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Tailor-made generation of insulin-producing cells from canine mesenchymal stem cells derived from bone marrow and adipose tissue

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Abstract

Trend of regenerative therapy for diabetes in human and veterinary practice has conceptually been proven according to Edmonton protocol and animal models. Establishing an alternative insulin-producing cell (IPC) resource is a challenge task for further clinical application. In this study, IPC generation from two practical canine mesenchymal stem cells (cMSCs), canine bone marrow-derived MSCs (cBM-MSCs) and canine adipose-derived MSCs (cAD-MSCs), was of interest. The results illustrated that cBM-MSCs and cAD-MSCs contained distinct pancreatic differentiation potential and required the tailor-made induction protocols. Effective generation of cBM-MSC-derived IPCs needed an integration of genetic and microenvironment manipulation using hanging-drop culture of *PDX-1*-transfected cBM-MSCs under three-step pancreatic induction protocol. However, this protocol was resource- and time-consuming. Another study on cAD-MSC-derived IPC generation found that IPC colonies could be obtained by low attachment culture under three-step induction protocol. Further Notch signaling inhibition during pancreatic endoderm/progenitor induction yielded IPC colonies with trend of glucose-responsive C-peptide secretion. Thus, this study showed that IPCs could be obtained from cBM-MSCs and cAD-MSCs by different induction techniques, and further signaling manipulation study should be conducted to maximize the protocol efficiency.

Introduction

Diabetes is not only a major metabolic disease affecting people around the world, but also the companion animals, mostly dogs and cats ¹⁻³. By pathophysiological basis, it is classified into 2 main types, type I and II, as characterized by absencing or presenting of intact beta-cells, respectively ^{2,4}. Type I diabetes is referred to an immune-mediated beta-cell destruction causing endogenous insulin depletion, while type II is related to insulin secretion defect and/or insulin resistance ^{1,4}. Although, diabetes treatment seems well-established, adverse events and compromised clinical efficiency have been periodically reported ^{3,5}. Trend of regenerative treatment has been introduced for addressing these issues starting from cadaveric islet transplantation in diabetes type I patients, namely “Edmonton protocol”, which resulted in long-term omitting of exogenous insulin administration ⁶⁻⁸. However, two main obstacles have been suggested, donor shortages and immunosuppressants’ side effects, making stem cell (SC)-based regenerative approach be the potential clinical candidate ⁶⁻⁹.

Concept of SC-derived insulin-producing cell (IPC) transplantation for treating diabetes has been conceptually approved in animal models. However, it comes with further challenges on finding potential candidate cell sources and establishing efficient IPC production platforms that are clinically applicable ¹⁰⁻¹². Although, the study on IPC production using human SCs has widely been studied and well-established, the knowledge in IPC generation aiming for veterinary application is still lacking. It has been a few reports suggesting the induction of canine somatic cells and canine mesenchymal MSCs (cMSCs) toward IPCs *in vitro* ^{13,14}. These generated IPCs were formed as cell aggregates attached to culture surface that might cause some difficulties during cell harvesting and processing for transplantation. To earn the clinical applicable IPCs, three-dimensional (3D) structure of IPCs floating or suspending in culture vessels would be

required to ease the harvesting and encapsulating processes ¹⁵. To address this issue, the integrative induction protocols aiming for the pancreatic differentiation of canine bone marrow-derived MSCs (cBM-MSCs) and canine adipose-derived MSCs (cAD-MSCs) were established in this study. The protocols were aimed for the delivery of 3D colony structure of the generated IPCs. Notch signaling manipulation was additionally conducted in the potential protocol for maximizing the induction efficiency. The results will be the crucial platform supporting the IPC generation which eventually benefits the establishment of clinical protocols for both veterinary and human applications.

Results

cBM-MSC and cAD-MSC characterization

The isolated cBM-MSCs (Figure 1A and B) and cAD-MSCs (Figure 1C and D) showed fibroblast-like appearance upon 2D culture. mRNA expression of stemness-related markers (*Rex1* and *Oct4*) and proliferation marker (*Ki67*) were detected (Figure 1E and F). MSC-related surface marker analysis by flow cytometry showed that both cells contained high proportion of Cd90⁺ cells, while the proportion of Cd73⁺ cells was relatively low. The expression of hematopoietic surface marker (Cd45) was considered absent in both cells (Figure 1G and H).

Both cells illustrated the *in vitro* osteogenic differentiation potential upon the 14-day induction protocol regarding ECM mineralization as demonstrated by Alizarin Red S and *Von Kossa* staining (Figure 1I and J) and osteogenic mRNA marker expression (*Alp*, *Runx2*, *Osx*, *Opn*, *Ocn*, and *Colla1*) (Figure 1K and L).

The results revealed the MSC-related characteristics of the isolated cBM-MSCs and cAD-MSCs.

Generation of IPCs from cBM-MSCs requires 3D culture condition

To generate IPC colonies from cBM-MSCs, three different culture techniques were investigated (Figure 2A-C). In all culture techniques, three pancreatic induction media were used as a microenvironmental manipulating/small molecule inducing approach. The results as illustrated in Figure 2D showed that suspending the cells in low attachment culture dish (Protocol I) was unable to deliver IPC colonies, while maintaining the cells using hanging-drop technique (Protocol II) could successfully generate IPC colonies with 50-200 μm in diameter. However, the colonies seemed loose cell aggregates. Further investigation was performed by maintaining the colonies collected from hanging-drop culture in Matrigel[®]-embedded culture condition (Protocol III). Although, the generated colonies were dense and compact, they could not maintain colony structure after gel digestion using Cell Recovery Solution[®] making them unable to be harvested for further functional testing.

