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Original Article

Characterization of Tibetan Mastiff pancreatic progenitor cells and differentiation into insulin-secreting cells

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Abstract: It is the first time to explore the biological characterization and differentiation of Tibetan Mastiff derived pancreatic progenitor cells. In this investigation, we isolated, purified, and cultured the pancreatic progenitor cells (PPCs) from islet tissue. PPCs could be cultured until passage 13 with a typical spindle shape and they were positive to the mesenchymal stem cells surface markers, while negative to the epithelial cells specific marker CK19. Immunofluorescence staining analysis was conducted and the results demonstrated that PPCs expressed NESTIN, NKX6.1, PDX1 and VIMENTIN. Results of karyotype indicates a stable property for PPCs. Differentiated insulin-secreting cells were detected through morphology observation, RT-PCR, immunofluorescence staining and dithizone staining. All results revealed the differentiation was successful and efficient.

Keywords: Tibetan mastiff, islet cells, insulin-secreting cells, pancreas tissue

Introduction

Tibetan Mastiff (TM) is kind of a dog, which mainly lived in the Qing-Tibet Plateau. The TM is considered to be the oldest and most ferocious and nowdays has developed as pet [1]. Due to the hard living conditions, settled in the Qing-Tibet Plateau for animals and humans are all challenges [2]. Therefore, the TM has a vital significance for the people living there. The TM can adapt well to high altitude and have outstanding strength and endurance [3]. The TM also has a powerful metabolic ability to endure the plateau hypoxia response [4].

Diabetes mellitus (DM) is a high blood sugar disorder, which is caused by the failure insulin supply of beta cells [5]. It can be divided into two types: type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) [6]. T1DM is an autoimmune disease characterized by endocrine beta cells, whereas T2DM is an insulin resistant disease [7-10]. The deficiency of functional beta cells is the major cause of hyperglycemia and related diseases [11]. Because of the long course of disease, the high cost, the high mortality rate and the disability of current treatment methods, it is urgent to find a new treatment direction for diabetes. Studies presented that transplantation of pancreas and expansion of functional beta cells can both regulate glucose homeostasis in vivo [12]. Organ transplantation is limited owing to the lack of donor organs and immune rejection, resulting in the expansion of functional beta cells in vivo as the best choice for the treatment of diabetes and other related diseases [6]. Beta cells are the only effective factors to regulate glucose homeostasis. They can also effectively repair the disorder of insulin production in diabetes mellitus and provide a new direction for the treatment [13].

Pancreas is consisted of endocrine region and exocrine region. There are many hormones such as insulin, glucose, somatostatin and pancreatic polypeptide secreted by pancreas [6]. The physiological and pathological changes of pancreatic tissue are closely related to life. Undifferentiated cells in pancreas are with self-

Table 1. Primer Sequences Used In RT-Polymerase Chain Reaction

Gene	Sequence (5' to 3')	Produce size	Tm (°C)
C-myc	Forward: GCGACTCGGAGGAAGAACAA	333	60
	Reverse: CGTTGTGTGTTCGCCTCTTG		
CD44	Forward: CCCCATTACCAAAGACCACGA	408	60
	Reverse: TTCTGCAGGTTCCGTGTCTC		
Vimentin	Forward: TTGGCACGTCTTGACCTTGA	431	59
	Reverse: TAGTTAGCAGCTTCGACGGC		
Nestin	Forward: TCCGGGAAGGAGTCTGTAGG	267	60
	Reverse: ACCCTCTGGGGACTCATCTC		
Insulin	Forward: CGGCTTCTTCTACACGCCTA	202	59
	Reverse: GCGCCCCTAGTTGCAGTAAT		
CK19	Forward: GGGCGATGTGCGAGCTGATAGTGA	189	58
	Reverse: AAAGGACAGCAGAAGCCCCAGAGC		
KRT7	Forward: GCAGGCTGAGATCGACAACA	296	60
	Reverse: TCACCACAGAGATGTTCACGG		
GAPDH	Forward: TCCATCTTCCAGGAGCGAGA	578	60
	Reverse: TCCGATGCCTGCTTCACTAC		

renewal ability and multipotency [14]. Current research indicated that pancreatic stem cells were mainly originated from ducal tissue, islets and acinus. Cells derived from ducal tissue appeared as cobblestone, while the cells which were isolated from islets were spindle [15]. Cells derived from pancreas have large advantages on diabetes treatment compared with other tissue cells. Firstly,the cells derived from pancreas could be differentiated into functional insulin-secreting cells more easily than other tissue cells. Secondly, the cells derived from pancreas could be with lower possibility of organ rejection. Thirdly, pancreas tissue has no ethical issues [6].

Cells studied in this research were isolated from pancreatic islets and expressed Nestin. Those cells named as pancreatic progenitor cells (PPCs) have a great significance in the development of islets [14, 16-18]. In this study, we characterized the PPCs of TM and evaluated the efficiency of insulin-secreting functional differentiation. We also supplied a new cell resource for the treatment of diabetes.

Materials and methods

Isolation and characterization of PPCs from Tibetan Mastiff pancreas

Tibetan Mastiff (TM) was supplied by Beijing breeding center according to the provisions of Animal Care Committee at Chinese Academy of Agricultural Sciences. All experimental protocols were approved by the Experimentation Committee of the Chinese Academy of Agricultural Sciences.

