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Differentiation of fetal pancreatic stem cells into neuron-like and islet-like cells *in vitro*★

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Abstract

Pancreatic stem cells were isolated and cultured from aborted human fetal pancreases of gestational age 14–20 weeks. They were seeded at a density of 1×10^4 in serum-free media for differentiation into neuron-like cells, expressing β-tubulin III and glial fibrillary acidic protein. These neuron-like cells displayed a synapse-like morphology and appeared to form a neuronal network. Pancreatic stem cells were also seeded at a density of 1×10^5 for differentiation into islet-like cells, expressing insulin and glucagon, with an islet-like morphology. These cells had glucose-stimulated secretion of human insulin and C-peptide. Results suggest that pancreatic stem cells can be differentiated into neuron-like and islet-like cells.

Key Words: fetal pancreas; pancreatic stem cells; differentiation; islet-like cells; neuron-like cells; neural regeneration

Abbreviations: PSCs, pancreatic stem cells; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor

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INTRODUCTION

During embryogenesis, the pancreatic and nervous systems develop from the endodermal and ectodermal layers, respectively[1]. Despite different embryologic origins, pancreatic and neural cells express numerous common enzymes and markers, as well as sharing some developmental control mechanisms[2-5]. Pancreatic stem cells (PSCs) can be differentiated into insulin expressing cells that recover hyperglycemia in diabetic animals[6-10]. They can also differentiate into neural cells that can be used to treat diabetic neuropathy[11-13]. Moreover, the fetal pancreas has been hypothesized to possess an extensive regeneration capacity compared with that of the adult pancreas. Thus, we decided to study the pluripotency of PSCs from fetal pancreases that were not fully mature, to determine if they can differentiate into pancreatic islet and neural cells *in vitro*.

RESULTS

PSCs exhibit stem cell characteristics

Freshly isolated islet cells were cultured in modified high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. After 48 hours, scattered adherent epidermal-like cells were observed (Figure 1A) and a large number of non-adherent cells were removed by a complete medium change. Culture medium was then replaced with DMEM-F12 supplemented with 2% fetal bovine serum (FBS). Cells reached 80% confluence after 10 days (Figure 1B) and proliferation was significantly enhanced by medium supplementation with 10 ng/mL epidermal growth factor (EGF) and 10 ng/mL basic fibroblast growth factor (bFGF), with cells reaching 80% confluence in 5–6 days. Epidermal-like cells were established with serial passaging and PSCs were similar to the primary stem cells after some passages. Furthermore, primary PSCs were identified by a noticeable expression of nestin by immunofluorescence (Figure 1C) and weak expression of pancreatic and duodenal homeobox (PDX)-1 (Figure 1D). This expression pattern indicated that stable self-renewable stem cells were established. After differentiation, neurons and neuroglial structures were generated, as well as mature round pancreatic islet cells. This indicated that the isolated PSCs were multipotent.

PSC differentiation into neuron-like cells

There were no significant changes in PSC morphology during the first 3 days following EGF, insulin-transferrin-selenium-A, and

glutamine treatment. In serum-free culture, PSC morphology gradually changed from fusiform-like to irregular polygon shapes, with bipolar and multi-polar cells, as well as synaptic-like structures and neural reticular formations similar to neuron and neuroglial morphologies. Occasionally, neural filament-like structures or large neuronal networks were also observed. The spontaneous differentiation rate of neural-like cells was 2–5% (Figures 1E, F).

PSC differentiation into islet-like cells

PSC differentiation into insulin-producing cells was performed by a two-stage protocol. During stage 1, cells decreased in size and became stretched with retraction. These spindle-like cells shortened and became semi-attached round epithelial-like cells (Figure 1G). During stage 2, cells became round and increasing islet-like clusters were observed (Figure 1H). By day 9 of differentiation, cells had detached and grew in suspension. Ultrastructural analysis by electron microscopy showed that differentiated cells contained small secretory granules, which are a characteristic of pancreatic endocrine cells.

