfecting islet single cells and then reaggregate them into islet spheroids. By doing this, I could deliver DNA without safety concerns such as immunogenicity or oncogenesis which is commonly accepted limitation of viral vectors. At the same time, I could overcome the limitation of low transfection efficiency which is one of the biggest problems in case of polymeric carriers.

According to the result of IL-10 expression in my study, the secretion was maintained continuously throughout the experiment. This pattern seems quite different from generally known expression pattern of PEI-mediated gene delivery which shows a peak on day 3 and decreases slowly after that. This difference might be caused by the difference of the gene source. In my study, I used signal peptide tagged gene which has greater expression compared to intact gene.

And by adapting surfaces with different contact angles, I tried to improve the functionality of islet spheroids. My hypothesis was that single cells will form more gap junctions within droplets hanging on the surface with greater contact angle, because they will get bigger driving force. According to the results, there was not significant improvement in functionality in normal polystyrene surface with smaller contact angle and polydopamine patterned superhydrophobic surface with greater one.

Until now, only a few researches have been done to investigate the physical influence on the formation and functionality of islet spheroids. Therefore, this research is meaningful as a stepping stone.

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## 7. 국문초록

제 1형당뇨의 효과적인 치료 방법으로 췌장소도세포이식이 주목을 받고 있다. 하지만 면역 거부 반응을 포함한, 이식 후의 해로운 상황 때문에 이식된 췌장소도세포가 그 기능을 상실하여 제대로 된 혈당 조절을 할 수 없는 상태가 된다. 이것을 해결하기 위한 방법으로 많은 연구가 진행되었고, 그 중에서도 이 논문에서는 유전자 전달과 변형 췌장소도세포에 대해 연구하였다.

췌장소도세포에 전달한 유전자는 interleukin-10으로, 선행 연구에 의하면 cytokine은 면역 조절 작용이 있기 때문에 이식 후 면역 거부 반응을 막는 용도로 쓰여 왔다. 본 연구에서는 polyethyleneimine이라는 양이온성 고분자 전달체를 이용하여 췌장소도세포에 유전자를 도입하여 그 분비량을 측정하였다.

일반적으로 췌장소도는 유전자 전달 효율이 매우 낮은 것으로 알려져 있는데 이를 개선하기 위해 췌장소도를 먼저 단일 세포로 만들고 그 상태에서 유전자 전달을 한 후, hanging drop 기술을 이용하여 다시 변형 췌장소도를 형성하였다. 먼저, hanging drop 기술을 이용한 변형 췌장소도 형성 조건을 최적화하였고, 이 과정이 세포에 미치는 기본적인 영향을 조사하였다. 이것을 바탕으로 접촉 각이 서로 다른 두 가지의 표면을 이용하여 만들어진 변형 췌장소도의 기능성을 비교하였다. 이 실험에서 쓰인 표면은 소수성에 큰 차이가 있기 때문에 물 방울의 접촉 각이 달라지므로 단일 세포를 묻치는데 필요한 미

세중력의크기가달라질것이다. 이는점점더 3D 입체에가까운상태에서 단일세포를몽치는환경을조성할수있고, 이것은세포간극의형성에도움을줄수있기때문에형성된변형채장소도의기능성에도영향을미칠것이라는가정하에실험을하였다. 접촉각이클수록변형채장소도가형성되는시간이줄어들었지만, 기대한바와는달리같은 hanging drop 기술의경우접촉각의차이와기능성의차이가비례하기는하지만통계학적으로의미있는상관관계를찾을수는없었다.

하지만, 아직물리적인환경의차이와변형채장소도의기능성의관계에대한연구가거의없는실정이고, 이론적으로보았을때접촉각이클수록 더욱조밀한변형채장소도를형성할수있는가능성이있기때문에, 본연구는디딤돌로서의역할을기대할수있다.

주요어: 채장소도세포

변형채장소도

Hanging drop 기술

폴리도파민코팅한초소수성표면

폴리에틸렌이민

인터류킨-10