Taiwanese Journal of Obstetrics & Gynecology 56 (2017) 306–311

Contents lists available at ScienceDirect

Taiwanese Journal of Obstetrics & Gynecology

journal homepage: www.tjog-online.com

Original Article

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

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ARTICLE INFO

Article history:
Accepted 21 February 2017

Keywords:
Cow
Islet-like cells
Placenta
Stem cells

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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http://dx.doi.org/10.1016/j.tjog.2017.04.007

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

covered with a mounting medium. After staining, the cells were observed with an inverted fluorescence microscope.

cDNA synthesis and polymerase chain reaction

Finally, the undifferentiated CPSCs (negative control) and differentiated islet-like cells were recovered using a 0.1% trypsin treatment. Total mRNA were extracted from the recovered cells using a RNeasy Micro kit, and the cDNA was reverse-transcribed from 1 μg mRNA using a QuantiTect Reverse Transcription kit (QIAGEN, 205411), according to the manufacturer's protocol. RT-PCR was also conducted, using a QuantiNova SYBR Green PCR kit (QIAGEN, 208054) as follows: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 5 s, and concluded with annealing at 60°C for 10 s. Details of the gene-specific forward and reverse primers for the cow islet-like cells are provided in Table 1.

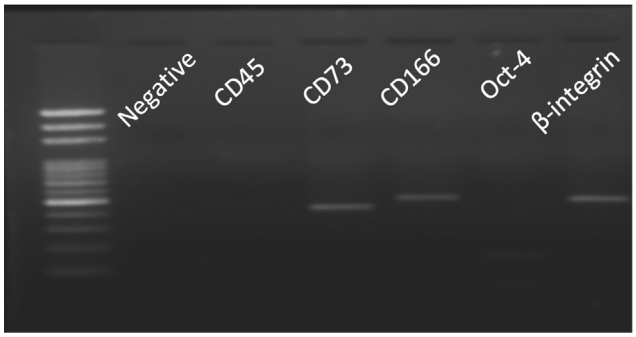

Fig. 2. CPSC identification through RT-PCR-based gene expression of CD45, CD73, CD166, Oct-4, and β-integrin. Notably, the CPSCs did not express the hematopoietic stem cell surface antigen marker (CD45), but did express mesenchymal stem cell surface antigen markers, such as CD73, CD166, and β-integrin; moreover, the CPSCs expressed the pluripotent marker, Oct-4.

Results

Cow placenta was successfully collected, and stem cells were harvested from the placental fragment (Fig. 1). The CPSCs expressed mesenchymal stem cell surface antigen markers, such as CD73, CD166, and β-integrin, and the pluripotent marker Oct-4 (Fig. 2). However, the CPSCs did not express the hematopoietic stem cell surface antigen markers, such as CD45 (Fig. 2), indicating that the CPSCs maintained their characteristics without contamination. After supplementation with 10 mM nicotinamide, 10 μM exendin-4, and 28 mM glucose, and being coated with poly-D-lysine, the CPSC colonies gradually formed (Fig. 3). The morphological transformation of CPSCs during the differentiation of islet-like spherical clusters was clearly observed (Fig. 3). DTZ staining confirmed that islet-like cells differentiated from CPSCs; notably, the clusters stained red because of insulin secretion (Fig. 4). Insulin secretion from the differentiated islet-like cells was also confirmed through immunocytochemical methods, where the cell clusters were stained fluorescent green with insulin antibodies (Fig. 5). Finally, the RT-PCR results revealed the expression of specific pancreatic genes, such as nestin, insulin, glucagon, pax-4, Nkx6.1, pax-6, and Fox, in the differentiated islet-like cells (Fig. 6).

Discussion

We successfully harvested stem cells from cow placenta, which was collected noninvasively without any complications or ethical concerns. These CPCSs expressed mesenchymal stem cell surface antigen markers, a finding that is consistent with a study by Lu et al. that demonstrated that cow-derived bone marrow mesenchymal stem cells express CD73 but do not express the hematopoietic cell surface antigen markers, such as CD45 [23]. Moreover, our results correspond with a research conducted by Chang et al., which revealed that human-placenta-derived stem cells express the Oct-4 gene, thus indicating that the CPSCs are pluripotent [37].

