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Original Article

Potential differentiation of islet-like cells from pregnant cow-derived placental stem cells

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ABSTRACT

Objective: Type 1 diabetes is an autoimmune disease that destroys islet cells and results in insufficient insulin secretion by pancreatic β-cells. Islet transplantation from donors is an approach used for treating patients with diabetes; however, this therapy is difficult to implement because of the lack of donors. Nevertheless, several stem cells have the potential to differentiate from islet-like cells and enable insulin secretion for treating diabetes in animal models. For example, placenta is considered a waste material and can be harvested noninvasively during delivery without ethical or moral concerns. To date, the differentiation of islet-like cells from cow-derived placental stem cells (CPSCs) has yet to be demonstrated.

Materials and methods: The investigation of potential differentiation of islet-like cells from CPSCs was conducted by supplementation with nicotinamide, exendin-4, glucose, and poly-D-lysine and was detected through reverse transcription polymerase chain reaction, dithizone staining, and immunocytochemical methods.

Results: Our results indicated that CPSCs are established and express mesenchymal stem cell surface antigen markers, such as CD73, CD166, β-integrin, and Oct-4, but not hematopoietic stem cell surface antigen markers, such as CD45. After induction, the CPSCs successfully differentiated into islet-like cells. The CPSC-derived islet-like cells expressed islet cell development-related genes, such as insulin, glucagon, pax-4, Nkx6.1, pax-6, and Fox. Moreover, CPSC-derived islet-like cells can be stained with zinc ions, which are widely distributed in the islet cells and enable insulin secretion.

Conclusion: Altogether, islet-like cells have the potential to be differentiated from CPSCs without gene manipulation, and can be used in diabetic animal models in the future for preclinical and drug testing trial investigations.

Introduction

Approximately 300 million patients are estimated to receive a diagnosis of diabetes by 2030 [1]. Therefore, it is crucial to develop strategies for the prevention and reduction of diabetes through blood sugar control. Type I diabetes is caused by the autoimmune destruction of insulin-producing pancreatic β-cells [2]. Currently, insulin injections are provided as therapy, which may be inconvenient for the patients with diabetes. The other therapeutic approach is islet transplantation from donors; however, the lack of donors is a considerable problem with this method. Stem cell therapy is an alternative treatment for diabetes [3]. Specifically, human-umbilical-cord- [4], skin- [5], and adipose-tissue-derived [6] stem cells, human-induced pluripotent stem cells [7], rat pancreatic-duct-derived stem cells [8], and duck pancreas-derived mesenchymal stem cells [9] have been reported to differentiate into insulin-producing cells. Genetically modified cells, such as those expressing the islet-development-related genes (e.g. microRNA (miR)-375, anti-miR-9, or miR-7 [7,10]) or those with pdx-1 transfection and epigenetic regulation [11,12] have been reported to induce differentiation into insulin-producing cells. Furthermore, stem cells cocultured with pancreatic islet cells [13,14], neonate

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pancreatic extracts [15], β-mercaptoethanol, and basic fibroblast growth factor [16], along with three-dimensional culture systems [8], have been reported to induce the differentiation of stem cells into insulin-producing cells. Therefore, stem cells from different tissues can differentiate into insulin-producing cells by supplementation with certain factors and gene manipulations.

The placenta connects the fetus and uterine wall during the gestational period to supply nutrients, produce hormones, eliminate waste, and facilitate gas exchange, and human-derived placental stem cells have been discussed [17]. With regard to the immunomodulation properties, placental stem cells inhibit T cell proliferation [18] and reduce the differentiation of T helper cell 1 and T helper cell 17 to control the proliferation and differentiation of dendritic cells [19]. In addition, placental stem cells, similar to amniotic fluid stem cells may reduce the incidence of graft-versus-host disease [20–22]. Altogether, placental stem cells have immunotolerant properties that are favorable for future clinical use.

Cows are large animals, and various cow-derived stem cells have been used in medical and agricultural applications, such as those from the liver [23], amniotic fluid [24], skin [25], fat tissue [26], bone marrow [27], muscles [28], umbilical cord [29], ovaries [30], lungs [31], breasts [32], and fetal brain [33]. However, the differentiation of insulin-producing cells from cow-derived placental stem cells (CPSCs) has yet to be reported. Without ethical and moral concerns, cow placenta can be harvested noninvasively. Moreover, high-glucose nicotinamide [34], exendin-4 [35], and nicotinamide [36] are capable of inducing the differentiation of insulin-producing cells from stem cells. Therefore, we investigated the potential differentiation of insulin-producing cells from CPSCs through supplementation with the aforementioned factors, which may facilitate further drug tests and medical explorations in the future.

Materials and methods

Source of placenta

Deciduas basilis of the placenta was removed from *Bos taurus* (Holstein bovine) after parturition using surgical scissors at a ranch at the National Pingtung University of Science and Technology.