Comparison of the pancreatic mRNA markers of the generated IPC colonies revealed that colonies from Protocol II expressed high pancreatic endoderm marker (*Pdx1*), but low pancreatic beta-cell markers (*Nkx-6.1*, *Isl-1*, *Glut-2*, and *Insulin*), comparing with those from Protocol III (Figure 2E and F). However, the mRNA expression of pancreatic-relating markers (*Glucagon* and *Glp1r*) was not detected in Protocol III (Figure 2G).

Further functional testing showed that IPC colonies collected from Protocol II secreted C-peptide under basal condition but could not produce a significant response upon low (5.5mM) and high (22mM) glucose stimulation. There was only trend of increased C-peptide secretion compared to basal control (Figure 2H).

Thus, generating IPCs from cBM-MSCs by microenvironmental manipulating/small molecule inducing approach required 3D culture condition. However, the generated IPCs showed limited function and maturity.

Overexpression of PDX1 fails to generate IPCs from cBM-MSCs

Further generating IPCs from cBM-MSCs using genetic manipulating approach was conducted by overexpression of the pancreatic commitment regulator, *PDX1*. Lentiviral vector carrying *PDX1* was transfected into cBM-MSCs at MOI 20, 30, and 50 (Figure 3A). The results showed that all transfected cells started forming loose cell aggregates since 48-hour post-transfection. Then, at 168-hour post-transfection, transfected cells at MOI 20 formed small-size cell clusters (< 50 μm in diameter), while those transfected at MOI 30 and 50 formed medium- to large-size cell clusters (100-200 μm in diameter). None of them formed floating colony-like structure (Figure 3B).

Further analysis on pancreatic mRNA markers showed that transfected cells at MOI 20 significantly illustrated high expression of pancreatic endoderm marker (*Pdx1*) and some of pancreatic beta-cell markers (*Maf-A*, *Glut-2*, and *Insulin*), comparing with those transfected at MOI 30 and 50 (Figure 3C and D). However, alpha-cell hormonal marker (*Glucagon*) was significantly expressed in MOI 20 transfection (Figure 3E), while *Glp1r* was not detected in all groups.

The results suggested that overexpression of *PDX1* could not successfully generate IPC colonies from cBM-MSCs in terms of pancreatic islet morphology and genotype.

Integration of PDX1 overexpression with 3D culture effectively generates IPCs from cBM-MSCs

In order to effectively generate IPCs from cBM-MSCs, combination of genetic and microenvironmental manipulating approaches was used. Cells were transfected with lentiviral vector carrying human *PDX1* at MOI 20 then maintained with three-step induction protocol under 3D culture condition (hanging-drop technique) (Figure 4A). The results illustrated that IPC colonies started forming since day 5 of the induction, and size of colonies at day 12 was approximately 100-200 μm (Figure 4B).

Pancreatic mRNA analysis showed that pancreatic endoderm marker (*Pdx1*) and pancreatic beta-cell markers (*Isl-1*, *Maf-A*, *Glut-2*, and *Insulin*) were significantly upregulated (Figure 4C and D). However, alpha-cell hormonal marker (*Glucagon*) was highly expressed (Figure 4E), while *Glp1r* was not detected. Functional testing also showed that IPC colonies secreted C-peptide under basal condition, but they could not produce a dose-dependent response upon low (5.5mM) and high (22mM) glucose stimulation (Figure 4F).

Thus, combination of genetic and microenvironmental manipulating approaches effectively generated IPCs from cBM-MSCs with high pancreatic mRNA marker expression along with the ideal islet morphology. However, their functional property was still limited.

Low attachment culture is efficient to generate IPCs from cAD-MSCs

To generate IPCs from cAD-MSCs, microenvironmental manipulating approach was used by suspending the cells onto low attachment culture dishes and maintaining in three-step induction media (Figure 5A). It was quite interesting that cells formed colony-like structure since day 3 of the induction, and the colonies became denser and bigger along the culture period

(Figure 5B). At day 12, approximately 834 colonies (median) were obtained from 1×10^6 seeding cells (Figure 5C), and the colony size was varied from $<50 \mu\text{m}$ to $> 700 \mu\text{m}$ (Figure 5D).

Analysis of pancreatic mRNA expression revealed that pancreatic beta-cell markers (*Nkx-6.1*, *Isl-1*, *Maf-A*, *Glut-2*, and *Insulin*) were significantly upregulated (Figure 5E). Alpha-cell hormonal marker (*Glucagon*) was a bit expressed, while *Glp1r* was downregulated (Figure 5F). Functional testing showed that IPC colonies secreted C-peptide under basal condition and showed trend of glucose-responsive C-peptide secretion upon high (22mM) glucose stimulation. However, it was not statistically significant compared to basal secretion (Figure 5G).