Changes in biomarkers in neuron-like and islet-like cells differentiated from PSCs

There was a significant expression of nestin in PMCs (Figure 1C), with weak levels of PDX-1 (Figure 1D). Differentiated neural cells expressed glial fibrillary acidic protein (Figure 2A) and β-tubulin III (Figure 2B), as well as microtubule-associated protein-2 (weak expression, not shown). After 9 days, differentiated islet cells expressed high levels of insulin (Figure 2C) and weak levels of glucagon (Figure 2D). Double immunofluorescent staining for insulin and glucagon revealed that insulin-positive cells did not immunostain for glucagon.

Islet-like structure of differentiated islet-like cells

Differentiated islet clusters were incubated with dithizone for 1 hour and a color change was observed due to a chelation reaction with zinc ions present in islet-like structures (Figure 2E).

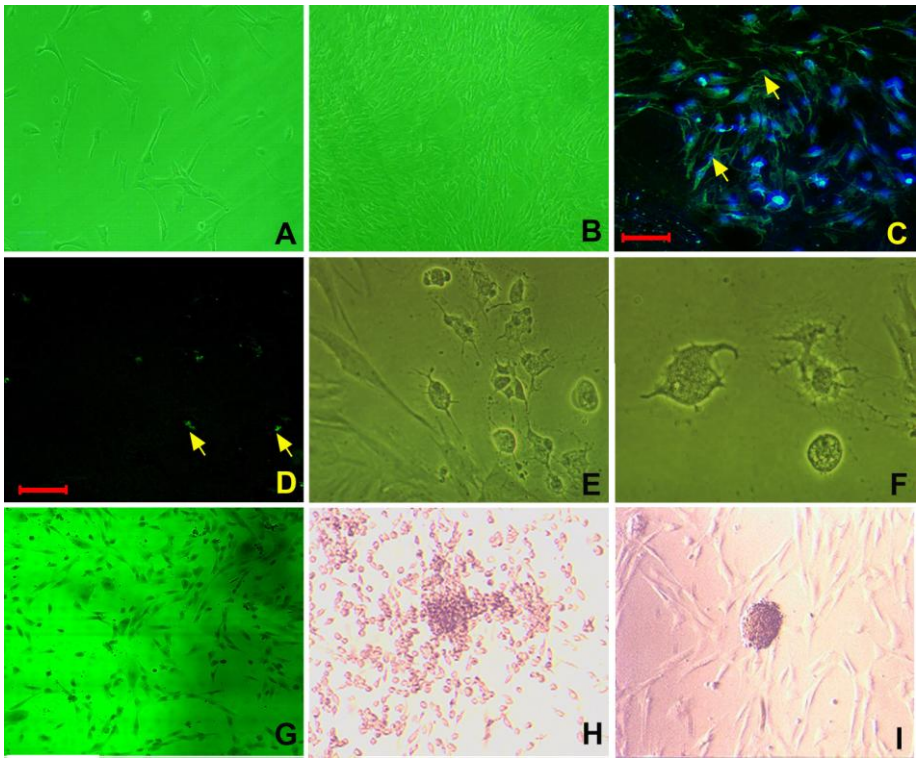

Figure 1 Morphological changes during the different pancreatic stem cell (PSC) differentiation stages and the identification of PSCs.

(A) Scattered adherent epidermal-like cells were observed after culturing of primary PSCs for 48 hours (× 100).

(B) Primary PSCs reached 80% confluence when cultured for 10 days (× 100).

(C) Undifferentiated human PSCs expressed a significant concentration of nestin (arrows, × 100).

(D) Undifferentiated human PSCs weakly expressed pancreatic and duodenal homeobox-1 (arrows, × 100).

(E, F) PSCs differentiated into neural cells with different shapes in different-density cell culture (× 200).

