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GLP-1 Could Improve the Similarity of IPCs and Pancreatic Beta Cells in Cellular Ultrastructure and Function

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ABSTRACT

Transplantation of functional insulin-producing cells (IPCs) provides a novel mode for insulin replacement, but is often accompanied by many undesirable side effects. Our previous studies suggested that IPCs could not mimic the physiological regulation of insulin secretion performed by pancreatic beta cells. To obtain a better method through which to acquire more similar IPCs, we compared the difference between IPCs of the GLP-1 group and IPCs of the non-GLP-1 group in the morphological features in cellular level and physiological function. The levels of insulin secretion were measured by ELISA. The insulin and glucagon-like peptide-1 (GLP-1) mRNA gene expression was determined by real-time quantitative PCR. The morphological features were detected by atomic force microscopy (AFM) and laser confocal scanning microscopy (LCSM). Intracellular Ca^{2+} levels and Glucagon-like peptide-1 receptor (GLP-1R) levels were determined by flow cytometer (FCM). We found that IPCs of the GLP-1 group had bigger membrane particle size and average roughness (R_a) than IPCs of the non-GLP-1 group but still smaller than normal human pancreatic beta cells. The physiology function of IPCs of the GLP-1 group were much closer to normal human pancreatic beta cells than IPCs of the non-GLP-1 group. GLP-1 could improve the similarity of IPCs from human adipose tissue-derived mesenchymal stem cells and pancreatic beta cells in cellular ultrastructure and function. J. Cell. Biochem. 114: 2221–2230, 2013.

KEY WORDS: INSULIN-PRODUCING CELLS; HUMAN NORMAL PANCREATIC BETA CELLS; ATOMIC FORCE MICROSCOPY; GLUCAGON-LIKE PEPTIDE-1

Diabetes mellitus (DM) is a degenerative disease which caused by selective destruction of pancreatic beta cells. Type 1 diabetes results from autoimmune destruction of insulin-producing beta cells [Herrath et al., 2007]. In type 2 diabetes, enhanced insulin secretion of beta cells can initially compensate obesity-related insulin resistance. However, in many type 2 diabetic individuals, these compensatory mechanisms are impaired, which lead to dysfunction and loss of insulin-producing pancreatic beta cells, culminating in the hallmark of DM [Kahn et al., 2006]. As stated by the WHO, there are 346 million individuals attacked by DM in the worldwide [Scully, 2012], and there is still no cure for diabetes. Hence, it is crucial to develop a novel therapy for DM. One of the current therapies for DM is to replace the destructive pancreatic beta cells by functional insulin-producing cells (IPCs). Although, the potential of embryonic stem cells or adult stem cells to differentiate into IPCs remains a feasible area of research [Soria et al., 2000; Assady et al., 2001; Lumelsky et al., 2001; Hori et al., 2002; Peek et al., 2004], there are still many undesirable side effects need to be disposed.

Qiping Shi and Simin Luo contributed equally to this study.

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Glucagon-like peptide-1 (GLP-1) is an incretin hormone with multiple functions, including the promotion of cell neogenesis, cytoprotection, and proliferation in several tissues [Drucker, 2003]. It has been proven that GLP-1 has many advantageous effects on the endocrine pancreas. GLP-1 can stimulate insulin and somatostatin secretion and inhibit the release of glucagon [Drucker, 2002]. Although the biological actions and signaling pathways of GLP-1 have been studied widely in isolated tissues, established cell lines, and in vivo, less is known about the effects, if any, of GLP-1, on the differentiation of stem cells into IPCs. Accordingly, we used mesenchymal stem cells from human adipose tissue to study the action of GLP-1 during differentiation into IPCs.

Atomic force microscopy (AFM) has helped overcome the disadvantages of conventional optical microscopes and electron microscopes (including scanning [SEM] and transmission [TEM] types), and has provided a means to capture three-dimensional images of surface morphology. However, morphological information, including roughness and volume, obtained by AFM are frequently affected by different kinds of artifacts, which include the microscope system itself, imaging mode, and external factors [Chen et al., 1998; Méndez-Vilas et al., 2002; Chen and Cai, 2006; Girasole et al., 2007]. One of the key components of the microscopic system is the AFM probe. The information obtained by AFM and the resolution of images strongly depend on the tip or probe used, whose factors mainly include surface topography of the scanning probe [Kitching et al., 1999] and asymmetric cantilever tips [Kaupp et al., 1995].

It is widely accepted that cellular structure is related to its function. We hypothesized that the differences in cell morphology between IPCs of the non-GLP-1 group and the beta cells may be the reason why IPCs of the non-GLP-1 group could not mimic physiological regulation of insulin secretion performed by pancreatic beta cells (Supplemental Material available online with this article; DOI: 10.1186/1556-276X-8-9023421382). Therefore, we compared the differences in morphology and function between normal human pancreatic beta cells and IPCs with or without the use of GLP-1. At the molecular stratum, we found that although both two group of IPCs could not release insulin match normal human pancreatic beta cells within the same stimulated condition, the cellular ultrastructure and physiology function of IPCs of the GLP-1 group were much closer to the beta cells than IPCs of the non-GLP-1 group, IPCs of the GLP-1 group could express more GLP-1R and have bigger membrane particles and R_a than IPCs of the non-GLP-1 group. Our findings propose that even though there was still so much difference between IPCs of the GLP-1 group and normal human pancreatic beta cells, but the use of GLP-1 could improve the similarity between these two kinds of cells no matter in cellular ultrastructure but also in physiological function.

MATERIALS AND METHODS

ISOLATION AND DIFFERENTIATION OF MSCs FROM HUMAN ADIPOSE TISSUE

Informed consent was obtained from participating donors according to procedures approved by the Ethics Committee at the Chinese Academy of Medical Sciences. Experiments were performed according to the ethical standards formulated in the Helsinki Declaration. Adipose tissue (2 g) was resected from the anterior abdominal wall under local anesthesia after making a small left paramedian incision below the umbilicus after obtaining informed consent of each donor.