In islet-like cell differentiation, glucose is involved in the growth of pancreatic β-cells via the phosphatidylinositol 3-kinase pathway and in the nuclear translocation to regulate the pdx-1 expression [38]. Nicotinamide and exendin-4 also stimulate pancreatic β-cell differentiation [39]. The other essential differentiation factor is the presence of an extracellular matrix (ECM) such as poly-D-lysine, which plays a crucial role in intercellular connection and support, water retention, compression resistance, cell migration, cell differentiation, and cell physiology. Studies have demonstrated that the ECM manipulates cell fate by influencing cell adherence and movement, as well as message deliverance, which consequently affect cell differentiation, cell cluster formation, and cell survival rate [40,41]. Islet-like cells differentiated from the stem cells were cultured in decellularized scaffolds, inclusive of vascular channels, to benefit islet-like cell development [42]. Furthermore, islet-like cells encapsulated by polymers [43] or pancreatic ECM [44] prior to transplantation have been successful in evading immune rejection. The present study revealed that poly-D-lysine enables the formation of a stable tertiary cell cluster structure and stimulates cell differentiation. Spherical clusters were also observed after

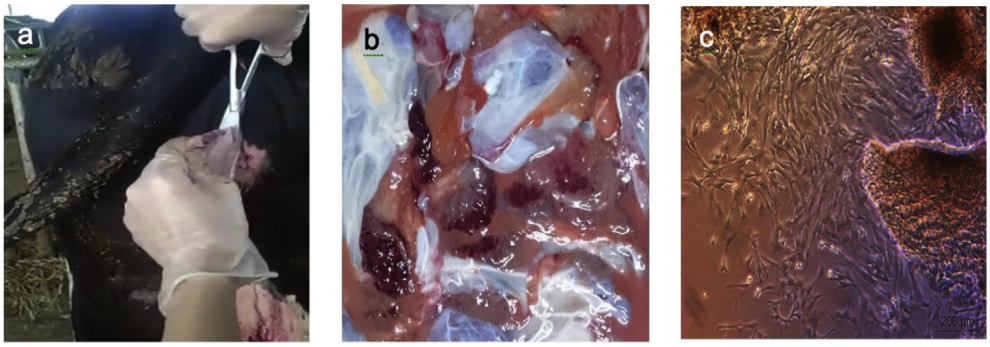

Fig. 1. Cow placenta collection and stem cell harvesting from placental fragments. Placenta (b) was cut using surgical scissors before it fell on the ground (a). Stem cells were harvested from the cow placental fragment (c).