To separate the islets from the pancreas, we isolated the islets using enzymatic treatment and mechanic operation under sterile condition. The islets were washed with phosphate-buffered saline (PBS) for more than 6 times. The islets were cut into small pieces after removing excess membranes and blood vessels. Then they were digested at 37°C for 15~20 min by 2.5 g/l trypsin (Amresco, USA). During digestion, the tissues were gently shaken every 5 minutes to make sure that the digestion was complete. We neutralized the digestion with DF12 (Gibco, USA) contained with 10% (v/v) fetal bovine serum (FBS; Gibco). After centrifuging, the pellets were obtained. We re-suspended them with a complete medium which was composed of 90% DF12, 10% FBS, 10 ng/ml leukaemia inhibitory factor (LIF, PeproTech, USA) and 1% penicillin/streptomycin. The medium was refreshed every 2 days.

RT-PCR analysis

Cellular mRNAs at three different passages were obtained by Trizol reagent (Invitrogen). Then the mRNAs were reversed as cDNA. The products employed as templates were amplified by polymerase chain reaction (PCR). Primers were presented in **Table 1**. The results were visualised through 2.5% agarose gel electrophoresis.

Immunofluorescence detection

Immumofluorescence (IF) staining was conducted on passage 3 cells. Cells were fixed with 4% paraformaldehyde at ambient temperature for 20 min. Then the cells were permeabilized for 10 min at room temperature with 0.1% Triton X-100 (Sigma) and blocked with 10% goat serum for 60 mim. The cells were stained with the following antibodies: NESTIN (1:200, Bioss), NKX6.1 (1:200, Abcam, USA), duodenal homeobox 1 (PDX1; 1:200, Abcam, USA)

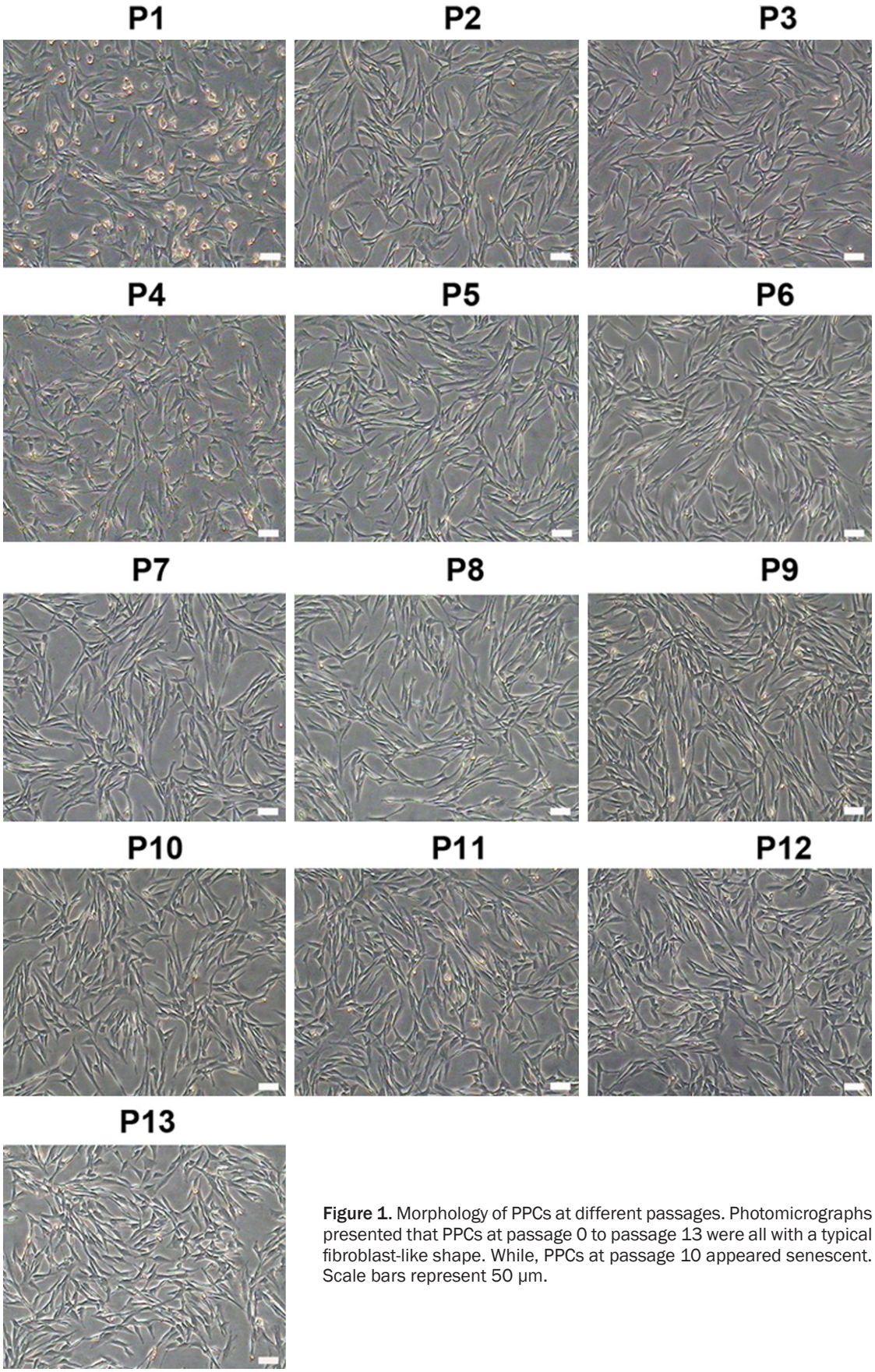


Figure 1. Morphology of PPCs at different passages. Photomicrographs presented that PPCs at passage 0 to passage 13 were all with a typical fibroblast-like shape. While, PPCs at passage 10 appeared senescent. Scale bars represent 50 μm.

