Bioengineered regenerative therapy for diabetes mellitus: establishment of canine mesenchymal stem cell-derived insulin producing cells.

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ชีววิศวกรรมฟื้นฟูสำหรับรักษาเบาหวาน: การสร้างเซลล์สังเคราะห์อินซูลินจากเซลล์ต้นกำเนิดมีเซนไคม์ของสุนัข

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แนวทางการรักษาแบบฟื้นฟูสำหรับเบาหวานในทางการแพทย์และสัตวแพทย์นั้น ได้รับการศึกษายืนยันเชิงหลักการตามวิธีการของเอ็ดมันทันและการศึกษาในสัตว์ต้นแบบ ซึ่งการศึกษาทางเลือกในการสร้างเซลล์สังเคราะห์อินซูลินนับว่าเป็นขั้นตอนสำคัญในการพัฒนาสู่การประยุกต์ใช้ทางคลินิก งานวิจัยนี้จึงมุ่งเน้นในการสร้างเซลล์สังเคราะห์อินซูลินจากเซลล์ต้นกำเนิดมีเซนไคม์ที่ได้มาจากไขกระดูกและเนื้อเยื่อไขมันของสุนัข ผลการศึกษาพบว่าเซลล์ต้นกำเนิดมีเซนไคม์ทั้งสองชนิดมีความสามารถและอาศัยวิธีการที่แตกต่างกันในการเปลี่ยนแปลงสู่เซลล์สังเคราะห์อินซูลิน โดยเซลล์ต้นกำเนิดมีเซนไคม์จากไขกระดูกสุนัขต้องอาศัยการผสมผสานวิธีการในการปรับพันธุกรรมและสิ่งแวดล้อมโดยใช้วิธีการ 3 ขั้นตอนในการเหนี่ยวนำเซลล์ที่ถูกบังคับการแสดงออกของยีนพีดีเอ็กซ์-1 ภายใต้การเพาะเลี้ยงแบบหยดแขวนซึ่งยุ่งยากและใช้เวลา ในขณะที่การเหนี่ยวนำเซลล์ต้นกำเนิดมีเซนไคม์จากเนื้อเยื่อไขมันสุนัขอาศัยวิธีการ 3 ขั้นตอน ภายใต้การเพาะเลี้ยงแบบยึดเกาะต่ำ โดยการยับยั้งสัญญาณนอทช์ในช่วงการสร้างเอ็นโดเดิร์มหรือเซลล์ตั้งต้นของตับอ่อน จะให้กลุ่มเซลล์สังเคราะห์อินซูลินที่มีแนวโน้มการตอบสนองต่อการกระตุ้นด้วยระดับกลูโคส ซึ่งแสดงให้เห็นว่าการสร้างเซลล์สังเคราะห์อินซูลินจากเซลล์ทั้ง 2 ชนิด อาศัยวิธีการที่แตกต่างกัน และการศึกษาเชิงกลไกเพิ่มเติมจะช่วยเพิ่มประสิทธิภาพของวิธีการดังกล่าว

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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. Generation of functional 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-consumed. Another study on cAD-MSC-derived IPC generation found that IPC-like colonies could be obtained by low attachment culture under three-step induction protocol. Further Notch signaling inhibition during pancreatic endoderm/progenitor induction yielded IPC-like colonies with trend of glucose-responsive C-peptide secretion. Thus, this study showed that IPC-like cells 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.

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CHAPTER I

INTRODUCTION

Importance and Rational

Currently, diabetes mellitus (1) is not only a major metabolic disease increasingly affecting more than 108 million people around the world, but also expandingly detected in companion animals, mostly dogs and cats, which is considered as a major cause for euthanasia especially in severe uncontrolled diabetic animals (2-4). Based on its pathophysiological, diabetes in both human and animal can be relatively classified into two main types comprising of type I and II, characterizing by the absence and the presence of intact beta-cells, respectively (2, 5-7). Type I diabetes is closely related to an autoimmune-mediated beta-cell destruction causing endogenous insulin depletion, and type II is related to a malfunction of insulin secretion and action (3, 5-8). Although, diabetes treatment seems well-established, adverse events and compromised clinical efficiency have been periodically reported (3, 7, 9, 10). Therefore, the trend of regenerative treatment has been introduced for addressing the problems by using cadaveric islet transplantation, namely "Edmonton protocol", in diabetes type I patients resulting in long-term omitting of exogenous insulin injection (11-13). However, two main obstacles have been suggested, donor shortages and

immunosuppressive drugs' side effects, making bioengineered SC-based regenerative approach be the potential clinical candidate (14-18).

Concept of SC-derived IPC transplantation has been experimentally approved *in vitro.* However, it comes with further big challenges on finding potential candidate cell source and establishing efficient IPC production that are clinically applicable for human and/or veterinary practices (19-21). Although, the production of IPCs from animal-derived pluripotent and multipotent SCs has largely been studied according to the benefits on direct veterinary clinical application and pre-clinical approval for further human protocol establishment, obstacles on maximizing the protocol's safety and efficiency are still of concern (20-26). In this regard, MSCs have been introduced and expandingly investigated due to their availability and less safety concern (27-32). However, the major problem on MSC application is the limited on differentiated potential compared with those pluripotent cells, embryonic SCs (ESCs) and induced pluripotent SCs (iPSCs), resulting in the intensive studies mainly focused on the maximization of differentiation-potential by genetic and/or microenvironmental manipulations (21, 24, 26, 33, 34).

In this study, the objective was focused on the establishment of genetic- and/or microenvironmental-based induction protocols for *in vitro* generation of MSC-derived IPCs from two practical canine MSCs (cMSCs), canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) and canine adipose-derived mesenchymal stem cells (cAD-MSCs), which eventually benefits

the establishment of clinical protocols for both veterinary and human applications. Additionally, further Notch signaling manipulation of the potential cMSC-derived IPCs was conducted to maximize the protocol efficiency.

Objectives

1. To generate insulin-producing cells (IPCs) from two practical canine mesenchymal stem cells (cMSCs), canine bone marrow-derived MSCs (cBM-MSCs) and canine adipose-derived MSCs (cAD-MSCs), using microenvironmental and/or genetic manipulation approaches *in vitro*.
2. To optimize the potential application and the production of cMSC-derived IPCs *in vitro*, focusing on Notch signaling.

Keywords (English)

canine bone marrow-derived mesenchymal stem cells (cBM-MSCs), canine adipose-derived mesenchymal stem cells (cAD-MSCs), insulin-producing cells (IPCs), Notch signaling

Keywords (Thai)

เซลล์ต้นกำเนิดมีเซนไคม์จากไขกระดูกสุนัข, เซลล์ต้นกำเนิดมีเซนไคม์จากเนื้อเยื่อไขมันของสุนัข, เซลล์สังเคราะห์อินซูลิน, สัญญาณนอทช์

Hypotheses

1. Two cMSCs, cBM-MSCs and cAD-MSCs, can be differentiated toward IPCs by microenvironmental and/or genetic manipulation approaches *in vitro*.

2. Manipulation on Notch signaling influences the cMSCs-derived IPCs potential application and production *in vitro*.

CHAPTER II

LITERATURE REVIEW

According to the problems in diabetes treatment informed above, trend of regenerative diabetes therapy has been introduced especially for DM type I. Pancreas transplantation was the first alternative treatment by transplanting the whole cadaveric pancreas into the DM type I recipient (35-37). Anywise, this procedure is hampered by the surgical morbidity which is leading to the coming of islets transplantation, so called "Edmonton protocol", as an implantation method of pancreatic islets collected from deceased donor and transplanted to DM type I patient, considered as a low morbidity transplantation procedure (11-13). Although, this protocol can attain insulin independent at approximately 2 years, there are some limitations i.e. adverse events of immunosuppressants, limited available of donors, and limited duration of insulin independent period (15-18).

Therefore, the new trend in regenerative medicine using stem cell-based therapy has been introduced (21, 38-40). Embryonic stem cells (ESCs), the stem cells derived from the inner cell mass (ICM) of embryo, (41, 42) show the pluripotent ability to differentiate toward any cell types in three germ layers (endoderm, mesoderm, and ectoderm) and the self-renewal ability to divide indefinitely into themselves which are retaining their pluripotency (43-45).