The adipose tissue was washed with phosphate-buffered saline (PBS) to remove blood cells and local anesthetics. It was minced in 0.1% collagenase I (Sigma) solution and incubated at 37°C while rotating at 35 rpm for 1 h before being centrifuged at 1,200 rpm for 10 min. After aspirating the supernatant, the cells were resuspended in proliferation medium and seeded into T-75 tissue culture flasks at a density of 2×10^6/ml. The cells were cultured at 37°C in 5% CO_2 for 10 days. The medium was changed every other day. The proliferation medium contained DMEM/F12 (Gibco), 10% fetal calf serum (Gibco), and antibiotics (1% penicillin/streptomycin [5,000 U/ml], Sigma). Once adherent cells were more than 70% confluent, they were harvested by 0.25% trypsin (Gibco) and 0.01% EDTA, counted, and checked for viability and sterility. In addition, flow cytometric analysis of cells was performed using antibodies for CD59, CD34, CD44, CD45, CD105, CD13, and HLA-DR (BD Biosciences).

Prior to differentiation into IPCs, the cells were divided into two groups. One group of hADSCs was placed for 6 days in differentiation medium I containing 500 ml IMDM (Hyclone), 2% B-27 (Invitrogen), 10 μg/L EGF (Peprotech), 10 μg/L bFGF (Peprotech) and antibiotics. These cells were then placed for 6 days in differentiation medium II containing 500 ml IMDM (Hyclone), 10 mM nicotinamide (Sigma), 2% B-27 (Invitrogen), 10 μg/L activin A (Peprotech), 10 μg/L betacellulin (Peprotech), 10 μg/L hepatocyte growth factor (Peprotech), and antibiotics.

The other group of cells were placed in differentiation medium I+ 10 μg/L GLP-1 (Peprotech) for 6 days, then placed in differentiation medium II+ 10 μg/L GLP-1 (Peprotech) for 6 days. The concentration of GLP-1 was according to the manufacturer's protocol which was the minimal effective concentration. The cells were expanded in culture at 2×10^4/cm^2.

CULTURE OF NORMAL HUMAN PANCREATIC BETA CELLS

Normal human pancreatic beta cells (HUM-CELL-0058) and expansion medium were obtained commercially (Wuhan Pricells Biotechnology & Medicine Co., Ltd, China). Expansion medium contained MED-0001 and 5 ng/ml rhEGF, 5 μg/ml rhinsulin, 5 μg/ml transferrin, 10 nM T3, 1.0 μM epinephrine, 5 μg/ml Hydrocortisone, 10% fetal bovine serum (FBS).

Fetal pancreases were cut into about 1 mm^3 pieces, and then digested with 1% collagenase type V at a ratio of 1 ml/g of wet pancreas. The cells were centrifuged at 35 rpm for 30 min at 38°C. The supernatant was carefully aspirated, and filtered out with a 150 μm mesh screen before centrifugation at 1,000 rpm for 10 min. The supernatant was aspirated, and the cells were cultured in DMEM/F12 + 20% FBS in T-25 tissue culture flasks at 37°C in 5%CO_2 for 6 h. Then, through gentle pipetting, nutrient solution was collected––a pure islet suspension––and transferred to a 15 ml centrifuge tube. The islet suspension was centrifuged at 1,000 rpm for 5 min. The supernatant as aspirated and the remaining sample was washed once in PBS, and centrifuged at 1,000 rpm for 5 min again. After aspirating the resulting supernatant, the sediment was resuspended in 3 ml 0.125% trypsin, then centrifuged at 35 rpm for 10 min at 37°C.

FBS (1 ml) was then used to terminate digestion. The solution was repeatedly mixed to create a homogenous cells suspension. Ficoll 400 was placed at the ratios of 250, 200, and 110 ml/L in separate 50 ml centrifuge tubes. The islet suspension was then placed slowly added to the separation liquid and centrifuged at 2,000 rpm for 20 min at 4°C. After centrifugation, cells within the interface lying between 11% and 20% were carefully isolated, washed with PBS containing 1% penicillin/streptomycin (5,000 U/ml) once, then centrifuged at 1,000 rpm for 5 min. The supernatant was discarded and, the cells were cultured in complete medium in T-25 tissue culture flasks that have been coated with collagenase at 37°C in 5% CO_2. Culture medium was changed every other day.

INSULIN ASSAY

Both normal human pancreatic beta cells and IPCs were preincubated in either Dulbecco's phosphate-buffered saline (D-PBS, without glucose), low glucose (5.5 mM), or high glucose (25 mM) DMEM (Gibco) for 1 h or 30 min. The buffers from six wells of cells were collected separately and their insulin levels were examined by ultrasensitive insulin enzyme-linked immunosorbent assay (ELISA, Product No.DRE10124; Shanghai Yanji Bio-technology Co., Ltd, China). Results were normalized by the number of cells in each well.

QUANTITATIVE GENE EXPRESSION ANALYSIS

Total RNA was collected from cells using TRIzol reagent (Invitrogen) and were treated with DNase. Total RNA (1 μg) was analyzed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in an ABI 7000 Real-Time PCR System (Applied Biosystems) using the Sybr-Green primers. Real-time PCR was performed using real-time PCR Taq core kit (Takara, China). The volume of the reaction was 50 μl and contained 25 μl Sybr-Green, 16 μl water, 5 μl cDNA, 2 μl sense primer (10 μmol/L), and 2 μl antisense primer (10 μmol/L). PCR conditions were set using manufacturer's protocol. The primers and PCR conditions for analysis of Insulin and GLP-1R expression are listed in Table I Table I. Expression levels were calculated relative to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All primers were from Invitrogen.

AFM MEASUREMENT

Cell samples were preincubated for 1 h or 30 min in D-PBS, low glucose DMEM (Gibco), or high glucose DMEM (Gibco). They were then washed in distilled water twice before being fixed with 2.5% glutaraldehyde for 20 min. The samples were washed in distilled water three times again, then air-dried for AFM scanning.

An Autoprobe CP AFM (Veeco) was used in contact mode to detect the immobilized IPCs and normal human pancreatic beta cells at room temperature. Silicon nitride tips (UL20B, Park Scientific Instruments) were employed in all AFM measurements. An optical microscope was used to help select the desired cells and direct the position of the AFM tip. Single-cell imaging was repeated for six cells, and each cell was scanned for three times. All images were analyzed by the instrument-equipped software (Image Processing Software Version 2.1) to gain information on the topography. "R_a" denotes the average roughness in the analytical area. All parameters were directly generated by the software IP2.1.