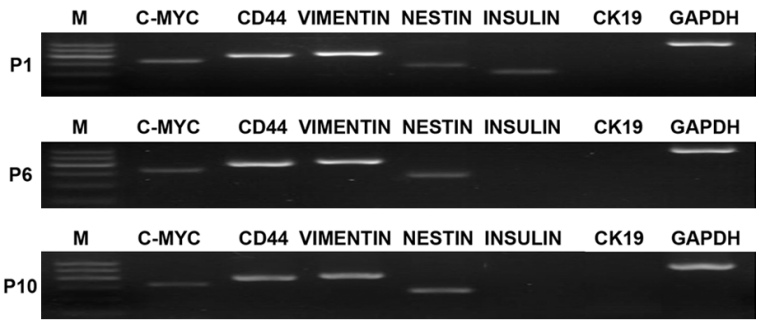


Figure 2. Specific surface markers of PPCs were detected by RT-PCR. Results showed the PPCs at passage 1 expressed C-myc, CD44, Vimentin, Nestin, Insulin, while did not express CK19. PPCs at passage 6 and 10 only expressed C-myc, CD44, Vimentin, Nestin.

and VIMENTIN (1:200, Abcam, USA) at 4°C overnight. FITC-conjugated secondary antibodies (1:200) were used at room temperature for 60 min. DAPI was used to counter stain nuclei under black condition. Results were presented by a con-focal optical system (Nikon, TE2000).

Karyotype analysis

PPCs were evaluated at 30% confluence. Firstly, cells were treated with hypotonic medium (0.075 mol/l KCl, 0.017 mol/l Na-citrate). Then the cells were fixed with ethanol: acetic acid (1:2:1) and stained with Giemsa (1-s2.0). Finally, counting the numbers of the chromosome was performed with Cytovision Applied Imaging.

Flow cytometry

Surface antigen expression of PPCs and differentiation were analyzed using flow cytometry. Cells were digested with 0.25% trypsin-EDTA and centrifugated. Then, the cells were fixed, washed and permeabilized. Cells were stained with the following antibodies: at 4°C over night. After washing with PBS, the cells were incubated with FITC-conjugated secondary antibody at 4°C for 1 h. For the control group, cells were incubated with PBS. Finally, the cells were washed and stained with DAPI at room temperature under black condition for 20 min. Results were evaluated by FACS using CellQuest Research Software.

Differentiation into insulin-secreting cells

To be induced into functional insulin-secreting cells, PPCs at passage 6 were digested and replated at 50% confluence. The method for induction was along with previous research (6.87 diabetes). Firstly, the cells were exposed to high glucose Dulbecco's modified Eagle medium (H-DMEM) 25 mmol/l, 10% FBS and 10-6 mol/l retinoic acid (Sigma Aldrich) for 24 h and followed by 2 day treatment with H-DMEM and 10% FBS. Secondly, the cells were changed into the medium containing low glucose DMEM 5.56 mmol/l, 10% FBS, 10 mmol/l nicotinamide (Sigma-Aldrich), 20 ng/ml epidermal growth factor (PeproTech, Rocky Hill, NJ), 50 ng/ml of FGF-10 (R&D, Minneapolis, MN), and 300 nmol/l of (-)-indolactam V (LC laboratories, Woburn, MA) for 9 days. Thirdly, the cells were cultured with the medium containing 90% L-DMEM, 10% FBS, 10 nmol/l exendin-4 (Sigma-Aldrich), and 50 ng/ml Activin A (PeproTech) for 7 days. For the control group, the cells were cultured with 90% DMEM/F12, 1% penicillin/streptomycin, 1% amphotericin B, and 10% FBS.