The results suggested that microenvironmental manipulating approach using low attachment culture was efficient to generate IPCs from cAD-MSCs in term of pancreatic islet characteristics. However, their functional property was still limited.

Notch signaling optimization generates potential cAD-MSC-derived IPCs

According to the results of IPC induction protocol efficiency, it has been suggested that generation of cAD-MSC-derived IPCs using microenvironmental manipulating approach seemed the most efficient protocol in terms of 1) morphological appearance and colony number, 2) pancreatic mRNA marker expression, and 3) functional property. In this regard, Notch signaling optimization was performed for generating the potential cAD-MSC-derived IPCs using protocol mentioned in our previous report ¹⁶.

cAD-MSC-derived IPCs were generated using optimized three-step induction protocol (Figure 6A) with Notch signaling manipulation using gamma-secretase inhibitor, DAPT, during definitive endoderm induction (DAPT-A) (Figure 6B) or pancreatic endoderm/progenitor induction (DAPT-B) (Figure 6C). The results showed that, in all conditions, cells started colony

formation since day 3 post-induction, then colony size and number were increased during the induction period (Figure 6D). Total colony counts (median) were 834, 691.5, and 504 colonies per batch (1×10^6 seeding cells) for control, DAPT-A, and DAPT-B, respectively (Figure 6E). It seemed that DAPT-B delivered more proportion of small-size colony ($<50 \mu\text{m}$ and $50\text{-}100 \mu\text{m}$), but statistical difference was not recognized due to variation among groups (Figure 6F).

Pancreatic mRNA analysis illustrated that cAD-MSC-derived IPCs from DAPT-B condition significantly showed lesser degree of pancreatic endoderm marker (*Pdx1*) and pancreatic beta-cell markers (*Isl-1*, *Maf-A*, *Glut-2*, and *Insulin*), comparing with those from DAPT-A condition (Figure 7A and B). However, alpha-cell hormonal marker (*Glucagon*) of DAPT-B group was much lower than that in DAPT-A group. *Glp1r* was downregulated in all conditions (Figure 7C). Interestingly, analysis of Notch target genes, *Hes-1* and *Hey-1*, showed that DAPT-B group showed significant upregulation of both genes comparing with others (Figure 7D). Functional testing showed that cAD-MSC-derived IPCs from DAPT-B condition yielded highest basal C-peptide secretion as well as the higher glucose-responsive C-peptide secretion upon low (5.5 mM) and high (22mM) glucose stimulation, comparing with control and DAPT-A groups. It should be noted that, due to variation within group, statistical difference within each group was not found (Figure 7E).

Taken together, the results suggested that cAD-MSC-derived IPCs could be efficiently generated using microenvironmental manipulating approach with Notch optimization. The obtained IPCs from Notch inhibition during pancreatic endoderm/progenitor induction showed pancreatic islet/beta-cell characteristics and positive trend of functional property.

Discussion

As the proof-of-concept evidences for treating diabetes by regenerative therapy have been reported in human and animal models ^{9,17-20}, MSCs have been proposed as one of the promising resources for generating clinical applicable IPCs ²¹⁻²⁴. In this study, the pancreatic differentiation potential of cBM-MSCs and cAD-MSCs was evaluated aiming for determining the feasibility of IPC formation *in vitro* and the potential of their clinical application. The cBM-MSCs and cAD-MSCs were isolated, cultured, and expanded using previous published protocols ²⁵⁻²⁸. Their characteristics were similar as described in previous reports including fibroblast-like structure, mRNA expression related to stemness and proliferation, MSC-related surface marker expression, and osteogenic differentiation potential ²⁶⁻³⁰. It should be noted that the expression of Cd73 in both MSCs was relatively low as mentioned in previous report ³¹. This evidence supported the consistency of the cMSCs' properties used in this report.

In term of IPC formation *in vitro*, various protocols employing either microenvironmental manipulation or genetic manipulation have been reported ^{11,32-36}. The strategies used in these studies usually relied on origin and pluripotency/multipotency of the cells ³⁷⁻⁴¹. It should be noted that pluripotent SCs, ESCs and iPSCs, contained high capability of pancreatogenesis *in vitro* ⁴²⁻⁴⁷. However, due to their ethical and safety concerns, MSCs have been proposed as an alternative source for IPC generation ^{10,11,17,33,46,48-50}.

Here, we illustrated that cBM-MSCs and cAD-MSCs could be differentiated toward pancreatic lineage *in vitro*. However, each cell type contained different pancreatic differentiation potential and required a tailor-made induction technique. For IPC generation by cBM-MSCs, it has been shown that microenvironmental manipulating approach with low attachment culture (2D culture) could not produce an islet-like cell aggregate *in vitro*, but it required 3D culture

technique for generating and maintaining the colony-like structure of IPCs. By using hanging-drop culture technique, cBM-MSCs formed cell aggregates since day 3 post-induction, then size of the colony was increased along with the expression of pancreatic mRNA markers. Further experiment showed that Matrigel[®]-embedded culture of the colonies derived from hanging-drop culture could give a dense colony structure and higher levels of pancreatic marker expression.