From these potentials, they provide a possibility in regenerative therapy using stem cells for replacing and restoring the damaged cells, tissues and organs (46, 47). MSCs are the multipotent stem cells, the daughter progenitor cells of pluripotent or ESCs, which can give rise to any cells in mesodermal lineage i.e. osteocyte, adipocyte, and chondrocyte (21, 48). Furthermore, this stem cell type can be used to overcome the limitation on the rejection between donors and recipient and the adverse effect from immunosuppressants since they can be collected from patient's own body (28-32). Various types of MSCs have been used for generation of IPCs *in vitro*. Bone marrow-derived mesenchymal stem cells (BM-MSCs) and adipose-derived mesenchymal stem cells (AD-MSCs), respectively collected from bone marrow and adipose tissue, had been reported as an *in vitro* IPCs inducible cells with various methods (22, 25, 26, 49, 50).

Recently, the study on the stem cell-based therapy for diabetes in companion animal has been proposed. Gabr and colleagues have reported a preliminary study about transplanted human BM-MSCs-derived IPCs into the chemically induced diabetes dogs. Although, the human IPCs were able to control the blood glucose of dogs after transplantation, the dogs had still to receive the immunosuppressive drugs along the experimental period due to the trigger by human insulin and C-peptide (proinsulin) in serum of transplanted dog on host cellular immunity that could against and caused the rejection of xenograft transplantation (51). This led to the adverse events related to the immunosuppressants.

From these findings, it clues an idea in using of canine MSCs (cMSCs) for reprogramming into IPCs which will be suitable for stem cell-based therapy for diabetes dog that capable to overcome some limitations like a transplant rejection and the immunosuppressant-related adverse events.

Pancreatic endocrine development

Naturally, pancreatic endocrine cells are generated during embryonic development by ESCs in ICM of blastocyst (52). In addition, the differentiation into endocrine cells is divided into 6 steps including 1) the pluripotent or multipotent stem cell stage 2) mesendoderm stage 3) definitive endoderm (DE) stage, an important germ layer in pancreas development which formed as a flat sheet of cells 4) pancreatic endoderm stage 5) pancreatic endocrine precursor stage and 6) pancreatic beta-cell or IPCs stage (53, 54). In each stage, they are stimulated by the numerous signaling which promotes the specific markers for helping in pancreatic developmental process (20). By diving into 6 sets of stage-specific marker genes and proteins, the lists of those markers are as follows: the pluripotent or multipotent stem cell (*Oct4, Nanog,* and *Rex1* etc.); mesendoderm (*Mixl1,* and *Brachyury* etc.); definitive endoderm (*Cxcr4, Goosecoid, FoxA2, Sox17*, and *Bmp2* etc.); pancreatic endoderm (*Pdx1*, and *Hnf6* etc.); pancreatic endocrine precursor (*Ngn3, Neurod1, Maf-B, Nkx-2.2*, and *Pax-4* etc.); and pancreatic beta-cell or IPCs (*Nkx-6.1*, *Maf-A*, *Insulin*, *Isl-1*, *Glut-2*, *Glp-1r*, and *Glucagon* etc.) (54-56).

IPCs induction by microenvironmental manipulation approach

The microenvironmental manipulation is an indirect differentiation method for an *in vitro* IPCs production in the way of activation or inhibition of embryonic signaling pathway using active small molecules to enhance the IPCs differentiation by changing phenotype and genotype of pluripotent or multipotent stem cells (20). In term of *in vitro* IPCs production protocol establishment using microenvironmental manipulation have been tried and reported in various studies with numerous strategies (22, 24, 57, 58). In 2005, D'Amour et al. had reported that the presence of activin A, a soluble molecule of transforming growth factor (TGF)-beta family, with low concentration of fetal bovine serum (FBS) could induce cells in definitive endoderm (DE) which is the first step of endoderm lineage. Human ESCs by up to 80% highly expressed DE-specific marker genes (*SOX17, GSC*, and *FOXA2*), while the expression of mesendoderm marker (*BRACHYURY*) was downregulated (57).

Subsequently, the multi-step IPCs induction/differentiation approach had been introduced. A five-stage IPCs induction protocol had been used for generating IPCs (59) by induction through five differentiation stages including 1) definitive endoderm (DE), an important germ layer in pancreas development which formed as a flat sheet of cells (53), 2) primitive gut tube, a second step in the developed of pancreas after DE (53), 3) posterior foregut, a place that pancreas is emerging from the gut tube (53), 4) pancreatic endoderm and endocrine precursor, and 5) hormone

expression endocrine cells. The low concentration of FBS with activin A, Wnt3a (a primitive streak marker, fibroblast growth factor (FGF) 10, KAAD-cyclopamine (CYC, the hedgehog-signaling inhibitor), B27 (an optimized serum for supported cells without differentiation), retinoic acid (RA), exendin-4 (Ex4), a glucagon-like protein (Glp)-1 receptor agonist, DAPT (a Notch inhibitor), IGF1 (insulin-like growth factor), and hepatocyte growth factor (HGF) were used in induction which showed the successfulness IPCs induction following the stage of pancreatic organogenesis and increased a 160-fold of *INSULIN* mRNA during stage 5 differentiation process compared with activin removal alone (57).

After that, Timper et al. demonstrated the initial prospect of human AD-MSCs into IPCs using various concentration of glucose in differentiated media (49). Then, Chandra et al. published a three-step differentiation protocol for induced murine AD-MSCs into functional islet-like cell aggregates (ICA) in define SFM. Following the pancreatic endocrine development, the three-stages differentiation protocol was used including 1) DE stage, 2) pancreatic endoderm stage, and 3) pancreatic endocrine precursor stage (24). By first step, murine AD-MSCs were resuspended in differentiated media in term of 'SFM-A' comprising high glucose SFM supplemented with bovine serum albumin (BSA) , insulin-transferrin-selenium (60), activin A, sodium butyrate, and beta-mercaptoethanol by plating suspended cells into ultralow attachment tissue culture plated. After that, SFM-A were replaced to 'SFM-B' for second stage comprising high glucose SFM with BSA, ITS,

and taurine. For the last stage, the media were changed to 'SFM-C' consisting of high glucose SFM with BSA, taurine, GLP-1, nicotinamide, and nonessential amino acids (NEAAs). Each stage had showed the successfulness on committing by the upregulation of specific marker genes in each stage (24). To check the maturation of produced ICAs, after incubated ICAs in differentiate glucose concentrations, the levels of insulin and C-peptide secretion in media were increasing in concentration dependent manner (24). Besides, the transplantation of ICAs in streptozotocin (STZ)-induced diabetic mice showed the normoglycemia after 2 weeks of transplantation and they could maintain the blood glucose level until 4 weeks (24). From previous reports, ITS and beta-mercaptoethanol showed the highly protection of stress during the AD-MSCs culture in SFM condition (61), and beta-mercaptoethanol could be enhancing the insulin secretion in insulin-secreting cells (24). Sodium butyrate is a histone deacetylase inhibitor as known as a chromatin rearrangement inducer that can alter gene transcription and reduce the proliferation helping in differentiation process (23, 62). Taurine is a non-essential amino acids (NEAAs) required for the functional beta-cell production by supporting the secretion of insulin (24, 63). Moreover, GLP-1 and nicotinamide are the supplement involving in beta-cell maturation by helping in functional beta-cell production, supplying the expansion of beta-cells and increasing beta-cell sensing in glucose-stimulated insulin secretion (24, 64, 65). Because of the highly efficiency on the differentiation of *PDX1*-positives cells, as known as the pancreatic endocrine precursor, toward insulin- or C-peptide-

positive cells using three-step differentiation protocol, this protocol had been used in various IPCs studies and had promoted the successful generation of IPCs from MSCs (58, 66).

Regarding the details described above, it can be suggested whether microenvironment manipulation approach will be able to stimulate the differentiation of pluripotent or multipotent cells toward pancreatic lineage and forming the functional beta-like cells or IPCs using *in vitro* experiment.