INTRACELLULAR CA^{2+} DETECTION

Fluo-3 AM is a fluorescent dye that can traverse the cell membrane and be cleaved into Fluo-3 by intracellular esterase activity. Fluo-3 can combine with Ca^{2+} specifically and has a strong fluorescence with an excitation wavelength of 488 nm. We detected the level of intracellular Ca^{2+} by using Fluo-3 AM. After being exposed to sugar-free medium or high glucose medium for about an hour, cells were harvested, washed three times with PBS, resuspended in Fluo-3 AM (5 μM), and then incubated for 30 min at room temperature in the dark. Detection of intracellular Ca^{2+} was executed by flow cytometry at 525 nm excitation wavelength.

LOCALIZATION OF GLP-1 RECEPTOR PROTEIN

After cells were fixed in 2.5% glutaraldehyde for 30 min and washed in PBS several times, they were treated for 30 min with PBS containing 5% goat serum in order to block non-specific binding. GLP-1 receptor (GLR-1R) was stained for 30 min with a fluorescein isothiocyanate-conjugated rat anti-GLP-1R antibody (Beijing Boi-synthesis Biotechnology CO., Ltd., China) at a 1:100 dilution in blocking solution. After several washes in PBS, localization of GLP-1R was visualized by a laser confocal scanning microscope (LCSM: 510 Meta Duo Scan, Carl Zeiss, Germany) using 545 nm (He-Ne) excitation. Emission was detected above 600 nm.

LEVEL OF GLP-1R PROTEIN DETECTION

The GLP-1 group (cells in media containing GLP-1), non-GLP-1 group (cells in media without GLP-1), and normal human pancreatic beta cells were harvested, washed three times in PBS, resuspended in a solution fluorescein isothiocyanate-conjugated rat anti-GLP-1R antibody (Beijing Boisynthesis Biotechnology CO., Ltd.) at a 1:100 dilution, and then incubated for 1 h at room temperature in the dark. The samples were then washed with PBS twice and resuspended in PBS. The level of GLP-1R was detected by Flow cytometry at 525 nm excitation wavelength. Each group analyzed contained 10,000 cells.

STATISTICAL ANALYSIS

All experiments were conducted at least in triplicate ($n = 3$), and results are expressed as the mean ± SD. Statistical analysis was conducted via analysis of variance (ANOVA). All the statistical tests were performed using the Statview (Version 10.0; SPSS, Chicago, IL, www.spss.com) program package. A P-value < 0.05 was taken as statistical significance.

TABLE I. Sequences and Reaction Efficiencies of Primers for Real-Time RT-PCR

Primer	Sense (5′–3′)	Antisense (5′–3′)
Insulin	5′-GCAGCCTTTGTGAACCAACA-3′	5′-TTCCCCGCACACTAGGTAGAGA-3′
GLP-1R	5′-TGGCTGTAGGTGGTGTTG-3′	5′-AGGATTCTTGAGCGTCTTT-3′

RESULTS

CELL ISOLATION AND CHARACTERIZATION

Human adipose tissue was obtained from four donors, two male and two female. Isolated hADSCs exhibited a spindle-shape, began to appear in culture, and reached 90% confluence in about 10–12 days. The second passage of hADSCs expanded rapidly and developed a uniform morphology that resembled of fibroblasts. The hADSCs maintained this morphology through repeated subcultures under non-stimulating conditions. FACS analysis of hADSCs at the third passage showed that these cultured cells were positive for CD13 (98.88%), CD44 (98.9%), CD59 (98.4%), and CD105 (71.24%). In addition, hADSCs exhibited low expression of CD34 (2.88%) and hematopoietic lineage marker CD45 (1.03%). Furthermore, expression of HLA-DR (0.98%) were not detected.

DIFFERENTIATION OF IPCs

Figure 1 shows that the expression of both insulin gene and GLP-1R gene massively increased. Insulin mRNA expression in the non-GLP-1 group and GLP-1 group increased 18- and 28-fold, respectively, from Day 0 to 12 ($P < 0.05$). Similarly, GLP-1R mRNA expression was 30- and 53-fold higher at Day 12 in the non-GLP-1 group and the GLP-1 group, respectively, compared with that at Day 0 ($P < 0.05$). Insulin cannot be used as a differentiating medium, so the insulin that appeared in media after glucose stimulation was synthesized de novo and secreted by IPCs. To verify whether two groups of IPCs could secrete insulin as a result of sensing physiological glucose concentrations as beta cells do, we first detected the quantity of insulin secretion in different glucose concentrations and under different stimulating time frames. ELISA (Table II Table II) showed that both beta cells and IPCs could secrete insulin after 30 min or 1 h of stimulation, with no difference existing between 30 min and 1 h stimulation in high glucose concentrations. Nevertheless, in low glucose concentrations, the amount of insulin was obviously lower than that resulting from high glucose stimulation for 30 min or 1 h. Excitingly, unlike IPCs of the non-GLP-1 group, IPCs of the GLP-1group could respond to low glucose concentrations after 30 min of stimulation like normal human