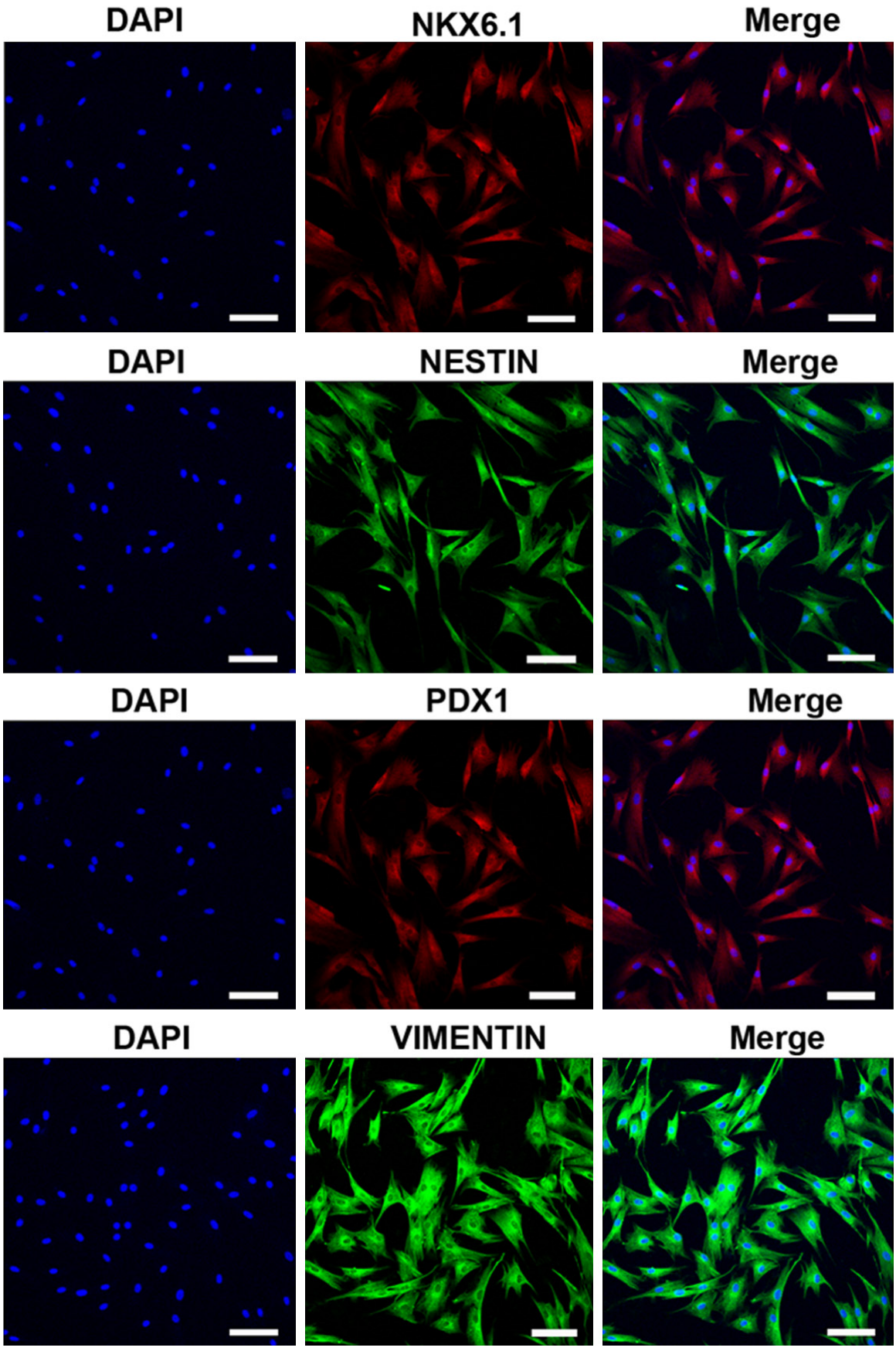
RT-PCR analysis and dithizione staining

Cellular mRNAs for the induced cells and the control group were obtained by Trizol reagent (Invitrogen). Then the mRNAs were reversed as cDNA. The products employed as templates were amplified by polymerase chain reaction (PCR). Primers were presented in Table. The results were visualized through 2.5% agarose gel electrophoresis.

Dithizone (DTZ, Sigma) were used to assess the differentiation. The induced cells were fixed with 4% paraformaldehyde at room temperature for 30 min, then they were treated with DTZ solution for 1 h at 37°C. DTZ solution were consisted of 50 mg DTZ and 5 ml dimethyl sulfoxide (DMSO, Sigma). Results were observed by microscope.

Immunofluorescence detection

Immunofluorescence (IF) staining was performed for induced cells and the control cells. Both cells were fixed with 4% paraformaldehyde at room temperature for 20 min. Then the cells were permeabilized for 10 min at room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PDX1
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VIMENTIN
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Figure 3. Immunofluorescence staining for PPCs antigens. DAPI was used to stain the nuclei. Results presented that PPCs were positive to the antigens of NKX6.1, NESTIN, PDX1 and VIMENTIN. Scale bars represent 200 um.

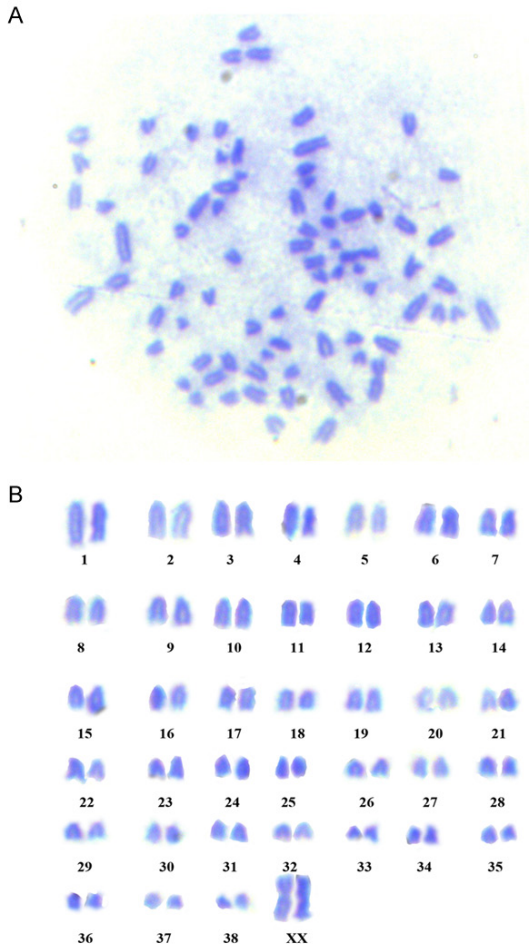


Figure 4. Karyotype analysis of Tibetan Mastiff derived PPCs. Tibetan Mastiff derived PPCs had 2n=78 diploid chromosome numbers. They were consisted of 38 pair of chromosomes and 1 pair of sex chromosomes. A XX type spread in metaphase was presented (n=100).