IPCs induction by genetic manipulation approach

Even though the microenvironmental manipulation are able to produce IPCs, but the amount of maturated IPCs is still low at approximately 10-15% (24, 66). To encourage the efficiency of differentiation protocol, genetic manipulation as a direct differentiation method, has been introduced and widely studied (67). In the last decade, the results on forcing the hierarchy expression of transcriptional factor-related beta-cell identity during embryogenesis i.e. *Pdx1, Ngn3, Maf-A*, and *Insulin*, showed the sufficiency to differentiate ESCs (68), MSCs (26, 50, 69), pancreatic duct cells (34), and Glucagonoma cell line (33) toward beta-like cells.

During pancreatic endocrine development, several important genes will be expressed to commit the cells into each stage (66). Therefore, the initial transduction of gene involving in beta-cell differentiation lineage will be able to optimize IPCs production (26, 33, 34, 50). The homeobox gene *Pdx1* (pancreatic and duodenal homeobox-1) is expressing in the first hierarchy of pancreatic

fate during organogenesis, and the cells expressing *Pdx1* gene can be giving rise to all three types of pancreatic tissue comprising exocrine, endocrine, and duct (20, 52). Thus, activation *Pdx1* is considered as the prerequisite for pancreatic differentiation *in vitro* which can enhance the progressive expression of more mature markers of the endocrine lineage including *Ngn3, Nkx-2.2, Nkx-6.1, Maf-A*, and *Maf-B* (20, 55, 56). Besides, the inactivation of *Pdx1* gene can affect in the loss of beta-cell or insulin positive cells, yet the glucagon positive cells had been rising in *Pdx1* mutant mice, Thus, the late age of mutant mice had developed to diabetic mice (70). In 2003, Ritz-Laser et al. had been using lentiviral vector-mediated transfection of *Pdx1* into glucagonoma (InR1G9) cells resulted in the inhibition of endogenous glucagon gene transcription, an alpha-cells' specific hormone for releasing glucose from the storage cells, while induced insulin gene expression in the InR1G9 cells (33).

From above information, IPCs formation using the direct differentiation for forcing stem cells toward pancreatic endocrine lineage has been published. In 2013, the non-integrated lentiviruses harboring *PDX1* gene were transduced to human AD-MSCs (50), and mouse BM-MSCs (26). The adherent spindle and fibroblast-like morphology of MSCs had been becoming the round ball-like appearance or three dimensions spherical or clusters aggregated colonies after transfection for 7-10 days. In addition, the transduced MSCs were upregulating the pancreatic lineage-related genes expression, that could be able to release insulin after stimulated with glucose (26, 50). The

potential of transduced cells from direct differentiation protocol had been reported. After the induction, diabetes rats were transplanted with *PDX1* transduced-human AD-MSCs. Blood glucose level was gradually decreased and normalize within 3 days (50). Furthermore, all of transplanted mice did not detect the tumor formation after treatment for 12 months of follow up (50). However, it was the transiently efficiency because the blood glucose level had been increased after 10 day of transplantation (50).

There were other interesting models supporting the efficiency of direct reprogramming toward IPCs by forcing the stem cell resources with multi-transcription factors. From previously reports, it had been presented that the insulin positive cells were appearing at day 3 after transfected with the separated of three adenoviral monocistronic vectors carrying *Pdx1, Ngn3*, and *Maf-A*. The results showed the increasing in the insulin positive cells production up to 20% (19). In 2015, Yamada and colleagues had reported the potential of a single polycistronic (*Ngn3/Pdx1/Maf-A*) adenoviral vector on differentiation mouse pancreatic duct cells (mPDCs) toward IPCs. *Pdx1* is necessary in both of pancreatic and beta-cell development, *Ngn3* (neurogenin 3) is essential for driving toward endocrine islet lineage (55), and *Maf-A* is a pancreatic islet beta-cell-specific transcription factor in beta-cell maturation (71). The results suggested that the combination of three factors could be helpfulness for reprogramming exocrine cells to IPCs by producing IPCs approximately up to 30% after 2 days of transfection also with the highly expression of pancreatic

endocrine beta-cell-specific marker genes and the enhancing on insulin releasing during glucose stimulation with various concentrations (34).

These reports illustrate that the direct differentiation by forcing one or set of transcription factors will be synergistically achieve normal pancreatic endocrine islet development with functional property.

The Notch pathway in beta-cell growth and differentiation

During pancreatic organogenesis, the plenty of transcription factors are regulating the hierarchy of gene expression which initiate and maintain downstream gene expression required for their unique phenotypes (55). Several molecules and signaling pathways play crucial roles in regulating transcription factors involved in pancreatic development i.e. Wnt/beta-catenin pathway which is essential in acinar cell, an exocrine cells for secreting digestive enzyme, and enrichment in beta-cell generation from pluripotent or multipotent stem cells to DE stage (59, 72, 73); hedgehog pathway which plays an important role in early pancreas development by the absence of hedgehog proteins resulted in the reduction of pancreatic mass and beta-cell population (72, 74, 75); and Notch pathway, a crucial signaling in regulating the expression of precious transcription factors in pancreatic endocrine precursor cells which will be driving to beta-cells (55, 72, 73, 76, 77)

Recently, Notch signaling was particularly interesting pathway in pancreatic endocrine lineage. The activation of Notch signaling cascades will be happening when Notch ligand is binding with Notch receptor on cell membrane, then Notch intracellular domain (NICD) of Notch receptor located in cytoplasm will be cleaved by gamma-secretase enzyme, translocate into nucleus and interact with the promoter region of targeted gene; *Hes-1* and *Hey-1* (78). Beside, during pancreatic lineage formation, endocrine cells will be becoming the cluster and forming aggregation which will be permitting cell-to-cell contact and cause the interaction of each other during development, from this incidents, there are leading to the activation of Notch signaling and causing the regulation of endocrine cell fate descended from *Pdx1* positive progenitors (79).

Previous studies had confirmed that Notch involved in control the endocrine progenitor fate toward beta-cell via the expression of *Ngn3*, a transcription activator for controlling endocrine cells fate driving into one of endocrine subtypes (beta or alpha-cell) in pancreatic endocrine precursor stage (52, 55, 80, 81). In addition, the inhibition of Notch using genetic modification methods in *in vivo* study (77, 82) or using N-(S)-phenyl-glycine-t-butyl ester (DAPT), gamma-secretase inhibitor, in *in vitro* study (66) had showed the interesting results. The study of Jensen and colleagues used the mice lacking function of *Hes-1*, a Notch targeted gene involving in cell fate regulation (78). From the experiment, they found a few *Pdx1* positive cells, an endocrine precursor cells, with the observation of pancreatic hypoplasia in *Hes-1* unfunctional mice, but they