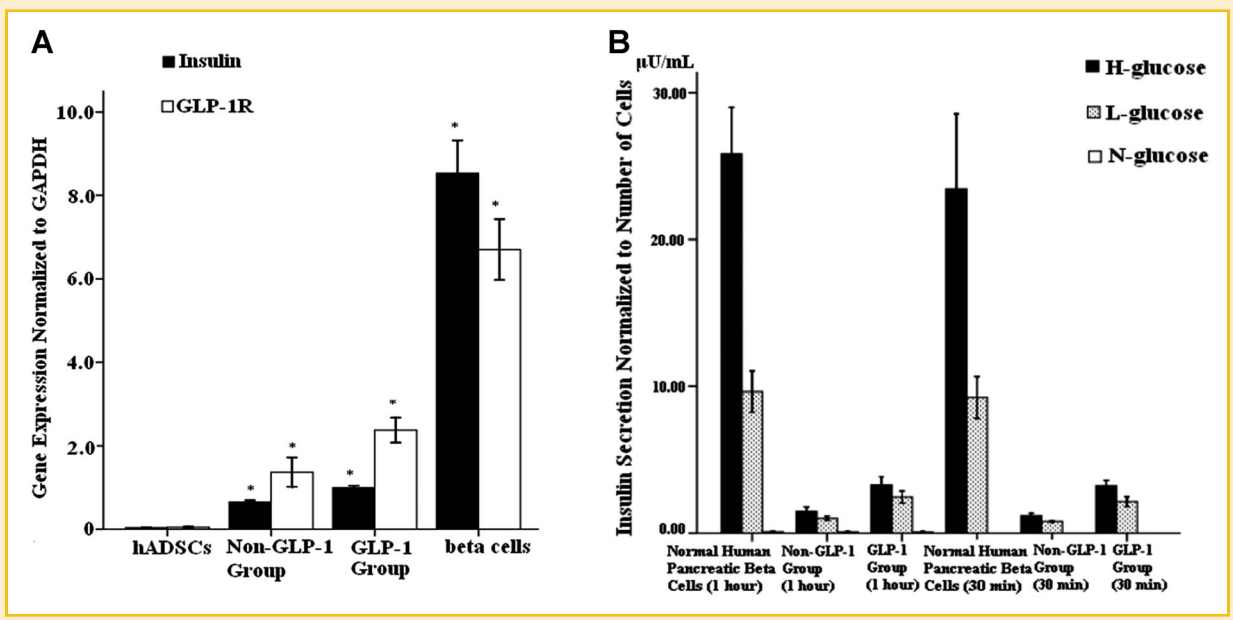

Fig. 1. Gene expression analysis and insulin secretion. A: Gene expression of hADSCs, IPCs of the non-GLP-1 group, IPCs of the GLP-1 group and normal human pancreatic beta cells. Insulin gene expression by these three groups of cells was 0.04 ± 0.004, 0.65 ± 0.036, 1.00 ± 0.037, 8.53 ± 0.636, respectively; CT value was 14.12 ± 0.45, 14.33 ± 0.37, 14.56 ± 0.09, 14.41 ± 0.31, respectively. GLP-1R gene expression was 0.05 ± 0.014, 1.37 ± 0.284, 2.37 ± 0.243, and 6.70 ± 0.584, respectively, CT value was 14.44 ± 0.39, 14.83 ± 0.21, 14.32 ± 0.40, 14.46 ± 0.33, respectively. Gene expression was normalized to GAPDH. B: Insulin secretion of the three groups of cells. Regardless of whether stimulation lasted 30 min or 1 h, both human normal pancreatic beta cells and IPCs could secret insulin. In addition, the secreted levels at these two time points were similar. Insulin secretion was normalized to the number of cells, $*P < 0.05$.

TABLE II. Insulin Secretion of Three Kinds of Cells (μU/ml)

	L-glucose (30 min)	L-glucose (1 h)	H-glucose (30 min)	H-glucose (1 h)
Normal human pancreatic β cells	10.43 ± 1.54	10.03 ± 1.06	25.64 ± 3.76	26.82 ± 3.51
Non-GLP-1 Group	0.31 ± 0.08	0.96 ± 0.23	1.53 ± 0.24	1.62 ± 0.21
GLP-1 Group	2.19 ± 0.20	2.47 ± 0.34	3.24 ± 0.29	3.29 ± 0.45

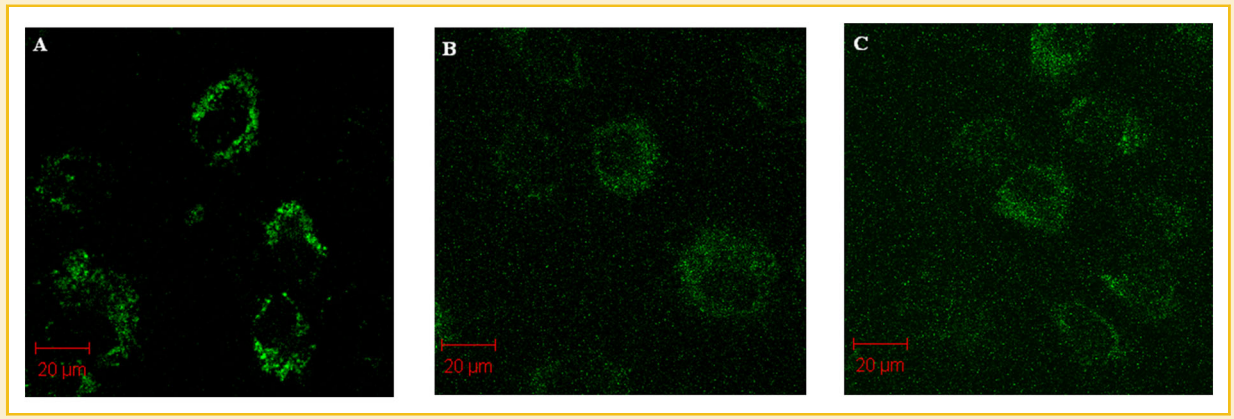

Fig. 2. GLP-1R protein was located on the cell membrane. Almost all normal human pancreatic beta cells and IPCs were positive for GLP-1R regardless of grouping. Immunofluorescent staining of normal human pancreatic beta normal cells with the GLP-1R antibody was more pronounced than the two groups of IPCs. A: Normal human pancreatic beta cells; (B) IPCs of the GLP-1 group; (C) IPCs of the non-GLP-1 group.

pancreatic beta cells, and the amount of insulin was similar to the amount resulting from 1 h of stimulation. Instead, IPCs of the non-GLP-1 group hardly secreted any insulin after low glucose stimulation for 30 min, and only secreted a little insulin after 1 h of stimulation in low glucose concentrations (Supplemental Table II). Our data demonstrated that insulin secretion from both normal beta cells and two groups of IPCs were regulated by glucose. However, only the IPCs of the GLP-1 group responded to glucose stimulation regardless of glucose concentration level, like normal human pancreatic beta cells.