(G) PSCs induced for 3 days contain shorter and rounder differentiated cells (× 100).

(H) PSCs induced for 9 days show islet-like cells and the formation of clusters (× 100).

(I) PSCs induced for 4 days with 500 ng/mL epidermal growth factor formed islet-like clusters (× 100).

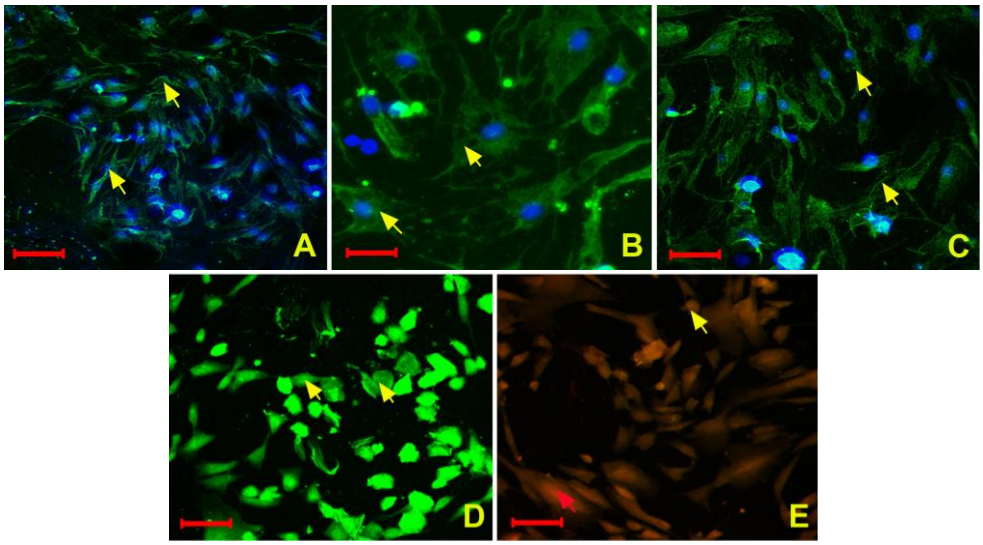

Figure 2 Expression of neural and pancreatic islet-like markers (× 100).

Differentiated neuron-like cells express glial fibrillary acidic protein (A, arrows) and β-tubulin III (B, arrows). Differentiated islet-like cells express insulin (C, arrows) and glucagon (D, arrows). (E) Differentiated islet-like clusters stained positive (arrows) with dithizone.

Insulin and C-pepide secretion in response to glucose stimulation in differentiated islet-like cells

Insulin and C-peptide secretion were not detected in undifferentiated PSCs. Cells at stage 1 differentiation released a small amount of insulin, while islet-like cells at stage 2 secreted significantly higher levels of insulin and C-peptide. Differentiated cells responded to various glucose concentrations. Insulin concentrations were 183.3 ± 28.3, 256.2 ± 41.5, and 298.4 ± 46.8 μIU/mL and C-peptide concentrations were 8.7 ± 4.9, 14.3 ± 4.3, 19.1 ± 6.4 μU/mL in response to 5.5, 16.7, and 25 mM glucose, respectively. Cellular insulin concentration was determined to be 6.8 ± 1.3 μIU/mL without stimulation, but C-peptide concentration was below detectable levels.