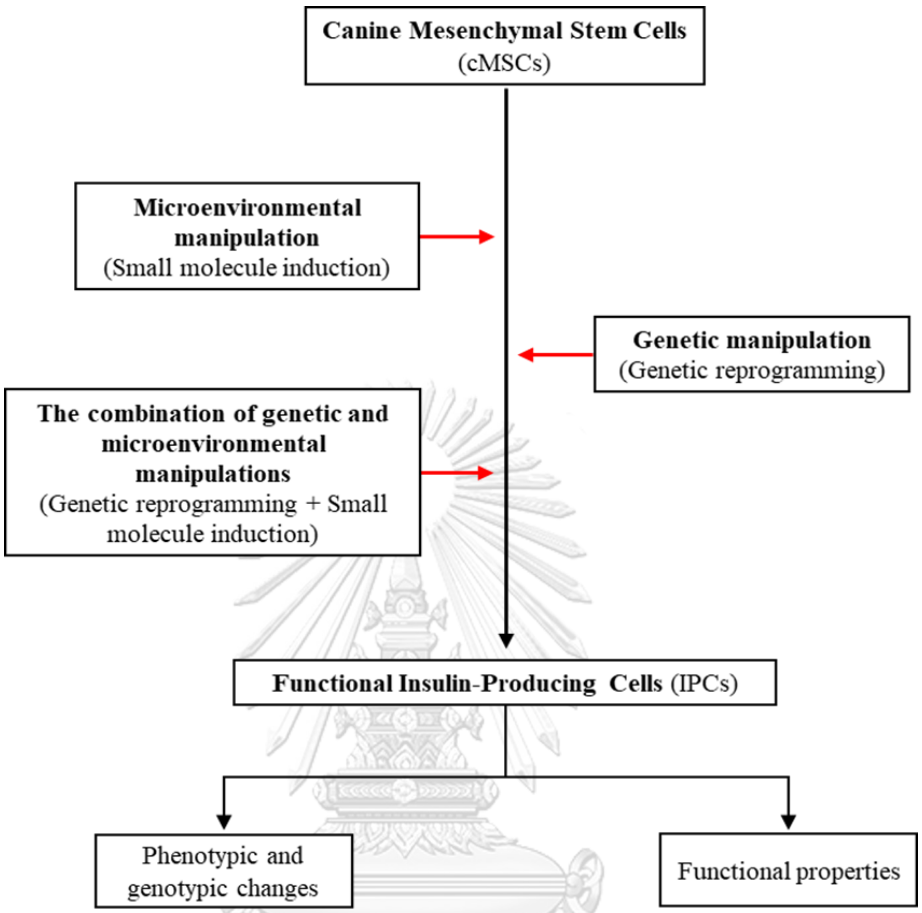
found the upregulation of *Ngn3* expression, a subset of *Pdx1*-positive pancreatic precursor cells (82). After that, Qu et al. had studied about the DAPT treatment with collected mice pancreas, and the results of pre-DAPT treatment showed the expression of Hes1 protein which caused by Notch activation and led to reduction of Ngn3 protein together with the increasing number of endocrine and ductal cells. However, after DAPT treatment they found the increasing of Ngn3 protein and regained the capacity in insulin-cell differentiation (77). Additionally, to estimate the effect of Notch on IPCs generation, Sawangmake and colleagues reported the using DAPT during three-step differentiation of human dental tissue-derived MSCs, and found that the Notch inhibition on second step (from pancreatic endoderm cells toward pancreatic endocrine precursor) showed high expression of *PDX1* and *NGN3*, together with the increasing number of IPCs colonies (66).

From these data, it can conclude that the activation of Notch in early stage may be enhancing the generation of endocrine precursors, then the inhibition on the following stage will be supporting the regulation of cell fate toward endocrine lineage. Therefore, the Notch activation in the appropriate time and the proper amount can be optimizing the potential of differentiation protocol.

For this regard, the objectives of study are aimed into two aspects consisting with **Objective 1)** the *in vitro* generation of insulin-producing cells (IPCs) from canine mesenchymal stem cells (cMSCs), employing microenvironmental manipulation approach by using the three-step

differentiation protocol (24, 58, 66), genetic manipulation approach by delivering a key pancreatic development regulating factor emphasizing on pancreatic and duodenal homeobox 1 (*PDX1*), or the combination of genetic and microenvironmental manipulation, and **Objective 2)** the most potential application and cMSCs-derived IPCs *in vitro* production will be optimizing, focusing on Notch signaling.

Conceptual Framework



CHAPTER III

METHODOLOGY

Cell isolation, culture, and expansion

Canine mesenchymal stem cells (cMSCs) were isolated from bone marrow and fat tissue of healthy dogs according to the inclusion criteria and owners' consent which had approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Science, Chulalongkorn University. Canine bone marrow-derived MSCs (cBM-MSCs) were isolated from heparin-containing bone marrow aspirate following the previous published protocol (83). Briefly, cells were washed twice with Hank's Balanced Salt solution (HBSS) (Thermo Fisher Scientific Corporation, USA), then resuspended and seeded in T-75 tissue culture flasks (Corning, USA) containing high glucose Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM /F-12) (Thermo Fisher Scientific Corporation, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific Corporation, USA), 1% GlutaMAX™) (Thermo Fisher Scientific Corporation, USA), and 1% Antibiotics-Antimycotic (Thermo Fisher Scientific Corporation, USA).

Canine adipose-derived MSCs (cAD-MSCs) were isolated from biopsied adipose tissues. Tissues were minced and incubated with Cell Recovery Solution® (Corning, USA) for 2 hours at 37℃, then passed through 70 µm strainer and washed twice with PBS. Pellet was resuspended and seeded in 60 mm culture dishes (Corning, USA). Cells were maintained in high glucose

Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific Corporation) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific Corporation, USA), 1% GlutaMAX™) (Thermo Fisher Scientific Corporation, USA), and 1% Antibiotics-Antimycotic (Thermo Fisher Scientific Corporation, USA).

Both cell types were maintained at 37°C in humidified atmosphere with 5% CO_2 and fresh air. Culture media was replaced every 48 hours. Cells were subcultures 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*, *Nanog*, and *Oct4*), proliferative marker (*Ki67*), mesenchymal cell surface markers (*Cd44*, *Cd45*, *Cd73*, and *Cd90*), and hematopoietic cell surface marker as a negative control (*Cd45*) by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). It should be noted that cell surface analysis by flow cytometry could not applied due to the unavailability of canine cross-reactive antibodies.

Cell differentiation potential was assessed using osteogenic induction protocol. Briefly, cells were seeded onto 24-well culture plate (Corning, USA) in a concentration of $2.5x10^5$ 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-glycerophosphate (66). Osteogenic differentiation potential was analyzed according to extracellular matrix (ECM) mineralization by Alizarin Red (84) and *Von Kossa* staining (85), and osteogenic-related gene marker expression (*Alp*, *Runx2*, *Osx*, *Opn*, *Ocn* and *Col1a1*) by RT-qPCR. Undifferentiated cells were used as a control.

IPC induction by microenvironmental manipulation

In this regard, three-step induction protocol modified from previous published reports was used (24, 58, 66). 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) (Sigma, USA), 1X insulin-transferrin-selenium (60) (Invitrogen, USA), 4 nM activin A (Sigma, USA), 1 nM sodium butyrate (Sigma, USA), and 50 μM β-mercaptoethanol (Sigma, USA). SFM-B was basal medium supplemented with 1% BSA, 1X ITS, and 0.3 mM taurine (Sigma, USA). SFM-C was basal medium containing 1.5% BSA, 1X ITS, 3 mM taurine, 100 nM glucagon-like peptide (GLP-1) (Sigma, USA), 1 mM nicotinamide (Sigma, USA), and 1X non-essential amino acids (NEAAs) (Sigma, USA).

Regarding culture maintenance, three different techniques were employed: low attachment, hanging-drop, and hydrogel (Matrigel®)-embedded culture techniques. For 2D low attachment culture, 60 mm non-treated culture dishes (Eppendorf, USA) were used. $1x10^6$ cells were collected and suspended onto each dish using three induction media mentioned above. For 3-dimensional (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 20,000 cells per 40 µL 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, USA). In this regard, 100-150 µL of hydrogel and induction medium mixture (1:1) was used to form a dome-like structure onto each well of 24-well culture plate (Coring, USA). Cell Recovery Solution® (Corning, USA) was used for gel digestion.

IPC induction by genetic manipulation

Overexpression of *PDX1* by lentiviral vector was used for genetic reprogramming of the cells. Lentivirus carried human pancreatic and duodenal homeobox 1 (*PDX1*), a transcription factor necessary for pancreatic development, was produced from the packaging of *pWPT-PDX1* (Addgene plasmid #12256, gift from Didier Trono) (http://n2t.net/addgene:12256; RRID: Addgene_12256) (33),

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-hours post-packaging and filtered through 0.45 µm 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 $5x10^4$ cells/well were seeded onto 24-well plates (Corning, USA) for 24 hours, then treated with 4 µg/mL polybrene infection/transfection reagent (Millipore/Chemicon, USA) for 30 minutes. Concentrated viral particles at MOI 20, 30, or 50 were used for each 24-hour-transfection course.

IPC induction by integration of genetic and microenvironmental manipulation

Integration of genetic and microenvironmental manipulation was performed by hanging-drop culture of *PDX1*-transfected cells using three induction media. *PDX1*-transfected cells at MOI 20 were seeded into GravityPLUS™ 96-well plate hanging-drop culture system at concentration of 20,000 cells per 40 µL per well. Series of induction media were substituted as described above.