temperature with 0.1% Triton X-100 (Sigma) and blocked with 10% goat serum for 60 mim. For the induced cells, they were stained with the following antibodies: C-CEPTIDE (1:200, Abcam, USA) and GLUCOSE (1:200, Abcam, USA) at 4°C overnight. For the control group, the cells were treated with PBS at 4°C overnight. FITC-conjugated secondary antibodies (1:200) were used at room temperature for 60 min. DAPI was used to counter stain nuclei under black condition. Results were presented by a con-focal optical system (Nikon, TE2000).

Statistical analysis

Statistical analyses of the data were performed with a one-way ANOVA followed by the Tukey-Kramer honestly significant difference test for the three sets of results. A *P*-value of less than 0.05 was considered significant. Statistical analyses were conducted with a JMP Statistical Discovery Software (SAS Institute, Cary, NC).

Results

Isolation and purification of PPCs

Cells isolated from islets formed a cell layer and presented an obvious fibroblast-like morphology (**Figure 1**). Cells first plated on the plastic after 16 h inoculating. In the beginning, PPCs at passage 0 took 3 days to proliferate to passage 1. Along with the time, PPCs grew rapidly and could be passaged every 1 or 2 days. Until PPCs proliferated to passage 10, they appeared as blebbing and karyopyknosis and had a lower proliferate ability. PPCs at passage 11 took 4 day to be proliferated to passage 12.

Reverse transcription-polymerase chain reaction of PPCs

Results were presented by agarose gel. We detected PPCs at passage 0, passage 6 and passage 10. The cells at passage 0 expressed C-MYC, CD44, VIMENTIN, NESTIN, INSULIN, but did not express epithelial marker CK19. The cells at passage 6 and passage 10 expressed C-MYC, CD44, VIMENTIN, NESTIN, but did not express INSULIN and epithelial marker CK19 (**Figure 2**).

Immunofluorescent detection of cell surface markers

Surface marker antigens for PPCs were analyzed by immunofluorescence staining. Results presented that PPCs were positive for NESTIN, NKX6.1, duodenal homeobox 1 and VIMENTIN (**Figure 3**).

Karyotype analysis

Tibetan Mastiff derived PPCs were diploid (2n =78), consisting of 38 pair of euchromosomes