Previous publications reported that small molecule induction could imitate the environment during pancreatic endocrine development^{10,16,43,51-56}. Generally, an *in vitro* pancreatic differentiation from SCs could be categorized into 6 differentiation stages: pluripotent/multipotent SCs, mesendoderm, definitive endoderm, pancreatic endoderm, pancreatic endocrine, and pancreatic beta-cells/IPCs^{15,57}. In this study, activin A was used to mimic the effects of endogenous noggin for shortcutting the definitive endoderm establishing step as described in previous reports^{32,57-62}. It was quite interesting that maintaining cBM-MSCs with pancreatic induction media in low attachment culture was unable to form colony-like structure which is the natural pancreatic islet topology and crucial for an *in vitro* pancreatic differentiation^{16,32,59,63-65}. Therefore, the 3D culture condition using hanging-drop and Matrigel[®]-embedded culture techniques were used for generating the cBM-MSC-derived IPC colony. It has been shown that hanging-drop culture was an efficient technique for embryoid body/cell colony formation *in vitro*⁶⁶⁻⁶⁹ along with the natural/synthetic hydrogel-embedded culture that was one of the effective culture techniques used for organoid formation and expansion⁷⁰⁻⁷⁴. In this study we demonstrated the successful IPC colony formation by these two culture techniques. However, it was quite difficult to collect and expand the IPC colonies since colony maintaining and medium changing for hanging-drop culture were time-consuming. In addition, treating the Matrigel[®]-embedded colonies with hydrogel digesting solution (Cell

Recovery Solution[®]) caused colony dissociation. Further functional assay could only be performed for IPC colonies derived from hanging-drop culture and found that the obtained IPC colonies could basally secrete C-peptide but not a significant response to glucose stimulation. Additional genetic manipulating approach was performed and showed that overexpression of *PDX1* at MOI 20 could enhance pancreatic beta-cell marker expression but was unable to produce 3D IPC colony.

These findings led to the integration of genetic and microenvironment manipulating approaches by hanging-drop culture of *PDX1*-transfected cBM-MSCs under three-step induction cocktails. The results demonstrated the effective formation of 3D IPC colonies with significant pancreatic marker expression along with the basal C-peptide secretion. Our findings were correlated to previous reports showing that *PDX1* was an essential gene in the first hierarchy of pancreatic organogenesis progressing toward beta-cell maturation^{57,75}. *PDX1*-positive cells were considered as the pancreatic progenitors for three pancreatic lineages, comprising endocrine, exocrine, and ductal cells¹⁰. It has been shown that the overexpression of *PDX1* by lentiviral vector into mouse MSCs could enhance IPC generation by triggering the morphological change from adherent spindle fibroblast-like cells toward the ball-like cell colonies^{33,76}. For cBM-MSCs, we found that 3D culture condition was required to form the IPC colony which was considered as the native pancreatic islet morphology^{35,63,65,77}. Thus, cBM-MSC-derived IPCs were able to obtain from the integrating protocol of genetic and microenvironmental manipulation. However, hanging-drop 3D culture technique was time- and labor-consuming, making it less clinical applicable.

Alternatively, cAD-MSCs have been proposed as the potential MSC candidate for regenerative diabetes therapy as mentioned in previous reports^{18,51,76,78-80}. We showed in this

study that cAD-MSC-derived IPC colonies could efficiently be generated from low attachment culture with the expression of crucial pancreatic mRNA markers. Functional assay showed a basal C-peptide release with trend of glucose-responsive c-peptide secretion in high glucose (22 mM) stimulation. Our finding was correlated with previous studies on the generation of pancreatic progenitors (PPs) and IPCs by AD-MSCs derived from human and animal resources ^{32,51,52,79,81}. Most of the IPC induction protocols used for AD-MSC induction relied on the concept of microenvironmental induction which reflect the trans-lineage differentiation potential of the cells ^{32,51,79,80}. In 2006, Timper et al. had initially proved the prospect of human AD-MSCs (hAD-MSCs) toward IPCs using a single step microenvironmental manipulating approach ⁸⁰. After that, Chandra et al. published a three-step microenvironmental manipulating protocol for inducing murine AD-MSCs (mAD-MSCs) toward islet-like cell aggregates (ICAs) ³². mAD-MSCs could be successfully committed toward each stage of pancreatic endocrine development regarding definitive endoderm, pancreatic endoderm, and pancreatic endocrine precursor as illustrated by the upregulating pancreatic markers in each stage. These findings supported the pancreatic differentiation potential of AD-MSCs derived from various species.

It has been suggested that the promising regenerative therapy for diabetes relies on the availability and the potential of stem cells used for generating PPs or IPCs, the efficiency of induction protocol, and the potential application on further established transplantation platform. One of the potential transplantation platforms is cell or colony encapsulation which requires the 3D colony structure of the IPCs that can be harvested after an *in vitro* production. This encapsulation platform can support and immobilize IPC colonies with the immunoisolating property against host immunity ¹⁵. By comparing the potential clinical application, it seemed that

cBM-MSC-derived IPCs showed less potential due to the complicated and time/labor-consuming induction protocol. Therefore, cAD-MSC-derived IPCs were further optimized.