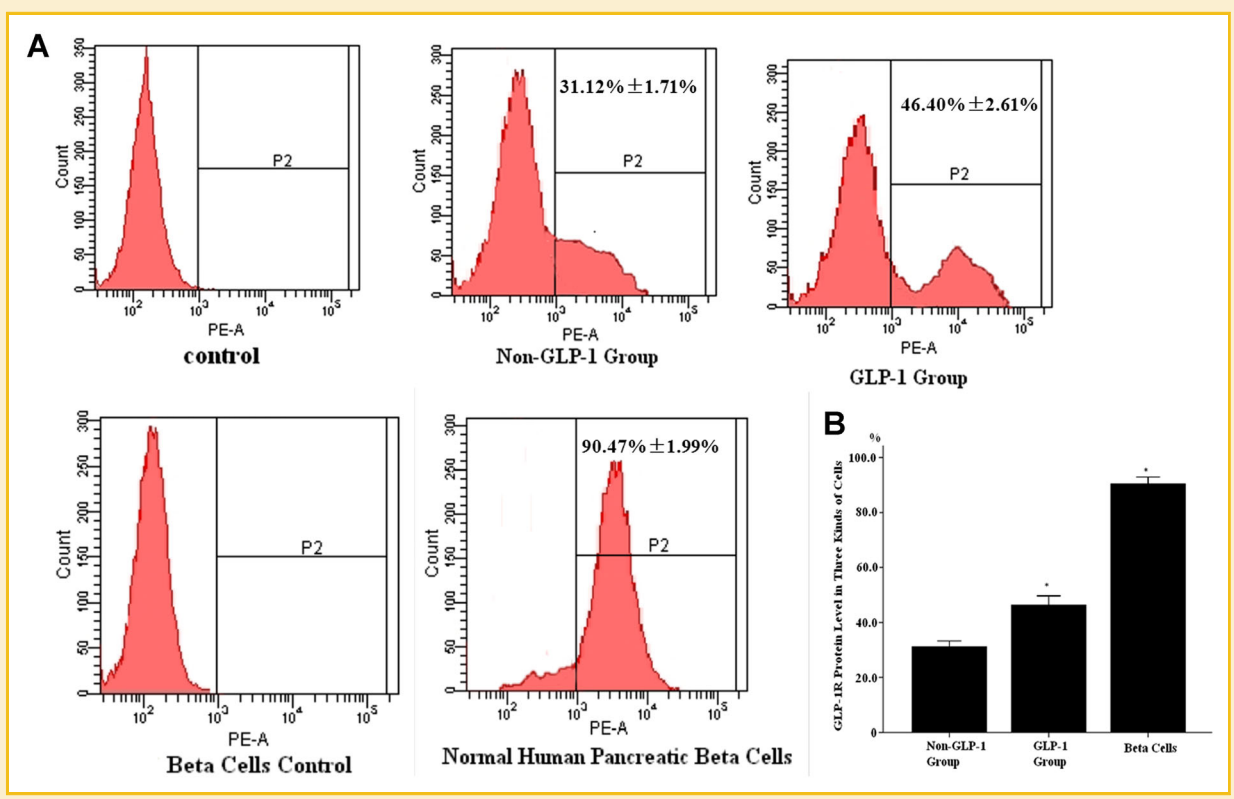

Fig. 3. GLP-1R level in normal human pancreatic beta cells and two groups of IPCs. A: FCM results: GLP-1R levels in the GLP-1 group were higher than those of the non-GLP-1 group. Both groups of IPCs expressed fewer GLP-1R than normal human pancreatic beta cells. B. Graphical plot of the mean values of GLP-1R level, $*P < 0.05$.