DISCUSSION

In this study, we used human fetal pancreases rather than heterogeneous islets or islet-depleted fractions used by other studies[7, 11-14]. Immature fetal pancreases, expressing PDX-1, CK19, nestin and other markers of regenerating and developing pancreas, possess excellent regenerative, proliferative, and differentiation abilities compared with those of the mature adult pancreas[15]. The EGF receptor is also expressed throughout the human fetal pancreas, and mice lacking EGF receptor develop abnormal pancreatic islets[16]. This study has shown that human fetal PSCs actively proliferate in serum-free medium supplemented with EGF and bFGF. PSCs were seeded at a high density for the formation of a three-dimensional cell structure, expressing insulin and glucagon. Upon glucose stimulation, these cells secreted C-peptide and insulin at similar quantities compared with those of native islets. PSCs were seeded at a low density for differentiation into neuron-like cells, expressing β-tubulin III and glial fibrillary acidic protein. We also observed that final C-peptide and insulin concentrations of stage 2 cultures varied by 2–4-fold. This may be due to cell-type variability and unequal proliferation/differentiation capacities. Thus, small variations in the initial composition of PSCs would translate into significant differences among differentiated cells. Our results suggest that high concentrations of EGF promote PSC differentiation into islet-like clusters. A previous study demonstrated that EGF is an important growth factor for the proliferation of various cell types, particularly fibroblasts and epithelial cells[17]. Combinatorial therapy with EGF and gastrin has also been shown to result in β-cell regeneration in rodents with chemically induced diabetes[18-19].

In summary, in this study, we used human fetal immature pancreases to examine the multipotent differentiation characteristics of pancreatic-derived stem cells. Under different differentiation conditions, PSCs were separately induced into neuron and islet-like cells. Serum-free and low-density cell culture contributes toward neural cell differentiation, while high-density cell culture is biased toward islet cell differentiation. This study may provide a new insight into potential sources of cells for therapeutic transplantation for diabetes and its related chronic neuropathy complications.

MATERIALS AND METHODS

Design

In vitro parallel experiment.

Time and setting

The experiment was performed at the Research Center of Stem Cell Engineering of Shandong Province, Central Laboratory of Yantai Yuhuangding Hospital, China from October 2008 to July 2010.

Materials

Human fetal pancreases of gestational age 14–20 weeks were provided by the Department of Obstetrics and Gynecology from spontaneously aborted fetuses (n = 6) with prior consent. Experimental procedures were approved by the Chinese Medical Ethics Committee.

Methods

PSC isolation and culture

Fetal pancreases were cut into small pieces in a sterile environment and then treated with 0.1% type IV collagenase for digestion into a single cell suspension. Freshly isolated islet cells were seeded at 1 × 10^6 cells/mL in high-glucose DMEM (Invitrogen, Grand Island, NY, USA) supplemented with 10% FBS (Invitrogen), 1 × B27 (Stemcell Co, Vancouver, BC, Canada), 2 mM glutamine, 100 U/mL penicillin and 100 mg/L streptomycin followed by incubation at 37°C. After 48 hours, non-adherent cells were removed *via* a total medium change followed by exchanging the medium to DMEM-F12 (Invitrogen) supplemented with 2% fetal bovine serum, 2 mM glutamine and 1× B27. Subsequently, cells slowly proliferated in serum-free medium and reached 80% confluence after 10 days. Cell proliferation was significantly enhanced by supplementing 10 ng/mL EGF (Invitrogen) and 10 ng/mL bFGF (Invitrogen) into culture medium, resulting in cells reaching 80% confluence after 5–6 days. PSCs were established after serial passaging and were identified by their epidermal-like morphology, with notable nestin immunofluorescent staining and weak PDX-1 expression.

Morphological observation

PSCs at various stages of differentiation were observed under an inverted microscope (Nikon, Tokyo, Japan) to assess morphological changes.

PSC differentiation

For neuron-like cell differentiation, passage 3 PSCs were seeded at a density of 1 × 10^4 cells/well in DMEM-F12 medium, supplemented with insulin-transferrin-selenium (Stemcell, Vancouver, BC, Canada), 2 mM glutamine and 10 ng/mL EGF for 6 days.