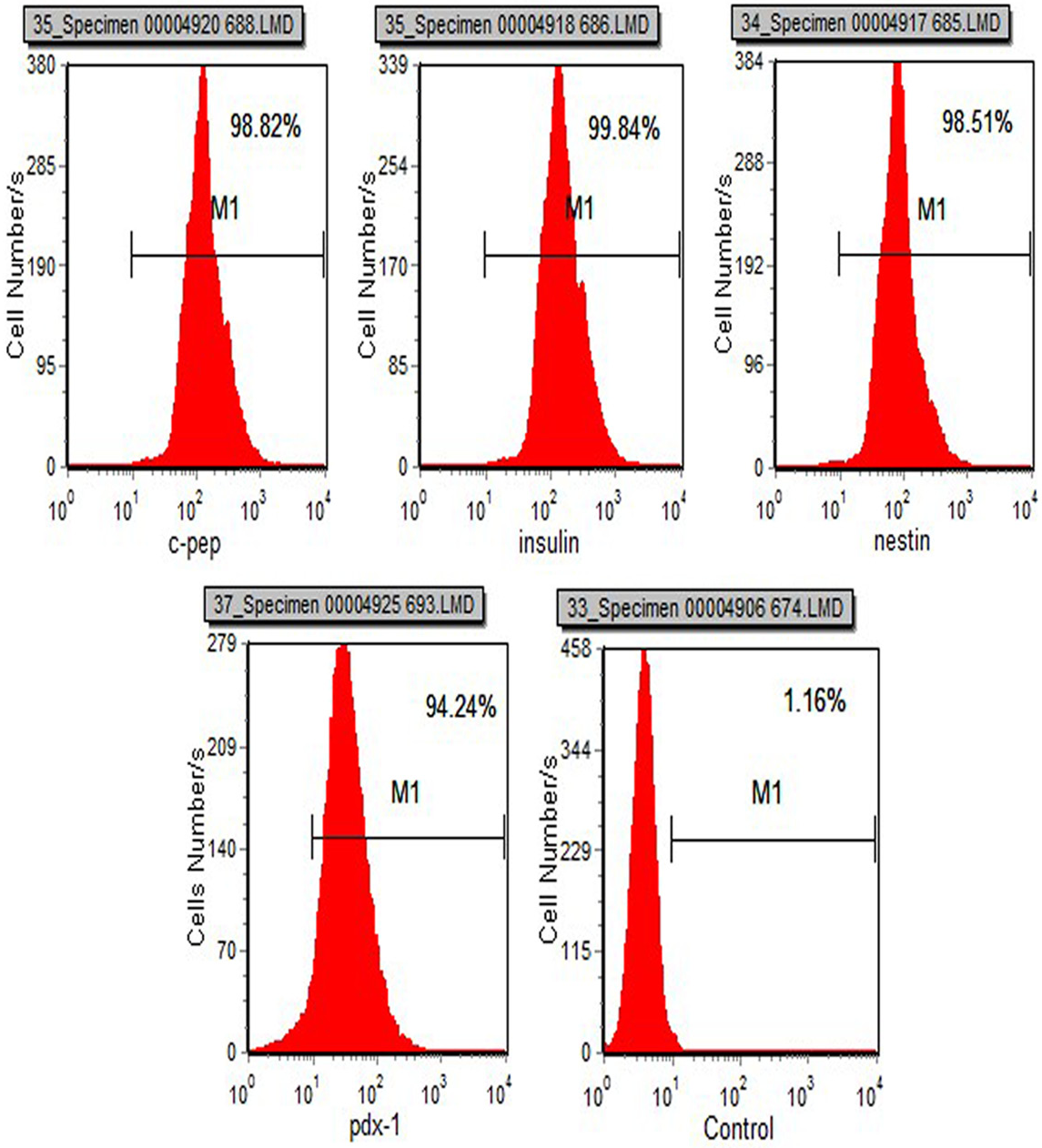


Figure 5. PPCs were stained with surface specific markers as C-Pep, insulin, nestin and pdx1 and analyzed by flow cytometry. The positive population was enclosed by the line.

and 1 pair of sex chromosomes. Results indicated that the hereditary property of the cells cultured in vitro was stable (**Figure 4**).

Positive rate detetction of PPCs

Surface specific antigens of PPCs were assessed by flow cytometry (**Figure 5**). Results presented that the positive rate of PPCs to C-Peptide was 98.82%, the positive rate of PPCs to insulin was 99.84%, the positive rate of PPCs to nestin was 98.51%, a positive rate of PPCs to pdx1 was 94.24%.

Differentiation of insulin-secreting cells

The differentiation of PPCs into insulin-secreting cells was evaluated through morphology observation, RT-PCR analysis and immunofluorescence staining (**Figures 6**, **7**). There was