Various factors and signaling have been studied for the potential effects on IPC generation *in vitro*. In this regard, Notch signaling was of interest due to its significant effect during pancreatogenesis both *in vivo* and *in vitro*⁸²⁻⁸⁵. cAD-MSC-derived IPCs were generated using optimized three-step induction protocol with Notch signaling manipulation by gamma-secretase inhibitor, DAPT, during definitive endoderm or pancreatic endoderm/progenitor induction. We found that Notch inhibition during pancreatic endoderm/progenitor induction benefited the cAD-MSC-derived IPC production in terms of high basal C-peptide secretion and positive trend of glucose-responsive C-peptide secretion. These findings were correlated with previous studies that Notch signaling played a biphasic role in pancreatogenesis during embryonic development. Downregulation of Notch is required for pancreatic endoderm commitment and *Pdx1*-positive pancreatic precursor expansion, while Notch upregulation is crucial for late-state pancreatic maturation⁸⁴⁻⁸⁶. Our previous study also showed that inhibition of Notch during pancreatic endoderm induction by human dental pulp stem cells (hDPSCs) resulted in high number of IPC colony production with high expression of *PDX1*, whereas the inhibition during maturation stage caused the impairment of glucose-responsive C-peptide secretion¹⁶.

During pancreatogenesis, endocrine precursors formed clusters which allowed cell-to-cell contact and the interaction so called “lateral inhibition”. This led to the activation of Notch signaling and the regulation of endocrine fate descended from *Pdx1*-positive progenitors⁸⁷⁻⁸⁹. Previous studies have confirmed the involvement of Notch signaling during endocrine progenitor fate commitment toward one of pancreatic endocrine subtypes (beta- or alpha-cells)⁸⁹⁻⁹¹. The

inhibition of Notch by *HES1* shRNA could induce the redifferentiation of expanded human beta-cell-derived cells following with the significant expansion of beta-cell *in vitro* and the upregulation of beta-cell-related genes ⁹². However, overactivation of Notch could limit the differentiation capability toward fully matured IPCs by inhibiting the expression of “pre-differentiation” gene by pancreatic progenitors ⁹³. These evidences also supported our findings that the cAD-MSC-derived IPCs could be generated *in vitro*, and the selective Notch signaling manipulation played the beneficial roles on colony production, pancreatic marker expression, and functional property.

Conclusion

In conclusion, we illustrated that cMSC-derived IPCs could be generated from cBM-MSCs and cAD-MSCs *in vitro*. However, these two cMSCs contained different pancreatic differentiation potential and required specific induction techniques. Further studies focusing on maturation and transplantation platform will fulfill the production of clinical applicable cMSC-derived IPCs.

Materials and Methods

Cell isolation, culture, and expansion

All protocols were conducted in accordance with guidelines and regulations approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Science, Chulalongkorn University (Animal Use Protocol No.1531072). cMSCs were isolated from bone marrow and fat tissue of healthy dogs according to the inclusion criteria. Informed Consent was taken from pets' owners for inclusion of dogs in the study. cBM-MSCs were isolated from

heparin-containing bone marrow aspirate following our previous published protocol²⁸. Briefly, cells were washed with Hank's Balanced Salt solution (HBSS) (Thermo Fisher Scientific Corporation, USA), then resuspended with high glucose Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM /F-12) (Thermo Fisher Scientific Corporation) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific Corporation), 1% GlutaMAX™ (Thermo Fisher Scientific Corporation), and 1% Antibiotics-Antimycotic (Thermo Fisher Scientific Corporation).

cAD-MSCs were isolated from biopsied adipose tissues. Tissues were minced and incubated with Cell Recovery Solution® (Corning, USA) for 2 hours at 37°C, then passed through 70 µm strainer and washed twice with PBS. Pellet was resuspended and seeded onto culture containers. Cells were maintained in high glucose DMEM (Thermo Fisher Scientific Corporation) supplemented with 10% FBS, 1% GlutaMAX™, and 1% Antibiotics-Antimycotic.

Both cell types were maintained at 37°C in humidified atmosphere with 5% CO₂ and fresh air. Culture media was replaced every 48 hours. Cells were subcultured when 80% confluence reached. Cells in passage 2-6 were used for the experiments.

Characterization of cBM-MSCs and cAD-MSCs

The isolated cells were characterized by assessing mRNA expression regarding stemness markers (*Rex1* and *Oct4*) and proliferative marker (*Ki67*) by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). MSC-related and hematopoietic surface markers were analyzed by flow cytometry. Cells were stained with mouse anti-Cd73 monoclonal antibody (Invitrogen, USA) and FITC-conjugated goat anti-mouse immunoglobulin (Ig) G secondary antibody (BioRad, USA), PE-conjugated rat anti-Cd90 monoclonal antibody (eBioscience, USA),

and FITC-conjugated mouse anti-Cd45 monoclonal antibody (BioLegend, USA). Mouse IgG Isotype (BioLegend), PE-conjugated rat IgG Isotype (BioLegend), and FITC-conjugated mouse IgG Isotype (BioLegend) were used as isotype control. FACScallibur flow cytometer with CellQuest software (BD Bioscience) was used for analysis.