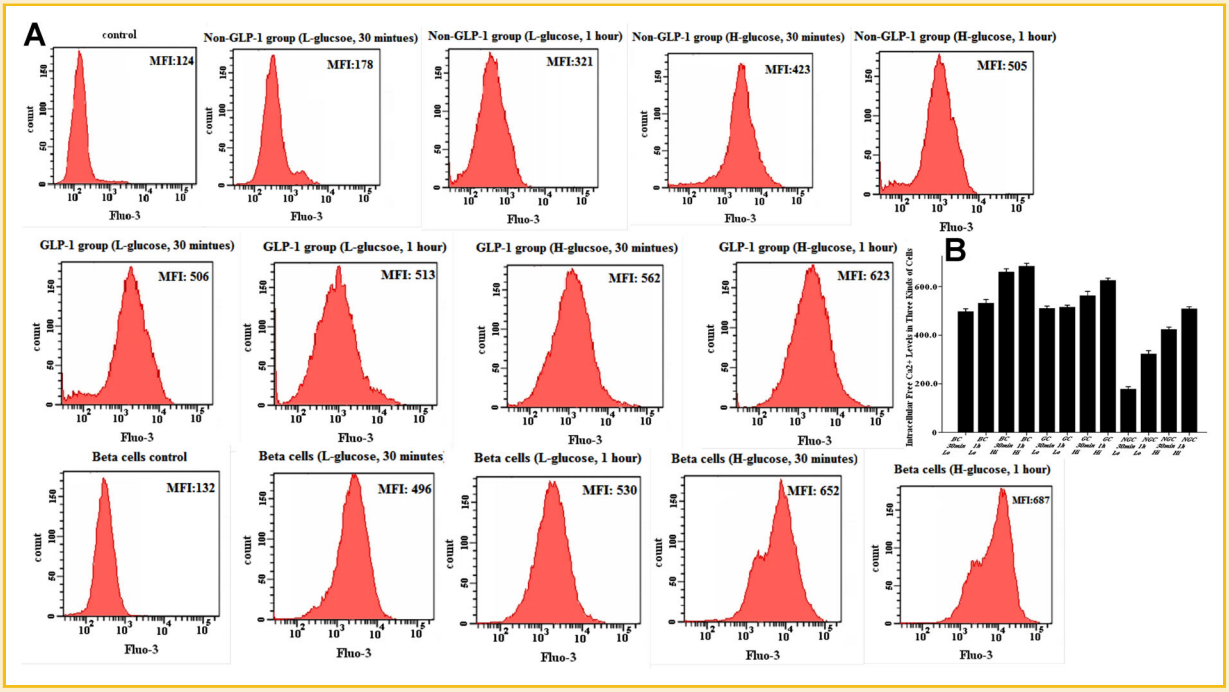

Fig. 4. Intracellular free Ca^{2+} levels in normal human pancreatic beta cells and two groups of IPCs. Regardless of cell type, when the cells were incubated in sugar-free medium, the level of intracellular free Ca^{2+} was low (N-glucose). After the cells were treated with high glucose and low glucose, intracellular free Ca^{2+} fluorescence increased dramatically in normal human pancreatic beta cells and IPCs of the GLP-1 group after both 30 min and 1 h of stimulation. In the non-GLP-1 group, the intracellular free Ca^{2+} levels increased a little only after 1 h of stimulation. N-glucose: no glucose stimulated group, D-PBS only; H-glucose: high glucose stimulation group, 25 mM; L-glucose: low glucose stimulation group, 5.5 mM. B. Graphical plot of the mean values of intracellular free Ca^{2+} levels. *BC 30min Lo*: beta cells 30 min low glucose concentration, *BC 1h Lo*: beta cells 1 h low glucose concentration, *BC 30min Hi*: beta cells 30 min high glucose concentration, *BC 1h Hi*: beta cells 1 h high glucose concentration, *GC 30min Lo*: cells of the GLP-1 group 30 min low glucose concentration, *GC 1h Lo*: cells of the GLP-1 group 1 h low glucose concentration, *GC 30min Hi*: cells of the GLP-1 group 30 min high glucose concentration, *GC 1h Hi*: cells of the GLP-1 group 1 h high glucose concentration, *NGC 30min Lo*: cells of the non-GLP-1 group 30 min low glucose concentration, *NGC 1h Lo*: cells of the non-GLP-1 group 1 h low glucose concentration, *NGC 30min Hi*: cells of the non-GLP-1 group 30 min high glucose concentration, *NGC 1h Hi*: cells of the non-GLP-1 group 1 h high glucose concentration.

GLP-1R PROTEIN WAS LOCATED ON THE CELL MEMBRANE

Given the important role of GLP-1R, we detected the expression and distribution of GLP-1R on cells through LSCM. We found that almost all the cells were positive for GLP-1R. Immunofluorescent staining showed that beta cells strongly expressed GLP-1R on their surface (Fig. 2A). There was little fluorescence on IPCs (Fig. 2B,C).

GLP-1R EXPRESSION ON NORMAL HUMAN PANCREATIC BETA CELLS WAS HIGHER THAN ON IPCS

Simultaneously, we analyzed the amount of GLP-1R on the cells by flow cytometry (Fig. 3). We observed that IPCs expressed less GLP-1R than beta cells ($90.47 \pm 1.99\%$). Additionally, the level of GLP-1R in the GLP-1 group ($46.40 \pm 2.61\%$) was slightly higher than that in the non-GLP-1 group ($31.12 \pm 1.71\%$).

INTRACELLULAR FREE CA^{2+} LEVELS WERE HIGHER IN HIGH GLUCOSE MEDIA

In regulated secretion, trigger signals such as hormones, neurotransmitters, and Ca^{2+} ions are the primary prerequisites for exocytosis. Improving the concentration of intracellular Ca^{2+} could promote exocytosis. With this in mind, we used Fluo-3 AM to measure the level of intracellular Ca^{2+} before and after stimulation with different concentration of glucose for varying time frames (Fig. 4). When the cells were incubated in sugar-free medium, the level of intracellular free Ca^{2+} was low. When the cells were stimulated by

TABLE III. Characteristic of Three Groups of Cells

	Normal human pancreatic β cells	GLP-1 group	Non-GLP-1 group
Length (μm)	57.31 ± 4.21	$67.61 \pm 2.28^*$	$76.32 \pm 3.14^*$
Width (μm)	35.73 ± 2.03	$37.62 \pm 0.93^*$	$41.42 \pm 1.53^*$
Height (nm)	513.45 ± 10.99	$452.11 \pm 16.19^*$	$412.42 \pm 16.87^*$

*Comparing with normal human pancreatic β cells, the difference was significant, $P < 0.05$.

high glucose for 30 min or 1 h, intracellular free Ca^{2+} levels increased dramatically, with no difference between 30 min and 1 h stimulation of the same group. However, only normal human pancreatic beta cells and IPCs of the GLP-1 group, exhibited similar increases in intracellular free Ca^{2+} after low glucose stimulation for 30 min or 1 h. Intracellular free Ca^{2+} levels in IPCs of the non-GLP-1 group hardly increased after 30 min of low glucose stimulation.

CELL MORPHOLOGY OF CELLS AS OBSERVED BY AFM

To compare the morphological changes between IPCs of the GLP-1 group and of beta cells before and after glucose stimulation, cells were separated into five groups: glucose-free culture medium group (D-PBS), 30 min low glucose stimulation group, 1 h low glucose stimulation group, 30 min high glucose stimulation group, and 1 h high glucose stimulation group. For each group, two coverslips containing five cells each were analyzed. There was not much difference in appearance between IPCs of the GLP-1 group and the beta cells observed via inverted microscope. Therefore, we analyzed the nanostructures of beta cells and IPCs through AFM in contact mode. IPCs of the GLP-1 group had similar morphological features to beta cells and IPCs of the non-GLP-1 group which appeared as polygons, ovals or circles. Two groups of IPCs were bigger than beta cells ($P < 0.05$; Table III).

Figure 5 shows a characteristic structure with many holes located in the cytoplasm in IPCs of the GLP-1 group-like beta cells and IPCs of non-GLP-1 group (Supplemental Figs. 2 and 3). The porous structure was also more obvious in glucose-stimulated group. Besides, we compared the R_a among three groups of cells. The statistical results suggested that the R_a of IPCs of the GLP-1 group was bigger than the non-GLP-1 group but smaller than the beta cells regardless of whether glucose stimulation was provided (Table IV). We measured the nanoparticle size of cells through AFM additionally. The data reveal that the nanoparticle size trends resembled those of the R_a values. The particle size of beta cells was bigger than all two groups of IPCs, regardless of whether they were subject to glucose stimulation. Furthermore, the R_a values of IPCs of the GLP-1 group were similar to each other when comparing 30 min stimulation with 1 h stimulation within the same glucose concentration ($P < 0.05$). These changes of R_a values in GLP-1 group were akin to human normal pancreatic beta cells. However, in the non-GLP-1 group we reported in the supplemental article, R_a values did not have such alterations when cells were stimulated for 30 min by low glucose concentrations. Particle size trends resembled those of the R_a values in all status. Meanwhile, due to the nanometer-scale resolution of AFM, we detected single membrane proteins and exposed details of the cellular surface structure. The membrane proteins of IPC of the GLP-1 group displayed a homogeneous granular distribution (Fig. 5 and Supplemental Figs. 2 and 3) like beta cells and IPCs of the non-GLP-1 group. Figure 5 also demonstrated that the cell surface style of IPCs of the GLP-1 group had been changed and the membrane