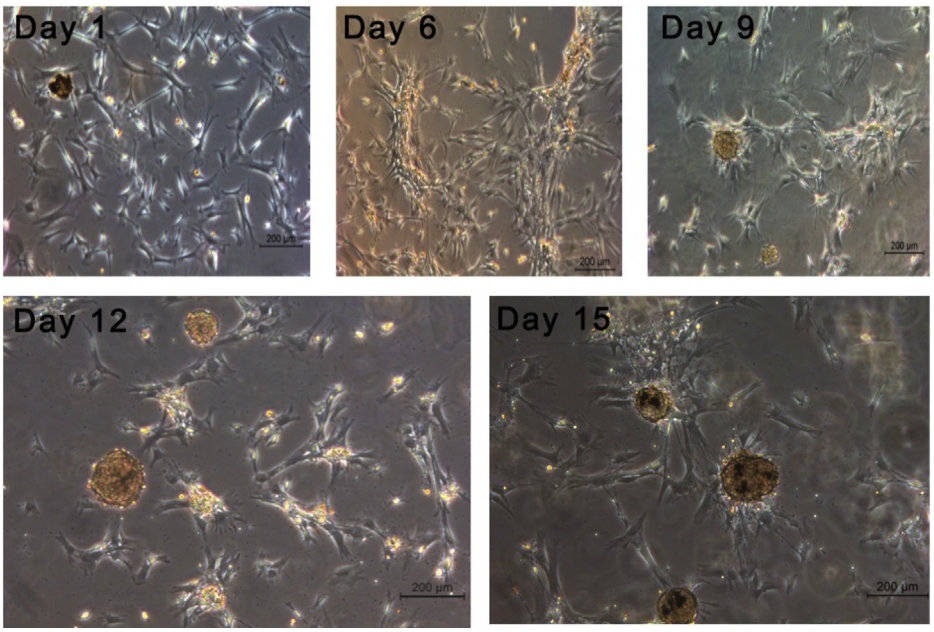

Fig. 3. Various differentiation periods of the CPSCs and their morphologies. CPSCs were first maintained in a spindle shape without considerable changes (Day 1), but CPSC colonies gradually formed (Days 6–9). Spherical cell clusters were clearly visible by Days 12 and 15.

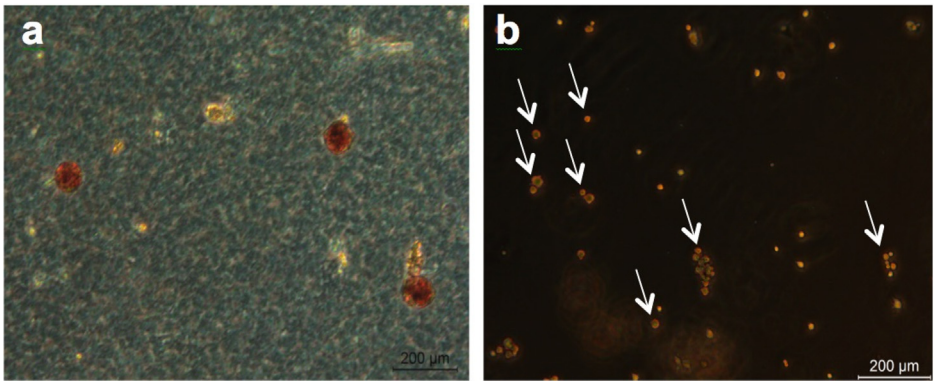

Fig. 4. DTZ staining confirmed islet-like cell differentiation from CPSCs after 15 days. The cell clusters (a) are red in color because of insulin secretion; clusters separated into single cells also stain red. The white arrows indicate red-colored DTZ staining (b).

induction [37]. Furthermore, Lucas-Clerc et al. verified that ECM increases islet cell gene expression and subsequently increases insulin secretion after islet cell differentiation [45].

Intact islet cells are composed of insulin- and glucagon-secreting cells. Notably, exendin-4 can potentially promote stem cell differentiation into glucagon-secreting and insulin-producing cells [35,46]. Our RT-PCR results indicated that the CPSCs supplemented with exendin-4 can not only differentiate insulin-expressing cells but also glucagon-expressing cells. In addition, the differentiated islet-like cells expressed pax-4, Nkx6.1, pax-6, and Fox genes, which is consistent with several other studies on islet-like cells differentiated from various stem cell sources [9,14,35,37]. Notably, nestin genes were expressed either before or after the differentiation of CPSCs, indicating that the CPSCs expressed nestin genes. Furthermore, Mehrfarjam et al. revealed that the nestin-positive mesenchymal stem cells have a higher islet cell differentiation rate [15]. Therefore, CPSCs are the preferred stem cell source for islet-like cell differentiation. Furthermore, we investigated the functionality of the differentiated islet-like cells through immunocytochemical detection of insulin antibody and by performing DTZ staining to substantiate insulin zinc ion expression. These outcomes indicated that the differentiated islet-like cells produced insulin protein.

In conclusion, we successfully differentiated islet-like cells from the CPSCs by supplementation with glucose, nicotinamide, exendin-4, and poly-D-lysine without gene manipulation. Xin et al. revealed that these cells with insulin-producing cell differentiation capabilities can be used to treat diabetic animal models after transplantation [34]; in short, islet-like cell transplantation offers an alternative therapy for diabetes. Future investigations are warranted to explore the applicability of CPSCs for preclinical treatment of diabetic animal models.