Cell differentiation potential was assessed using osteogenic induction protocol. Briefly, cells were seeded onto 24-well culture plate (Corning, USA) in a concentration of 2.5×10^5 cells/well. After 24 hours, cells were maintained in osteogenic induction medium for 14 days. The osteogenic induction medium was a growth medium supplemented with 50 mg/mL L-ascorbic acid, 100 mM dexamethasone, and 10 mM β -glycerophosphate¹⁶. Osteogenic differentiation potential was analyzed according to extracellular matrix (ECM) mineralization by Alizarin Red S⁹⁴ and *Von Kossa* staining⁹⁵, and osteogenic-related mRNA marker expression (*Alp*, *Runx2*, *Osx*, *Opn*, *Ocn* and *Colla1*) by RT-qPCR. Undifferentiated cells were used as control.

IPC induction by microenvironmental manipulation

In this regard, three-step induction protocol modified from previous published reports was used^{16,32,59}. Briefly, cells were trypsinized and resuspended in a series of three pancreatic induction media, namely serum-free medium (SFM)-A, SFM-B, and SFM-C, respectively. Cells were consequently maintained in SFM-A for 3 days (72 hours), SFM-B for 2 days (48 hours), and SFM-C for 5 days (120 hours). SFM-A was SFM-DMEM/F12 or SFM-DMEM (basal medium) supplemented with 1% bovine serum albumin (BSA, Cohn fraction V, fatty acid free) (Sigma-Aldrich, USA), 1X insulin-transferrin-selenium (ITS) (Invitrogen), 4 nM activin A (Sigma-Aldrich), 1 nM sodium butyrate (Sigma-Aldrich), and 50 μ M beta-mercaptoethanol

(Sigma-Aldrich). SFM-B was basal medium supplemented with 1% BSA, 1X ITS, and 0.3 mM taurine (Sigma-Aldrich). SFM-C was basal medium containing 1.5% BSA, 1X ITS, 3 mM taurine, 100 nM glucagon-like peptide (GLP)-1 (Sigma-Aldrich), 1 mM nicotinamide (Sigma-Aldrich), and 1X non-essential amino acids (NEAAs) (Sigma-Aldrich). Gamma-secretase inhibitor (DAPT) was used in some experiments at 25 μ M.

Regarding culture maintenance, three different techniques were employed: low attachment, hanging-drop, and hydrogel (Matrigel®)-embedded culture techniques. For 2-dimensional (2D) low attachment culture, 60 mm non-treated culture dishes (Eppendorf, USA) were used. 10^6 cells were collected and suspended onto each dish using three induction media mentioned above. For 3D hanging-drop culture, GravityPLUS™ 96-well plate hanging-drop culture system (PerkinElmer, USA) was used. Cells were suspended in induction media and seeded into hanging-drop wells at concentration 2×10^4 cells per 40 μ L per well. Another protocol was 3D hydrogel-embedded culture. Cell colonies obtained from hanging-drop culture were collected and embedded in hydrogel (Matrigel® Matrix: growth factor reduced type) (Corning). In this regard, 100-150 μ L of hydrogel and induction medium mixture (1:1) was used for forming a dome-like structure onto each well of 24-well culture plate (Corning). Cell Recovery Solution® was used for gel digestion.

IPC induction by genetic manipulation

Overexpression of *PDX1* by lentiviral vector was used for genetic manipulating approach. Lentivirus carrying *PDX1* was produced from the packaging of *pWPT-PDX1* (Addgene plasmid #12256; gift from Didier Trono) (<http://n2t.net/addgene:12256>; RRID: Addgene_12256)³⁴, *psPAX2* (Addgene plasmid #12260; gift from Didier Trono) (<http://n2t.net/addgene:12260>;

RRID: Addgene_12260), and *pMD2.G* (Addgene plasmid #12259; gift from Didier Trono) (<http://n2t.net/addgene:12259>; RRID: Addgene_12259) in human embryonal kidney (HEK 293FT) cells. The supernatant containing lentiviral particles were collected at 48- and 72-hour post-packaging and filtered through 0.45 μ m filter. Viral particles were harvested using Plasmid Midiprep Plus Purification Kit (Gene Mark Bio, Taiwan) and then freshly concentrated by Amicon[®] Ultra Centrifugal Filter (Merck Millipore, USA).

For transfection protocol, cells at concentration of 5×10^4 cells/well were seeded onto 24-well plates for 24 hours, then treated with 4 μ g/mL polybrene infection/transfection reagent (Merck Millipore) for 30 minutes. Multiplicity of infection (MOI) at 20, 30, or 50 were used for each 24-hour-transfection course.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was used for mRNA analysis. The total RNA was collected using TRIzol-RNA isolation reagent (Thermo Fisher Scientific Corporation) and extracted by DirectZol-RNA isolation kit (ZymoResearch, USA) according to the manufacture's protocol. RNA was converted to complementary DNA (cDNA) using ImProm[™] Reverse Transcription System (Promega, USA). The amplification of targeted genes was carried out by FastStart Essential DNA Green Master (Roche Diagnostics, Switzerland) using CFX96[™] Real-Time PCR Detection System (BioRad) with specific amplification primers. Glyceraldehyde 3-phosphate dehydrogenase, *Gapdh*, was used as the reference gene. Relative mRNA expression of target genes was normalized with reference gene and control group. The primer sequences were listed in Supplementary Table S1.