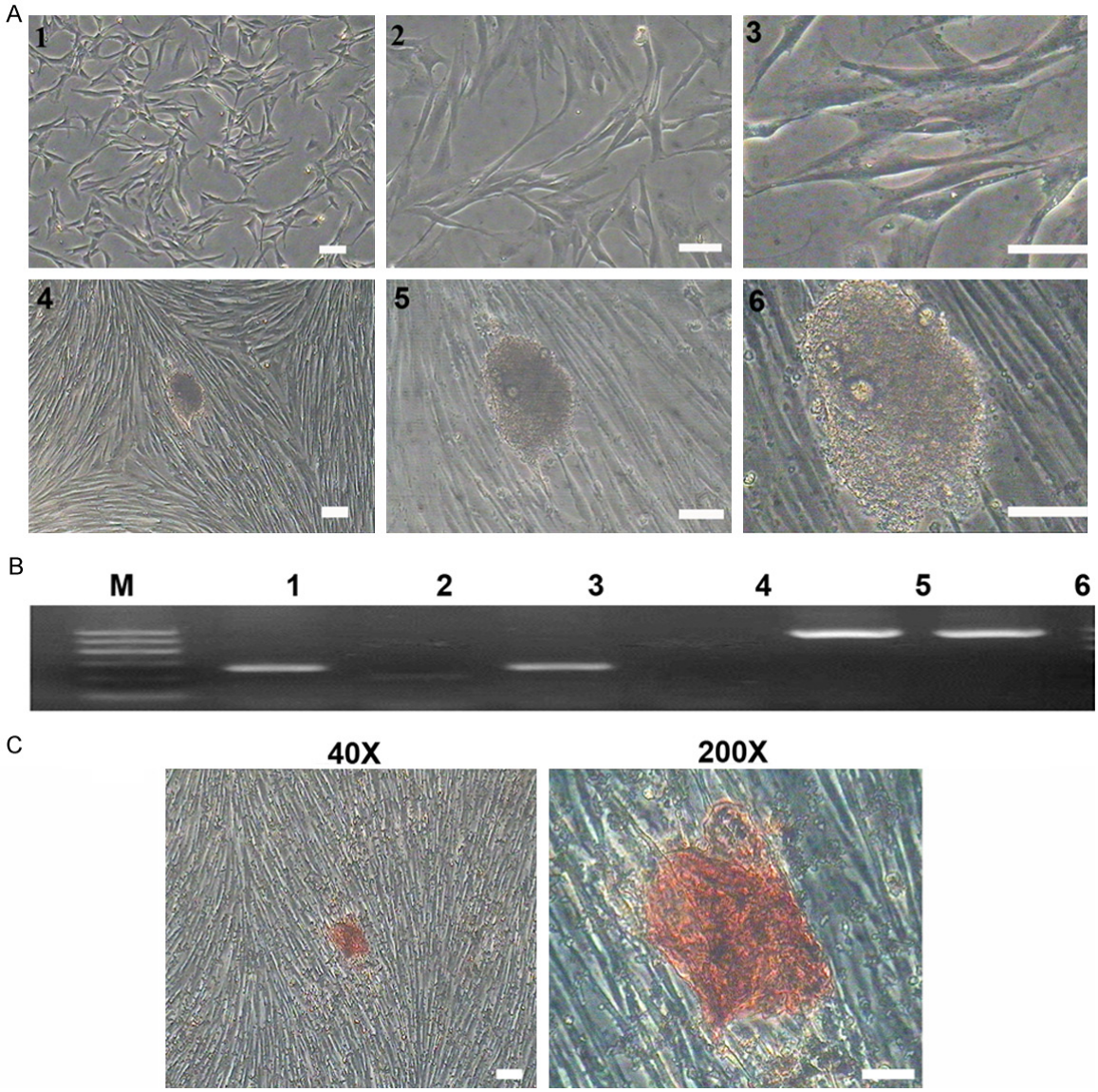
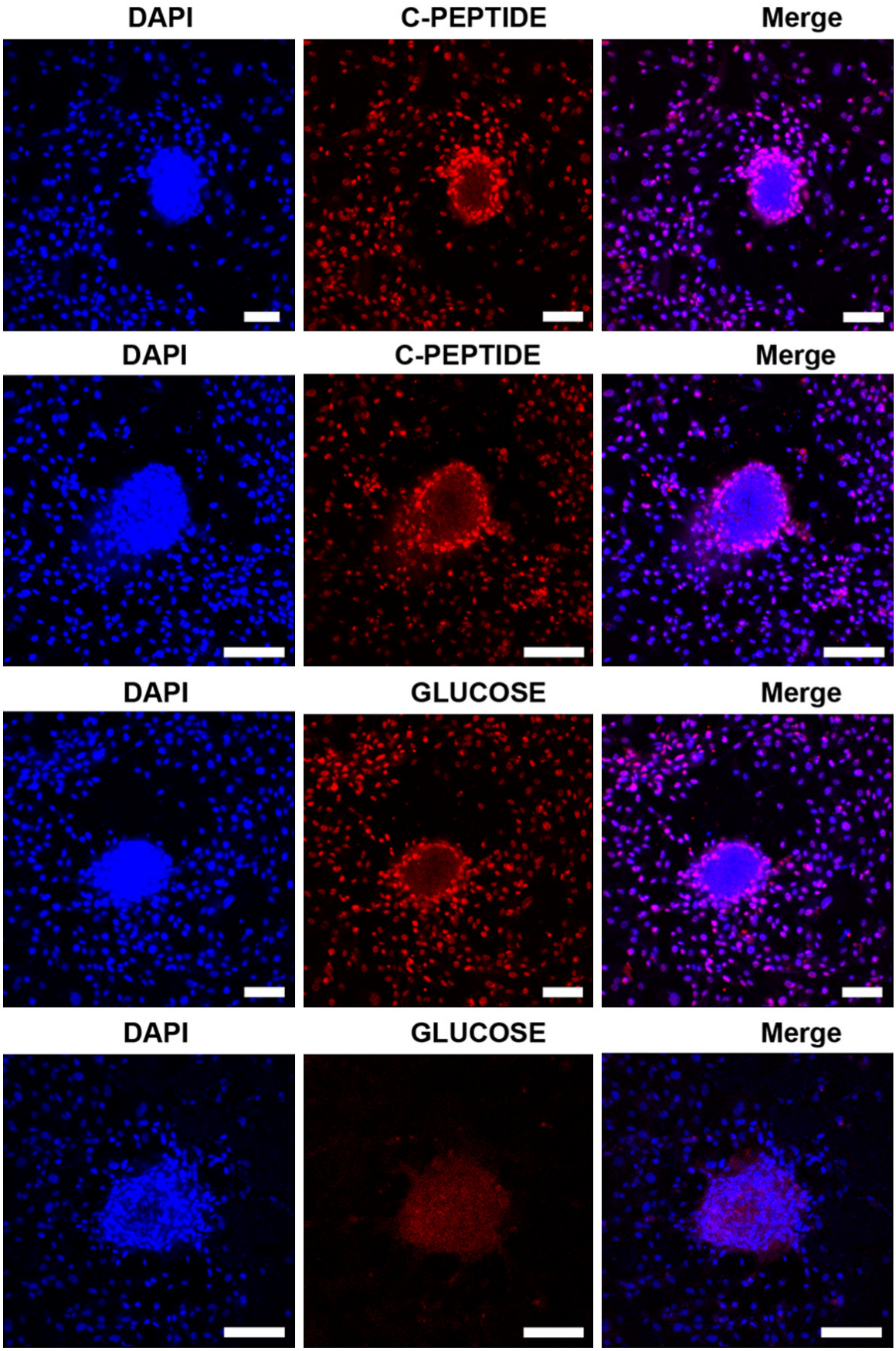