Reverse transcription-quantitative polymerase chain reaction

Reverse transcription-quantitative polymerase chain reaction (RT-qCR) was used for mRNA analysis. The total RNA was collected using TRIzol-RNA isolation reagent (Thermo Fisher Scientific Corporation, USA), and extracted by DirectZol-RNA isolation kit (ZymoResearch, USA) according to the manufacture's protocol. Then, RNA was converted to complementary DNA (cDNA) using ImProm^{TM} 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, USA) with specific amplification primers. Glyceraldehyde 3-phosphate dehydrogenase, *Gapdh*, was used as the reference gene. mRNA expression of target genes was normalized with reference gene and control group. The primer sequences were listed in Table 1.

Table 1 Primer sequences

Genes	Accession number	Sequences	5' 3'	Length (bp)	Tm (℃)
Stemness genes					
Zinc finger protein 42 (Zep42 or *Rex1)*	XM_003639567.1	Forward	AGGTTCTCACAGCAAGCTCA	199	59.24
		Reverse	CCAGCAAATTCTGCGCACTG		60.73
POU class 5 homeobox 1 (Pou5f1 or *Oct4)*	XM_538830.1	Forward	AGGAGAAGCTGGAGCAAAACC	100	60.55
		Reverse	GTGATCCTCTTCTGCTTCAGGA		59.50

Proliferation marker					
Proliferation marker protein Ki-67 (Ki67)	XM_014108788.1	Forward	GTGCAACTAAAGCACGGAGA	124	58.49
		Reverse	GAGATTCCTGTTTGCGTTTTCGT		58.49
Stem cell surface markers					
Cd44 molecule (Cd44)	NM_001197022	Forward	CCCCATTACCAAAGACCACGA	148	60
		Reverse	TGGGATTTGAGGTTTCCGCA		59.89
5'-nucleotidase, ecto (Nt5e or *Cd73)*	XM_532221.5	Forward	GGCAACCTGATTTGTGATGCT	142	59.42
		Reverse	AGGTAATTGTGCCGTTGTTCC		59.12
Thy-1 cell surface antigen (Thy1 or *Cd90)*	NM_001287129.1	Forward	AGGACGAGGGGACATACACA	109	59.96
		Reverse	ATGCCCTCACACTTGACCAG		59.96
Endoglin (*Eng* or *Cd105*)	XM_005625330.2	Forward	CGAGGAGTCTGTCACCGGAAA	118	61.76
		Reverse	GCGCCAAAGGTGATACCCAG		61.38
Protein tyrosine phosphatase, receptor type, C (*Ptprc* or *Cd45*)	XM_005622282.1	Forward	GTTTCCAGTTCTGTTTCCCCAG	137	59.38
		Reverse	CATTGGTCACAATTCACGGTATCA		59.61
Osteogenic markers					
Alkaline phosphatase (*Alp*)	NM_001197137.1	Forward	CCTGCCAGATAACTGCCTCT	168	59.16
		Reverse	GTGGAGACACCCATCCCATC		59.82

Runt-related transcription factor 2 (*Runx2*)	XM_005642335.1	Forward	GGAAGAGGCAAGAGTTTCACC	209	58.84
		Reverse	GTGCTCACTTGCCAACAGAA		58.89
Sp7 transcription factor (*Spp* or *Osx*)	XM_844688.3	Forward	GCGTCCTCCCTGCTTGAG	122	60.13
		Reverse	GCTTTGCCCAGTGTCGTTG		60.01
Secreted phosphoprotein 1 (*Spp1* or *Opn*)	XM_003434024.2	Forward	GCCACAGAGCAAGGAAAACTC	180	59.73
		Reverse	CTGCTTCTGAGATGGGTCAGG		60.13
Bone gamma-carboxyglutamate protein (*Bglap* or *Ocn*)	XM_547536.4	Forward	GCCAGCCTATGGTCTCCTCTG	249	61.90
		Reverse	CCACCAGCTCCTTCTGTTCTCT		54.55
Collagen type I alpha 1 chain (*Col1a1*)	NM_001003090.1	Forward	CCAGCCGCAAAGAGTCTACAT	150	60.41
		Reverse	CTGTACGCAGGTGACTGGTG		60.67
Pancreatic markers					
Pancreatic endoderm marker					
Pancreatic and duodenal homeobox 1 (*Pdx1*)	NM_001284471.2	Forward	AAGTCTACCAAGGCTCACGC	201	60.04
		Reverse	GTGCCTCTCGGTCAAGTTCA		59.97
Pancreatic beta-cell or insulin-producing cells (IPCs) markers					
NK6 homeobox 1 (*Nkx-6.1*)	XM_544960.5	Forward	CAGGAGTTATGCAGAGCCCG	111	60.53
		Reverse	ACGTGGGTCTCGTGTGTTTT		60.11
ISL LIM homeobox 1 (*Isl-1*)	XM_848628.4, XR_001315955.1	Forward	TGGCTTACAGGCAAACCCAG	171	60.54
		Reverse	GACATCGACGCCACTTCACT		60.39

V-maf avian musculoaponeurotic fibrosarcoma oncogene homolog A (*Maf-A*)	XM_003431814.3	Forward	GCTTCAGCAAGGAGGAGGTC	136	60.39
		Reverse	CTCTGGAGCTGGCACTTCTC		60.11
Solute carrier family 2 (facilitated glucose transporter), member 2 (*Slc2a2* or *Glut-2*)	XM_545289.5	Forward	ACTCATCACAGGACGTGGAG	108	59.11
		Reverse	AGCTGAGTGTAGCGGTGAAG		59.76
Insulin (*Ins* or *Insulin*)	NM_001130093.1	Forward	TGGTAGAGGCTCTGTACCTGG	235	60.34
		Reverse	CGCCCCTAGTTGCAGTAATTC		59.06
Pancreatic-relating markers					
Glucagon (*Gcg* or *Glucagon*)	NM_001003044.1	Forward	TCCAATCGCGGTGTCAGAAG	197	60.39
		Reverse	ACCCTGAGAATGACGCTTGT		59.31
Glucagon-like peptide 1 receptor (*Glp1r*)	XM_014118246.1	Forward	CACGGTGGGCTATACACTCTC	116	59.93
		Reverse	AGGACGCAAACAGGTTCAGG		60.54
Notch targeted genes					
Hes Family BHLH Transcription Factor 1 (*Hes-1*)	XM_025478075.1	Forward	GAGAAGGCGGACATTCTGGA	137	59.46
		Reverse	ACCTCGTTCATACACTCGCTG		60.14
Hes Related Family BHLH Transcription Factor with YRPW Motif 1 (*Hey-1*)	NM_001002953.1	Forward	ACCTGAAAATGCTGCACACG	195	59.69
		Reverse	GCTGGGAGGCGTAGTTGTTA		59.75

Reference gene					
Glyceraldehyde 3-phosphate dehydrogenase (Gapdh)	NM_001003142.1	Forward	CCAACTGCTTGGCTCCTCTA	100	59.38
		Reverse	GTCTTCTGGGTGGCAGTGAT		59.67

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 (66, 86, 87). KRBH buffer solution contained 120mM NaCl, 5mM KCl, 2.5mM $CaCl_2$, 1.1mM $MgCl_2$, 25mM $NaHCO_3$, and 10mM HEPES. IPCs were gently collected and maintained with KRBH buffer solution at 37$^{\circ}C$ 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, USA) 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 (Millipore, USA) according to the manufacturing protocol. Secreted c-peptide levels were then normalized with total DNA (9) and incubation time (minutes).

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 sample groups, while Kruskal-Wallis test was used for three or more sample group comparison. The significant difference was considered when p-value < 0.05.

CHAPTER IV

RESULTS and DISCUSSION

RESULTS

cBM-MSCs and cAD-MSCs characterization

The isolated cBM-MSCs (Fig 1A and B) and cAD-MSCs (Fig 1C and D) showed fibroblast-like structure upon 2-dimensional (2D) culture. mRNA expression of stemness-related markers (*Rex1*, *Nanog*, and *Oct4*) and proliferation marker (*Ki67*) were detected (Fig 1E and F). MSC-related markers (*Cd44*, *Cd73*, *Cd90*, and *Cd105*) were also expressed, while hematopoietic cell surface marker (*Cd45*) was not detected (Fig 1G and H).