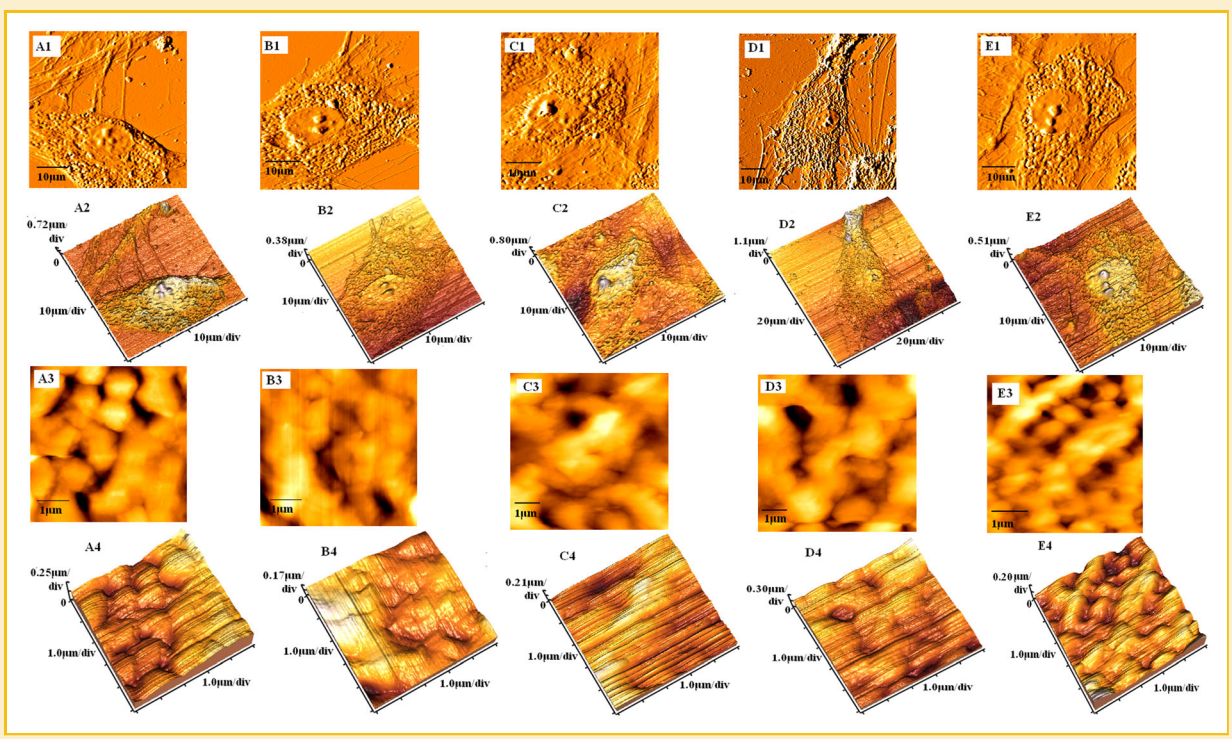

Fig. 5. Morphological changes of IPCs of the GLP-1 group, as detected by AFM. Treated with D-PBS (A1–A4), treated with high glucose medium for 1 h (B1–B4), treated with high glucose medium for 30 min (C1–C4); treated with low glucose medium for 1 h (D1–D4), treated with low glucose medium for 30 min (E1–E4) A1, B1, C1, D1, and E1 show the morphology of the whole cell; A3, B3, C3, D3, and E3 show surface ultrastructures on corresponding cells in images A2, B2, C2, D2, and E2; A4, B4, C4, D4, and E4 show 3D structures of the cells.

TABLE IV. Morphological Features of Three Groups of Cells

	Normal human pancreatic β cells	Non-GLP-1 group	GLP-1 group
R_a (nm)			
N-glucose	110.21 ± 9.32	29.43 ± 1.98	46.15 ± 3.16
H-glucose (30 min)	137.12 ± 7.32*	43.42 ± 2.01*	64.38 ± 4.97*
H-glucose (1 h)	138.65 ± 10.98*	48.55 ± 8.09*	75.28 ± 5.56*
L-glucose (30 min)	118.34 ± 45.45*	31.98 ± 2.01	45.32 ± 4.18
L-glucose (1 h)	125.45 ± 14.62*	37.51 ± 3.09*	54.45 ± 2.20*
Particle size (nm)			
N-glucose	221 ± 6.3	150.32 ± 4.97	180 ± 8.0
H-glucose (30 min)	349.65 ± 8.83*	227.54 ± 6.42*	247 ± 7.58*
H-glucose (1 h)	362 ± 9.4*	245.32 ± 11.43*	261 ± 9.6*
L-glucose (30 min)	225.63 ± 11.23*	158.43 ± 5.67	192 ± 14.4*
L-glucose (1 h)	232.34 ± 15.21*	173 ± 10.34	207 ± 11.51*

*Comparing with N-glucose, the difference was significant, $P < 0.05$.

proteins presented some extent of aggregation after glucose stimulation which resembled of the beta cells.

DISCUSSION

Transplantation of either isolated pancreatic beta cells or the whole pancreas provides a mode of insulin replacement, but is often accompanied by many disadvantageous side effects. IPCs, however, offer the potential to treat diabetes by providing a potential replacement for impaired pancreatic beta cells without the limitations of current therapeutic modalities. We observed many differences between IPCs and beta cells in cellular function as well as in ultrastructure of the cell membrane.