Functional analysis for IPCs

Glucose-stimulated C-peptide secretion (GSCS) was used for functional analysis of IPCs. Two glucose concentrations were used, 5.5 and 22 mM. Krebs-Ringer bicarbonate HEPES (KRBH) at pH 7.4 was used as physiological buffer solution according to previous reports^{16,97,98}. KRBH buffer solution contained 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1.1 mM MgCl₂, 25 mM NaHCO₃, and 10 mM HEPES. IPCs were gently collected and maintained with KRBH buffer solution at 37°C for 60 minutes as basal C-peptide secretion (0 mM glucose), then respectively incubated in 5.5 mM (99 mg/dL) and 22 mM (396 mg/dL) glucose (Sigma-Aldrich) for 60 minutes each. Buffer solution in each incubation period was collected for measuring C-peptide concentration using canine C-peptide enzyme-linked immunosorbent assay (ELISA) kit (Merk Millipore) according to the manufacturing protocol. Secreted C-peptide levels were then normalized with total DNA (mg) and incubation time (minutes). Total DNA was measured by DNeasy Blood and Tissue Kit (Qiagen, CA) and Qubit fluorometer (Thermo Fisher Scientific).

Statistical analysis

The results were illustrated as whisker and box plot (N=4). Statistical analysis was determined using SPSS statistics 22 software (IBM Corporation, USA). Mann-Whitney *U* test was used for comparing two independent samples, while Kruskal Wallis test and pairwise comparison were used for three or more group comparison. The significant difference was considered when *p*-value < 0.05.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

WR performed most of experiments, collected data, analyzed and interpreted data, and wrote manuscript. SN and KP provided study material and collected data. TO and PP supervised the project. CS conceived and designed the experiments, analyzed and interpreted data, wrote and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Figure Legends

Figure 1 cBM-MSC and cAD-MSC characterization. Morphological appearances of cBM-MSCs (A and B) and cAD-MSCs (C and D) were observed under phase-contrast microscope with magnification of 40X and 200X. mRNA expression regarding stemness and proliferation markers (E and F) were determined by RT-qPCR. mRNA expression was normalized with reference gene. The MSC-related surface markers were analyzed using flow cytometry (G and H). Osteogenic differentiation potential at day 14 post-induction was determined by Alizarin Red S and *Von Kossa* staining (I and J). Osteogenic mRNA marker expression was analyzed by RT-qPCR (K and L). mRNA expression was normalized with reference gene and undifferentiated control. Bars indicated significant difference (*, p -value < 0.05).

Figure 2 Generation of cBM-MSC-derived IPCs by microenvironmental manipulation.

Diagrams of three culture techniques used for the generation of cBM-MSC-derived IPCs were showed: I) low attachment (A), II) hanging-drop (B), and III) hydrogel-embedded (C) culture techniques. Morphological appearances of cells undergone each of induction technique were observed under phase-contrast microscope with magnification of 100X and 200X (D). mRNA markers relating to pancreatic endoderm (E), pancreatic beta-cell (F), and pancreatic-relating markers (G) were analyzed by RT-qPCR. mRNA expression was normalized with reference gene and undifferentiated control. Functional testing by glucose-stimulated C-peptide secretion (GSCS) was illustrated (H). Bars indicated significant difference (*, p -value < 0.05; **, p < 0.01).

Figure 3 Generation of cBM-MSC-derived IPCs by genetic manipulation. Diagram of the *PDX1* transfection for the generation of cBM-MSC-derived IPCs was showed (A).

Morphological appearances of cells undergone each of transfection condition were observed under phase-contrast microscope with magnification of 40X, 100X and 200X (B). mRNA markers relating to pancreatic endoderm (C), pancreatic beta-cell (D), and pancreatic-relating markers (E) were analyzed by RT-qPCR. mRNA expression was normalized with reference gene and undifferentiated control. Bars indicated significant difference (*, p -value < 0.05; **, p < 0.01; ***, p < 0.001).

Figure 4 Generation of cBM-MSC-derived IPCs by integrating genetic and microenvironmental manipulation. Diagram of culture technique used for the generation of cBM-MSC-derived IPCs was showed (A). Morphological appearances of cells undergone

induction technique were observed under phase-contrast microscope with magnification of 40X and 200X (B). mRNA markers relating to pancreatic endoderm (C), pancreatic beta-cell (D), and pancreatic-relating markers (E) were analyzed by RT-qPCR. mRNA expression was normalized with reference gene and undifferentiated control. Functional testing by glucose-stimulated C-peptide secretion (GSCS) was illustrated (F). Bars indicated significant difference (*, p -value < 0.05).

Figure 5 Generation of cAD-MSC-derived IPCs by microenvironmental manipulation.

Diagram of culture technique used for the generation of cAD-MSC-derived IPCs was showed (A). Morphological appearances of cells undergone induction technique were observed under phase-contrast microscope with magnification of 40X and 200X (B). Total colony number (C) and colony size proportion (D) were evaluated. mRNA markers relating to pancreatic beta-cell (E), and pancreatic-relating markers (F) were analyzed by RT-qPCR. mRNA expression was normalized with reference gene and undifferentiated control. Functional testing by glucose-stimulated C-peptide secretion (GSCS) was illustrated (G). Bars indicated significant difference (*, p -value < 0.05).

Figure 6 Generation of cAD-MSC-derived IPCs with Notch signaling manipulation.