Both cells illustrated an osteogenic differentiation potential upon an *in vitro* 14-day induction regarding ECM mineralization as demonstrated by Alizarin Red S and *Von Kossa* staining (Fig 1I and J) and osteogenic mRNA marker expression (*Alp*, *Runx2*, *Osx*, *Opn*, *Ocn,* and *Col1a1*) (Fig 1K and L). The expression of stemness-, proliferation- and MSC-related markers were normalized with *Gapdh*, a reference gene.

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

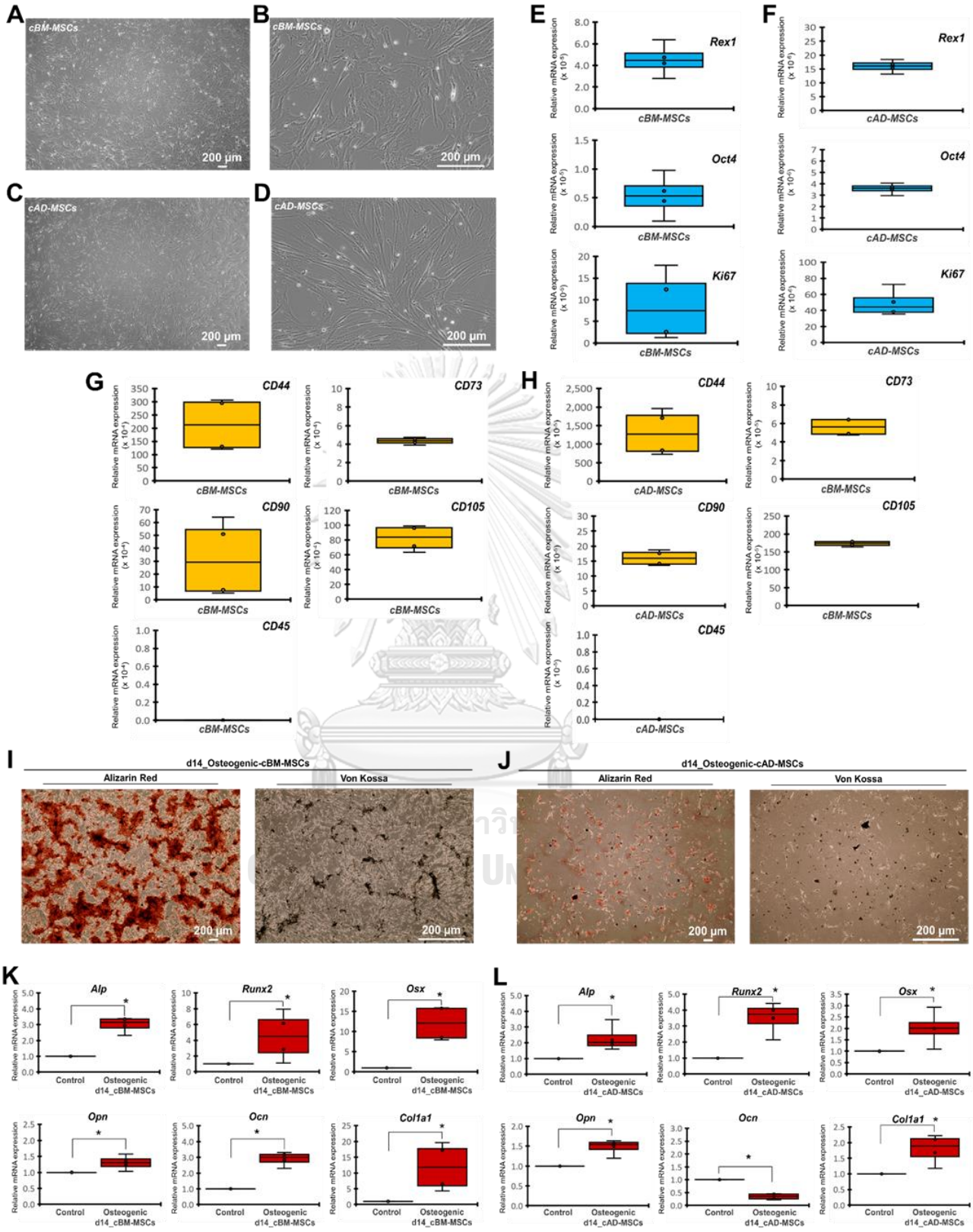

A
cBM-MSCs
200 µm
B
cBM-MSCs
200 µm
C
cAD-MSCs
200 µm
D
cAD-MSCs
200 µm
E
Rex1
Oct4
Ki67
cBM-MSCs
F
Rex1
Oct4
Ki67
cAD-MSCs
G
CD44
CD73
CD90
CD105
CD45
cBM-MSCs
H
CD44
CD73
CD90
CD105
CD45
cAD-MSCs
I
d14_Osteogenic-cBM-MSCs
Alizarin Red
Von Kossa
200 µm
J
d14_Osteogenic-cAD-MSCs
Alizarin Red
Von Kossa
200 µm
K
Alp
Runx2
Osx
Opn
Ocn
Col1a1
Control
Osteogenic d14_cBM-MSCs
L
Alp
Runx2
Osx
Opn
Ocn
Col1a1
Control
Osteogenic d14_cAD-MSCs

Figure 1 cBM-MSCs and cAD-MSCs 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), and surface markers (G and H) were determined by RT-qPCR. mRNA expression was normalized with reference gene. 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).

Generation of IPC-like cells from cBM-MSCs requires 3D culture condition

To generate IPC-like colonies from cBM-MSCs, three different culture techniques were investigated (Fig 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 Fig 2D showed that suspending the cells in low attachment culture dish (Eppendorf, USA) (Protocol I) was unable to deliver IPC-like colonies, while maintain the cells using hanging-drop technique (GravityPLUSTM plate, PerkinElmer, USA) (Protocol II) could successfully generate IPC-like 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 (Matrigel® Matrix, Corning, USA) (Protocol III). Although, the generated colonies were dense and compact, they could not maintain colony structure after gel digestion (Cell Recovery Solution®, Corning, USA) 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 (Nkx6.1, Isl-1, Maf-A, Glut-2, and Insulin), comparing with those from Protocol III (Fig 2E and F). However, the mRNA expression of pancreatic-relating markers (Glucagon and Glp-1r) were not detected in Protocol III (Fig 2G).

Further functional testing showed that IPC-like 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 (Fig 2H).