We successfully extracted hADSCs from human adipose tissue. These isolated and cultured cells were positive for CD59, CD13, and CD44; negative for and HLA-DR; and exhibited low expression of CD34 and hematopoietic lineage markers CD45. Then we differentiated hADSCs into IPCs in vitro without using insulin. IPCs were divided into two groups according to the differentiated media used (with or without GLP-1). We compared the glucose-induced insulin secreted function of two groups of IPCs and beta cells in vitro. Our results display an interesting phenomenon. Unlike IPCs of the non-GLP-1 group which could secrete a little bit of insulin only when stimulated for 1 h in low glucose concentrations, IPCs of the GLP-1 group could release a certain amount of insulin regardless of whether they were stimulated for 30 min or 1 h like beta cells with high or low glucose concentrations. The data indicates that IPCs of the GLP-1 group could better mimic the physiological regulation of insulin secretion performed by pancreatic beta cells than IPCs of than non-GLP-1 group.

Many studies have reported that IPCs from any kind of stem cells can be manipulated to express and secrete insulin [Lumelsky et al., 2001, Assady et al., 2001; Hori et al., 2002; Soria et al., 2000]. IPCs have also been shown to reduce blood sugar to different degrees after they were transplanted in diabetic animals [Zalzman et al., 2003; Zhao et al., 2005; Kajiyama et al., 2010]. Our results also proved that hADSC-derived IPCs could secrete a little bit of insulin after glucose stimulation. However, we also found that the use of GLP-1 for the differentiation of hADSCs into IPCs could better mimic the physiological regulation of insulin secretion like pancreatic beta cells, but the use of GLP-1 did not improve obviously the ability of these cells to secrete insulin. In fact, there was only a little amount of insulin that could be detected in the media of these cells after glucose stimulation. On the other hand, the same glucose stimulation resulted in detection of much more insulin in the media of the beta cells.

In order to assess the morphological properties of IPCs of the GLP-1 group, we still used AFM to detect the cellular ultrastructure. The morphology of IPCs of the GLP-1 group was similar to IPCs of the non-GLP-1 group and beta cells, as observed via AFM. IPCs of the GLP-1 group were also contained visibly porous features in the cytoplasm and the porous structures were organized and well-distributed around the nuclei. In the general, there was not much difference between the GLP-1group and the non-GLP-1 group in cellular ultrastructure. Nevertheless, we compared the cell membrane particle size and R_a of the membrane surface before or after glucose-stimulation of IPCs of the GLP-1 group, IPC of the non-GLP-1 group and the beta cells. Our results revealed that both membrane particle size and R_a of IPCs of the GLP-1 group were bigger than IPCs of the non-GLP1 group, but smaller than the beta cells. Unlike IPCs of the non-GLP-1 group that the membrane particle size and R_a did not change obviously when stimulated for 30 min in low glucose concentrations, when IPCs of the GLP-1 group were stimulated by glucose, the membrane particle size and R_a were higher than those not stimulated. Types, structure, and quantity of membrane protein molecules directly influenced the inclines and declines of the membrane surface which affected the magnitude of cellular R_a [Brammer et al., 2009]. We conjectured that the reason for the bigger membrane particle size and R_a in IPCs of the GLP-1 group might be owing to their bigger membrane protein content which caused by the use of GLP-1.

Yue et al. [2006] concluded that GLP-1 induced differentiation of nestin-positive progenitor ES cells into IPCs was achieved by upregulation of PDX-1 expression. GLP-1 can also direct human ES cell differentiation into IPCs via hedgehog, cyclic adenosine monophosphate, and phosphatidylinositol-3-kinase pathways [Hui et al., 2010]. GLP-1R is transmembrane protein that serves as a key regulator of insulin secretion and a major therapeutic target for treatment of diabetes [Murphy and Bloom, 2007]. It is a family B peptide hormone G protein coupled receptor primarily expressed in

pancreatic beta cells. GLP-1 binding to GLP-1R, induces signal activation that, amplifies glucose-induced insulin release. Sanz et al. demonstrated that adipogenesis was inhibited when GLP-1 was present in the adipogenic medium. Additionally, they found that hMSCs expressed GLP-1R mRNA and protein, which is consistent with the observed agonistic actions. They believed GLP-1 inhibited the early steps of adipogenesis in hMSCs through GLP-1R signal transduction [Sanz et al., 2010]. Here, we also tested the quantity and distribution on GLP-1R of cells. Our results showed that the three groups of cells expressed GLP-1R, and the levels of the GLP-1 group were slightly higher than those of the non- GLP-1 group. However, GLP-1R expression of the two groups of differentiated cells was significantly lower than that of the beta cells. Hence, we speculate that when hADSCs differentiated into IPCs in normal IPC differentiation medium without GLP-1, IPCs could express GLP-1R, for it is a significant transmembrane protein in pancreatic beta cells. On the other hand, when GLP-1 was present in the IPC differentiation medium, GLP-1R were expressed in hADSCs until they differentiated into IPCs. This provided the difference between the GLP-1 group and non-GLP-1 group which caused the difference in membrane particle size and R_a between two kinds of IPCs.

Beta cells secrete insulin through exocytosis. Ca^{2+} ions can regulate insulin secretion as a trigger signal. Increasing the level of intracellular Ca^{2+} can intensify exocytosis [Mundorf et al., 2000; Machado et al., 2009; Borges et al., 2012]. We measured the concentration of intracellular free Ca^{2+} in IPCs and beta cells. The results showed that in both normal human pancreatic beta cells and the cells of the GLP-1 group, the level of intracellular free Ca^{2+} was significantly higher after glucose stimulation than before. The data also hinted that exocytosis in IPCs and beta cells had increased after glucose stimulation. Interestingly, after low glucose stimulation for 30 min, the level of intracellular free Ca^{2+} in IPCs of the non-GLP-1 group remained low, similar to levels observed when no glucose stimulation was performed. This data the observations made by AFM and the insulin secretion experiment were consistent.

In summary, our data proved that IPCs of the GLP-1 group could release insulin after low and high glucose stimulation for 30 min and 1 h like normal human pancreatic beta cells. The cellular ultrastructure and function of IPCs of the GLP-1 group resembled more closely those of the normal human pancreatic beta cells than those exhibited by the IPCs of the non GLP-1 group. GLP-1 could improve the similarity of IPCs from human adipose tissue-derived mesenchymal stem cells and pancreatic beta cells in cellular ultrastructure and function.

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