Figure 6. Differentiation of PPCs into insulin-secreting cells. A. Morphology detection. The induced cells formed an islet-like cluster at 7 day after induction. 1, Magnification, ×40; 2, Magnification, ×100; 3, Magnification, ×200. B. RT-PCR analysis. Electrophoretogram revealed that the induced cell both expressed KRT7 and Insulin genes, while the control group cells only expressed KRT7 gene. 1, 2 were the induced group; 3, 4 were the control group; 5, 6 were GAPDH (an internal control). C. Dithizone staining of induced cells. Islet-like cluster was stained with dithizone and dyed scarlet. Pictures were magnificated to 40× and 200×.

no obvious change observed for the first week. Until the 10th day, there was little islet-like clusters appeared. Along with the differentiation, more and larger islet-like clusters were observed, which was typical islet morphology (**Figure 5A**). We also detected the differentiation through RT-PCR and dithizone staining (**Figure 6B**, **6C**). Results presented that the induced group cells both expressed KRT7 and INSULIN, while the control group cells only expressed KRT7. Dithizone staining results demonstrated that the induced cells were positive to dithizone and dyed scarlet. C-PEPTIDE and INSULIN were specific antigens of insulin-secreting cells and they were detected by immunofluorescence staining (**Figure 7**). Results indicated that the induced cells were both positive to C-PEPTIDE and INSULIN.

Discussion

Stem cells should fulfill the following two standards: (1) Cells could proliferate themselves without induction for a long time; (2) They could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GLUCOSE
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Figure 7. Detection of differentiation by immunofluorescence staining. C-PEPTIDE and GLUCOSE staining were both located in the cytoplasm. DAPI was used to stain the nuclei (blue), and C-PEPTIDE and GLUCOSE were used to stain the cytoplasm (red). They were both magnificated to 100× and 200×.

be differentiated into multiple cell types [19]. In this study, we isolated PPCs from islet tissue with trypsin and inoculated them in the plate with a aleukaemia inhibitory factor (LIF). LIF could inhibit cell differentiation and promote self-renewal ability [20]. LIF was usually used in stem cells medium to restrain spontaneous differentiation. Because pancreas tissue contained many different cells, it was important for us to isolate islet tissue. PPCs could renew themselves from passage 0 to passage 13 with a stable spindle-shape. Results of RT-PCR presented that PPCs expressed the mesenchymal stem cells surface markers, while did not express the epithelioid cells specific marker. PPCs derived from Tibetan Mastiff also had a normal karyotype as well as an ability to differentiate into insulin-secreting cells. All results revealed that PPCs we isolated were highly homogenous and multipotent.

PPCs could also be differentiated into insulin-secreting cells by a three-step method in this study. Owing to many different protocols for insulin-secreting cells differentiation, this three-step method was high efficient and able to prevent transfection. The reprogramming protocols were immediate and effective, but due to the unsafe exogenous genes, they were unsafe. In this three-step method, H-DMEM and many other factors were used. High glucose was an inducer in islet induction, and H-DMEM was a medium consisted of DMEM and 23.3 mmol/l glucose [21]. H-DMEM was added at the first step to begin induction. At the second step, the medium was added by L-DMEM, nicotinamide, epidermal growth factor (EGF), FGF-10 and indolactam V (LV). Islet clusters were appeared and aggregated mainly at this stage. LC could maintain the differentiation more efficient [8]. Nicotinamide and lower glucose could avoid the cells dying or differentiating [11]. Nicotinamide could promote the production of insulin-secreting cells and protect cells from high concentration glucose [24]. EGF was a factor which was fit for PPCs proliferation, maturation and PDX-1 positive cells growth [25]. At the third stage, exendin-4 and Activin A were employed as inducers. Exendin-4 was a glucagon-like peptide-1 analog and it could induce PPCs into insulin-secreting cells and inhibit insulin-secreting cells apoptosis [25, 26]. Active A diverted the induction into a PAX-4 expressing pancreatic lineage and not a glucagon producing cells (αcells) [27]. The successful induction of PPCs into insulin-secreting cells was assessed by RT-PCR detection, DTZ staining and immunofluorescence staining.

The first pancreas transplantation was conducted in 1966. At present, the pancreas transplantation was performed with a kidney transplantation. Though the transplantation has a 77% survival rate for the 1st year, it was still accompanied by immunosuppressive and organ injection. Islet transplantation provided a safe method. However, it was still restricted by the shortage of donor organs and unable to proliferate in vitro. Diabetes could be cured through stem cell treatment. Therefore, PPCs were considered as the most perfect stem cell resource for the treatment of diabetes and degenerative diseases. Functional differentiation into insulin-secreting cells of PPCs has been considered as a remarkable breakthrough. Through stem cell therapy was a hot spot in the current research, there were still lots of challenges need to overcome. How to obtain mature insulin-secreting cells efficiently? How to obtain high inductivity to prevent teratoma formation? How to figure out the molecular mechanisms under the differentiation? Hence, we should focus on the mechanisms of differentiation and figure out each stage of differentiation both in morphology and molecular level. It was of great significance for us to explore the differentiation mechanism and signial pathways contained in it.

Conclusion

In this article, we isolated, purified and characterized PPCs from Tibetan Mastiff derived pancreatic islet tissue and differentiated them into insulin-secreting cells using a three-step method. This was the first time to explore Tibetan Mastiff derived PPCs. It had great significance to the diabetes patients.

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Disclosure of conflict of interest

None.

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