For islet-like cell differentiation, passage 3–5 PSCs were seeded at a density of 1 × 10^5 cells/well and differentiated into insulin-secreting cells by a modified 2-stage protocol. Stage 1 (pre-differentiation): PSCs were cultured in DMEM-F12 medium supplemented with 10 ng/mL bFGF, 10 ng/mL EGF, 1 × B27 and 2 mM glutamine for 3 days; Stage 2 (insulin-secreting cell maturation): differentiated cells were cultured in DMEM-F12 supplemented with 20 mM nicotinamide, 1 × B27 and 2 mM glutamine for 6 days. An increase in EGF concentration from 10 to 500 ng/mL during pre-differentiation induced the formation of islet-like clusters.

Immunostaining of cell surface markers at different stages

Approximately 1 × 10^3 PSCs or cells from each differentiation stage were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 minutes at room temperature and incubated at 4°C overnight with primary antibodies. The primary antibodies used and their dilutions were as follows: mouse anti-human nestin monoclonal antibody (1:200; Chemicon, Temecula, CA, USA); rabbit anti-PDX-1 polyclonal antibody (1:500; Chemicon); mouse anti-human insulin monoclonal antibody (1:100; R&D System, Minneapolis, MN, USA); rabbit anti-glucagon polyclonal antibody (1:250; Chemicon); rabbit anti-glial fibrillary acidic protein polyclonal antibody (1:100; Sigma, St. Louis, MO, USA); mouse anti-human microtubule-associated protein-2 monoclonal antibody (1:100; Sigma); mouse anti-human myelin basic protein monoclonal antibody (1:50; Sigma); and mouse anti-human β-tubulin III monoclonal antibody (1:100; Sigma). Labeled-cells were washed three times with PBS and incubated at 37°C for 1 hour with diluted fluorescently labeled secondary antibodies as follows: goat anti-mouse IgG-FITC (1:100; Sigma); and sheep anti-rabbit IgG-Cy3 (1:60; Sigma). Following this, cells were counter-stained with 4', 6-diamidino-2-phenylindole dihydrochloride (2 μg/mL; Sigma) for 30 minutes. Cells were visualized and photographed with a confocal microscope (MPS60, Leica, Solms, Germany). Immunostaining was performed with secondary antibodies alone as negative controls.

Islet-like cell structure identification by dithizone staining

After inducing PSCs to differentiate into islet-like cells for 9 days, the cells were harvested with culture medium in 1.5 mL tubes, centrifuged, washed with PBS and placed in 35 mm Petri dishes for dithizone staining (Sigma). Routine staining was performed by adding 10 μL dithizone stock to islet cells suspended in 1 mL Krebs-Ringer bicarbonate buffer (pH 7.4) and incubated at 37°C for 1 hour, following which they were observed using an inverted microscope.

Insulin and C-peptide expression in differentiated islet-like cells

Insulin and C-peptide release assays[20] were performed in 0.5 mL serum-free DMEM and incubated for 1 hour. An electrochemiluminescence immunoassay[20] was used to measure the release of intracellular insulin and C-peptide into the medium in response to various glucose concentrations and to measure cellular insulin concentration after ultrasonic cell lysis, using an Elecsys 1010 insulin and C-peptide kit (Roche, Penzberg, Germany). To test whether insulin and C-peptide released from PSC-differentiated endocrine cells was glucose-dependent, three glucose concentrations (5.5, 16.7, and 25 mM) were used for stimulation. Pre-differentiated PSCs treated with the same conditions were used as a control.

Statistical analysis

Data were represented as mean ± SD. Results were analyzed by independent samples *t* test and one-way analysis of variance. A value of $P < 0.05$ was considered statistically significant.

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Author contributions: Xiufeng Hua was responsible for the research design, data analysis and article writing. Yanwei Wang and Peiwen Lian completed the cell culture and Shouxin Zhang help with laser scanning confocal microscopy and statistical processing. Jianyuan Li, Haiyan Wang, Shulin Chen and Wei Gao assisted with the data analysis and article writing as well as statistical processing.

Conflicts of interest: None declared.

Ethical approval: Experimental procedures were approved by Chinese Medical Ethics Committee.

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