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

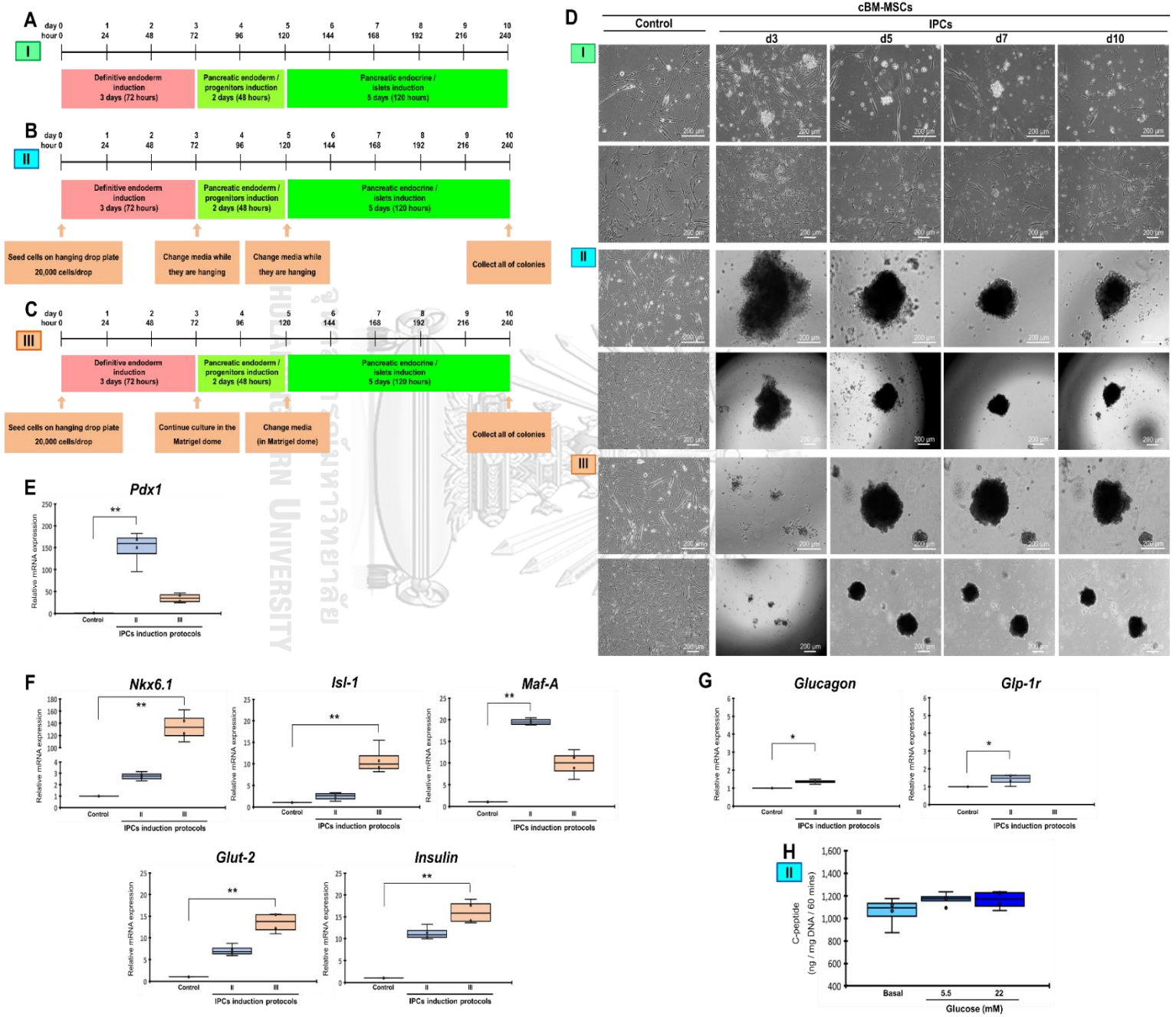

A
day 0 hour 0
1 24
2 48
3 72
4 96
5 120
6 144
7 168
8 192
9 216
10 240
I
Definitive endoderm induction 3 days (72 hours)
Pancreatic endoderm / progenitors induction 2 days (48 hours)
Pancreatic endocrine / islets induction 5 days (120 hours)
B
II
Seed cells on hanging drop plate 20,000 cells/drop
Change media while they are hanging
Change media while they are hanging
Collect all of colonies
C
III
Continue culture in the Matrigel dome
Change media (in Matrigel dome)
D
cBM-MSCs
Control
IPCs
d3
d5
d7
d10
200 µm
E
Pdx1
Relative mRNA expression
IPCs induction protocols
Control
F
Nkx6.1
Isl-1
Maf-A
Glut-2
Insulin
G
Glucagon
Glp-1r
H
C-peptide (ng / mg DNA / 60 mins)
Basal
5.5
22
Glucose (mM)

Figure 2 Generation of cBM-MSC-derived IPC-like cells by microenvironmental manipulation. Diagrams of three culture techniques used for the generation of cBM-MSC-derived IPC-like cells 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).

Overexpression of PDX1 fells to generate IPC-like cells from cBM-MSCs

Generating IPC-like cells from cBM-MSCs using genetic manipulating approach was conducted by overexpression of the pancreatic commitment regulator, PDX1. Lentiviral vector carrying human *PDX1* (Addgene plasmid #12256, gift from Didier Trono) was transfected into cBM-MSCs at MOI 20, 30, and 50 (Fig 3A-C). 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 (Fig 3D).

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 (Fig 3E and F). However, alpha-cell hormonal marker (*Glucagon*) was significantly expressed in MOI 20 transfection (Fig 3G), while *Glp-1r* was not detected in all groups (data not shown).

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

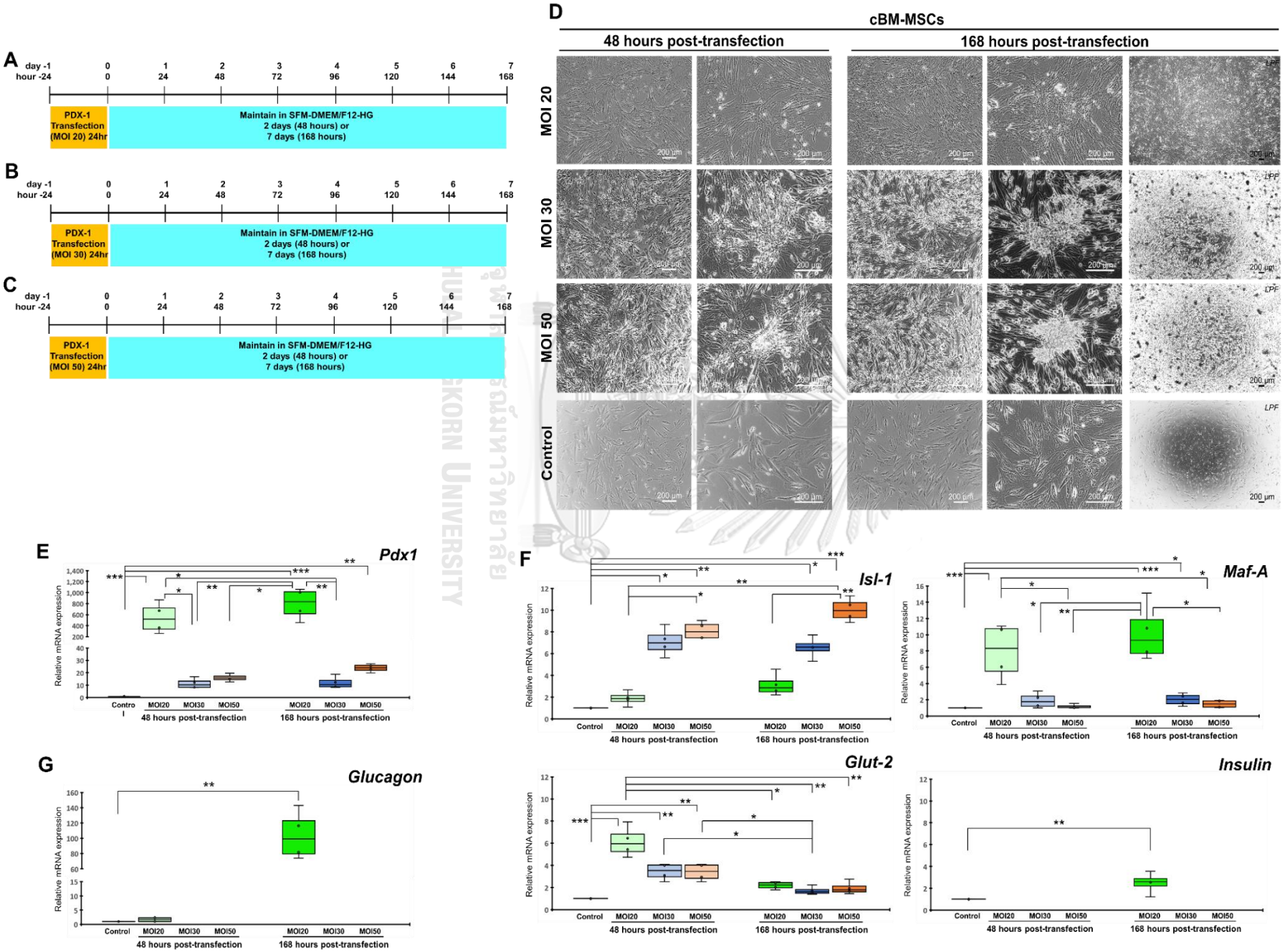

A
day -1 0 1 2 3 4 5 6 7
hour -24 0 24 48 72 96 120 144 168
PDX-1 Transfection (MOI 20) 24hr
Maintain in SFM-DMEM/F12-HG
2 days (48 hours) or
7 days (168 hours)
B
day -1 0 1 2 3 4 5 6 7
hour -24 0 24 48 72 96 120 144 168
PDX-1 Transfection (MOI 30) 24hr
Maintain in SFM-DMEM/F12-HG
2 days (48 hours) or
7 days (168 hours)
C
day -1 0 1 2 3 4 5 6 7
hour -24 0 24 48 72 96 120 144 168
PDX-1 Transfection (MOI 50) 24hr
Maintain in SFM-DMEM/F12-HG
2 days (48 hours) or
7 days (168 hours)
D
cBM-MSCs
48 hours post-transfection
168 hours post-transfection
MOI 20
MOI 30
MOI 50
Control
200 µm
LPF
E
Pdx1
Relative mRNA expression
Control
MOI20
MOI30
MOI50
48 hours post-transfection
168 hours post-transfection
F
Isl-1
Maf-A
Glut-2
Insulin
G
Glucagon

Figure 3 Generation of cBM-MSC-derived IPC-like cells by genetic manipulation.