Diagrams of Notch signaling manipulation used for the generation of cAD-MSC-derived IPCs were showed (A-C). Morphological appearances of cells undergone each of induction technique were observed under phase-contrast microscope with magnification of 40X and 200X (D). Total colony number (E) and colony size proportion (F) were evaluated.

Figure 7 Generation of cAD-MSC-derived IPCs with Notch signaling manipulation. mRNA markers relating to pancreatic endoderm (A), pancreatic beta-cell (B), pancreatic-relating markers (C), and Notch target genes (D) were analyzed by RT-qPCR. mRNA expression was normalized with reference gene and undifferentiated control. Functional testing by glucose-stimulated C-peptide secretion (GSCS) was illustrated (E). Bars indicated significant difference (*, p -value < 0.05; **, p < 0.01; ***, p < 0.001).

Figures

Figure 1

cBM-MSC and cAD-MSC characterization. Morphological appearances of cBM-MSCs (A and B) and cAD-MSCs (C and D) were observed under phase-contrast microscope with magnification of 40X and 200X. mRNA expression regarding stemness and proliferation markers (E and F) were determined by RT-qPCR. mRNA expression was normalized with reference gene. The MSC-related surface markers were analyzed

using flow cytometry (G and H). Osteogenic differentiation potential at day 14 post-induction was determined by Alizarin Red S and Von Kossa staining (I and J). Osteogenic mRNA marker expression was analyzed by RT-qPCR (K and L). mRNA expression was normalized with reference gene and undifferentiated control. Bars indicated significant difference (*, p-value < 0.05).

Figure 2

Generation of cBM-MSC-derived IPCs by microenvironmental manipulation. Diagrams of three culture techniques used for the generation of cBM-MSC-derived IPCs were showed: I) low attachment (A), II)

hanging-drop (B), and III) hydrogel-embedded (C) culture techniques. Morphological appearances of cells undergone each of induction technique were observed under phase-contrast microscope with magnification of 100X and 200X (D). mRNA markers relating to pancreatic endoderm (E), pancreatic beta-cell (F), and pancreatic-relating markers (G) were analyzed by RT-qPCR. mRNA expression was normalized with reference gene and undifferentiated control. Functional testing by glucose-stimulated C-peptide secretion (GSCS) was illustrated (H). Bars indicated significant difference (*, p-value < 0.05; **, p < 0.01).

Figure 3

Generation of cBM-MSC-derived IPCs by genetic manipulation. Diagram of the PDX1 transfection for the generation of cBM-MSC-derived IPCs was showed (A). Morphological appearances of cells undergone each of transfection condition were observed under phase-contrast microscope with magnification of 40X, 100X and 200X (B). mRNA markers relating to pancreatic endoderm (C), pancreatic beta-cell (D), and pancreatic-relating markers (E) were analyzed by RT-qPCR. mRNA expression was normalized with reference gene and undifferentiated control. Bars indicated significant difference (*, p-value < 0.05; **, p < 0.01; ***, p < 0.001).

Figure 4

Generation of cBM-MSC-derived IPCs by integrating genetic and microenvironmental manipulation. Diagram of culture technique used for the generation of cBM-MSC-derived IPCs was showed (A). Morphological appearances of cells undergone induction technique were observed under phase-contrast microscope with magnification of 40X and 200X (B). mRNA markers relating to pancreatic endoderm (C), pancreatic beta-cell (D), and pancreatic-relating markers (E) were analyzed by RT-qPCR. mRNA expression was normalized with reference gene and undifferentiated control. Functional testing by glucose-stimulated C-peptide secretion (GSCS) was illustrated (F). Bars indicated significant difference (*, p-value < 0.05).

Figure 5

Generation of cAD-MSC-derived IPCs by microenvironmental manipulation. Diagram of culture technique used for the generation of cAD-MSC-derived IPCs was showed (A). Morphological appearances of cells undergone induction technique were observed under phase-contrast microscope with magnification of 40X and 200X (B). Total colony number (C) and colony size proportion (D) were evaluated. mRNA markers relating to pancreatic beta-cell (E), and pancreatic-relating markers (F) were analyzed by RT-qPCR. mRNA expression was normalized with reference gene and undifferentiated control. Functional testing by glucose-stimulated C-peptide secretion (GSCS) was illustrated (G). Bars indicated significant difference (*, p-value < 0.05).

Figure 6

Generation of cAD-MSC-derived IPCs with Notch signaling manipulation. Diagrams of Notch signaling manipulation used for the generation of cAD-MSC-derived IPCs were showed (A-C). Morphological appearances of cells undergone each of induction technique were observed under phase-contrast microscope with magnification of 40X and 200X (D). Total colony number (E) and colony size proportion (F) were evaluated.

Figure 7

Generation of cAD-MSC-derived IPCs with Notch signaling manipulation. mRNA markers relating to pancreatic endoderm (A), pancreatic beta-cell (B), pancreatic-relating markers (C), and Notch target genes (D) were analyzed by RT-qPCR. mRNA expression was normalized with reference gene and undifferentiated control. Functional testing by glucose-stimulated C-peptide secretion (GSCS) was illustrated (E). Bars indicated significant difference (*, p -value < 0.05; **, p < 0.01; ***, p < 0.001).

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