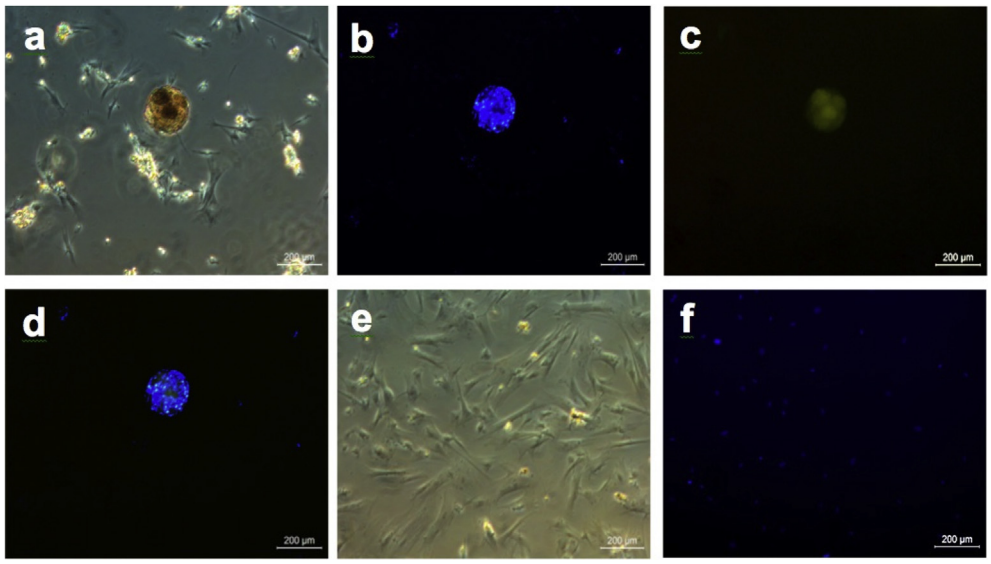

Fig. 5. Immunocytochemical analysis of insulin protein expression, which confirmed the differentiation of islet-like cells from CPSCs. Differentiated islet-like cell clusters are shown (a) as stained with DAPI to confirm their nuclear position (b). Other differentiated islet-like cells were stained green with the insulin antibody (goat antimouse fluorescein isothiocyanate-conjugated insulin) (c). Notably, (c) and (d) were merged to determine the relative nuclear position (d). Undifferentiated CPSCs that did not express the insulin protein were considered the negative control group (e, f).

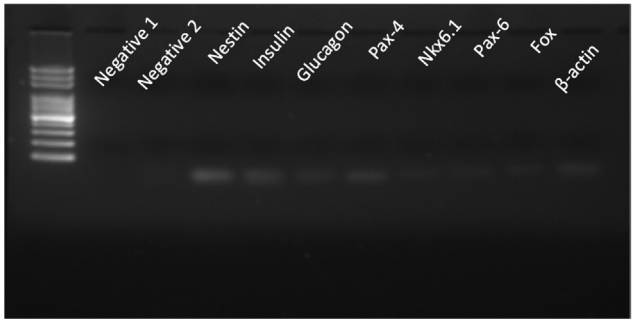

Fig. 6. Differentiated islet-like cells express typical pancreatic genes, such as nestin, insulin, glucagon, pax-4, Nkx6.1, pax-6, and Fox. These genes are markers for a mature pancreas, islet development, endocrine cell development, and glucose regulation. Negatives 1 and 2 are the CPSCs with RNase-free water and undifferentiated CPSCs, respectively, which are considered the negative control groups.

Conflict of interest

No conflict of interest.

Acknowledgments

This study was funded by the Ministry of Science and Technology of Taiwan (MOST 105-2313-B-020-001-MY2 and MOST 104-2314-B-182A-136), and Chang Gung Memorial Hospital (CMRPG3D1683, CMRPG3G0181).

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