Isolation and culturing of CPSCs

The placental tissue was first immersed in 75% alcohol for 30 s and then washed with Dulbecco's phosphate-buffered saline (DPBS) several times. Next, the bovine placenta was mechanically fragmented after digestion with 0.25% trypsin (Gibico) and incubation at 37°C for 3 min. The alpha minimal essential medium (αMEM) was added to the mix to terminate the trypsin reaction. The mix was centrifuged at 2000 rpm for 10 min to obtain the tissue fragments, which were subsequently seeded in a 10-cm dish (91 cm^2) with the αMEM containing 10% fetal bovine serum (FBS), 0.1% penicillin, and 2.2 g sodium hydrogen carbonate; it solution was maintained at 38.5°C and in 5% CO_2 for 7 days. After achieving 80% confluence of passage one, the cells were cultured in αMEM with 20% FBS and subcultured using 0.1% trypsin. The CPSCs were characterized through reverse transcription polymerase chain reaction (RT-PCR). Finally, gene-specific forward and reverse primers for CD45, CD73, CD166, Oct-4, and β-integrin were used consecutively. The specific primers designed for the CPSCs are presented in Table 1.

Islet-like cells differentiation from CPSCs

The CPSCs of passage five were cultured at 10^4 cells/cm^2 and were supplemented with a islet-like cell differentiation medium, which consisted of the αMEM medium with 10% FBS, 10 mM nicotinamide, 10 μM exendin-4, and 28 mM glucose. The dishes were coated with poly-D-lysine before cell seeding, and the differentiation medium was monitored daily and replaced every 3 days. For 15 days, several clusters were observed with an inverted fluorescence microscope. The functionality of the differentiated islet-like cells was assessed through dithizone (DTZ) staining, immunocytochemical methods, and RT-PCR.

Table 1
Forward and reverse primer sequences (5′–3′).

Gene	Primer sequences (5′–3′)
Cow CD45-F	CTACCCAACCTTCTACTCAA
Cow CD45-R	TTCACATCCAGGAGGTTC
Cow CD73-F	CAATGGCACGATTACCTG
Cow CD73-R	GACCTTCAACTGCTGGATA
Cow CD166-F	TATCAGGATGCTGGAAAC
Cow CD166-R	TAGCCAATAGACGACACC
Cow Oct-4-F	CTCTTTGGAAAGGTGTTCAG
Cow Oct-4-R	GTCTCTGCCTTGCATATCTC
Cow β-integrin-F	GAAACTTGGTGGCATCGT
Cow β-integrin-R	CTCAGTGAAGCCCAGAGG
Cow β-actin-F	GCCCTGAGGCTCTCTTCCA
Cow β-actin-R	CGGATGTCGACGTCACACTT
Cow Insulin-F	GCAGAAGCGTGGCATCGT
Cow Insulin-R	GGGCAGGCCTAGTTACAGTAGTTC
Cow Nestin-F	TCCCTGCCCTGCTGTAGATG
Cow Nestin-R	TTGGCTTCAGCCCACATGA
Cow PDX1-F	AGCAGAGCCGGAGGAGAAC
Cow PDX1-R	CCTGGAGATGTATTTGTTGAAAAGG
Cow Pax4-F	ATTCCAGCGTGGGCAGTATC
Cow Pax4-R	TCGGTTGGAAAACCAGATCCT
Cow Glucagon-F	TCCCAGAAGAAGTCAACATCGTT
Cow Glucagon-R	CGGGTGGCAAGACTATCGA
Cow Fox-F	CCACCTGAAGCCGGAACA
Cow Fox-R	GGGCTGGTGGTGGTGATG
Cow Nkx6.1-F	GCTCGCTTGGCCTATTCGT
Cow Nkx6.1-R	CCATCTCGGCTGCATGCT
Cow Pax6-F	TCAGCACCAGCGTCTACCAA
Cow Pax6-R	TGTTTGTGAGGGCTGTGTCTGT

Dithizone staining

Ten milligrams of DTZ (Sigma) was dissolved in 10 mL dimethyl sulfoxide (Sigma) and stored at −20°C (stock solution). The solution was then diluted with DPBS to a ratio of 1:10 before staining. After the morphology of islet-like cell clusters was observed, the cell clusters were incubated in the dithizone staining solution at 38.5°C for 15 min. Notably, because insulin is chelated by zinc ions, the dye combines with the zinc ions and stains bright red.

Immunocytochemical methods

After differentiation, islet-like cells and undifferentiated CPSCs (for negative control) were washed with DPBS and fixed with 4% paraformaldehyde for 15 min, followed by another three washings. DPBS containing 0.25% Triton-X and 1% BSA were used to block and permeabilize at room temperature for 30 min. Primary antibodies (mouse antibovine insulin, SPM531; diluted 1:100 in DPBS containing 1% BSA) were then added to the cells and incubated overnight at 4°C. In addition, cells were incubated with secondary antibodies without primary antibodies to create negative controls. Subsequently, all cells were washed with DPBS containing 1% BSA and incubated with secondary antibodies (goat antimouse Alexa Fluor 488; diluted 1:100 in DPBS containing 1% BSA) at room temperature for 1 h in dark. Subsequently, cells were washed three times, incubated with 4′-6-diamidino-2-phenylindole (DAPI), and