Diagrams of three *PDX1* transfection condition for the generation of cBM-MSC-derived IPC-like cells were showed: MOI 20 (A), MOI 30 (B), and MOI 50 (C). Morphological appearances of cells undergone each of transfection condition were observed under phase-contrast microscope with magnification of 40X, 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. Bars indicated significant difference (*, *p*-value < 0.05; **, $p < 0.01$; ***, $p < 0.001$).

Integration of PDX1 overexpression with 3D culture efficiently generates IPC-like cells from cBM-MSCs

In order to efficiently generate IPC-like cells from cBM-MSCs, combination of genetic and microenvironmental manipulating approaches were 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 using GravityPLUS^{TM} plate) (Fig 4A). The results illustrated that IPC-like colonies started forming since day 5 of the induction, and size of colonies at day 12 was approximately 100-200 µm (Fig 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 (Fig 4C and D). However, alpha-cell hormonal marker (*Glucagon*) was highly expressed (Fig 4E), while *Glp-1r* was not detected (data not shown). Functional testing also showed that IPC colonies secreted C-peptide under basal condition and could produce a significant response upon low (5.5mM) glucose stimulation. However, the significant response upon high (22mM) glucose stimulation was not accomplished (Fig 4F).

Thus, combination of genetic and microenvironmental manipulating approaches efficiently generated IPCs from cBM-MSCs with pancreatic islet characteristics and functional property.

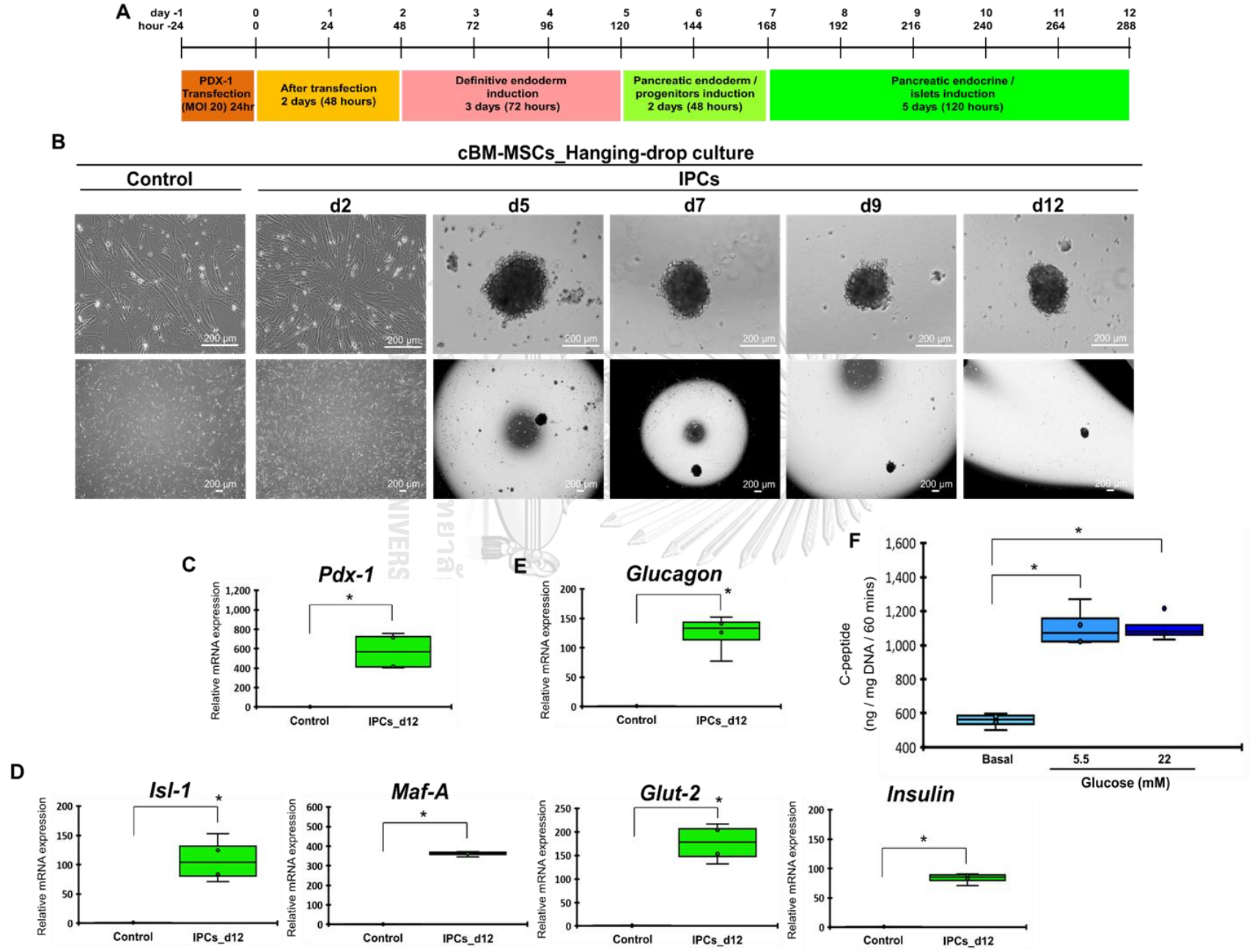

A
day -1
hour -24
0
0
1
24
2
48
3
72
4
96
5
120
6
144
7
168
8
192
9
216
10
240
11
264
12
288
PDX-1 Transfection (MOI 20) 24hr
After transfection 2 days (48 hours)
Definitive endoderm induction 3 days (72 hours)
Pancreatic endoderm / progenitors induction 2 days (48 hours)
Pancreatic endocrine / islets induction 5 days (120 hours)
B
cBM-MSCs_Hanging-drop culture
Control
IPCs
d2
d5
d7
d9
d12
200 μm
C
Pdx-1
Relative mRNA expression
Control
IPCs_d12
E
Glucagon
F
C-peptide (ng / mg DNA / 60 mins)
Basal
5.5
22
Glucose (mM)
D
Isl-1
Maf-A
Glut-2
Insulin

Figure 4 Generation of cBM-MSC-derived IPC-like cells by integration of genetic and microenvironmental manipulation.

Diagram of culture technique used for the generation of cBM-MSC-derived IPC-like cells 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).

Low attachment culture is efficient to generate IPC-like cells from cAD-MSCs

To generate IPC-like cells from cAD-MSCs, microenvironmental manipulating approach was used by suspending the cells onto low attachment culture dish and maintaining in three-step induction media (Fig 5A). It was quite interesting that cells formed colony-like structure since day 3 of the induction, and the colonies become denser and bigger along the culture period (Fig 5B). At day 12, approximately 834 colonies (median) were obtained from $1x10^6$ seeding cells (Fig 5C), and the colony size was varied from <50 µm to > 700 µm (Fig 5D).

Analysis of pancreatic mRNA expression revealed that pancreatic beta-cell markers (*Nkx6.1, Isl-1, Maf-A*, *Glut-2*, and *Insulin*) were significantly upregulated (Fig 5E). Alpha-cell hormonal marker (*Glucagon*) was a bit expressed, while *Glp-1r* was downregulated (Fig 5F). Functional testing showed that IPC-like 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 significant (Fig 5G).

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

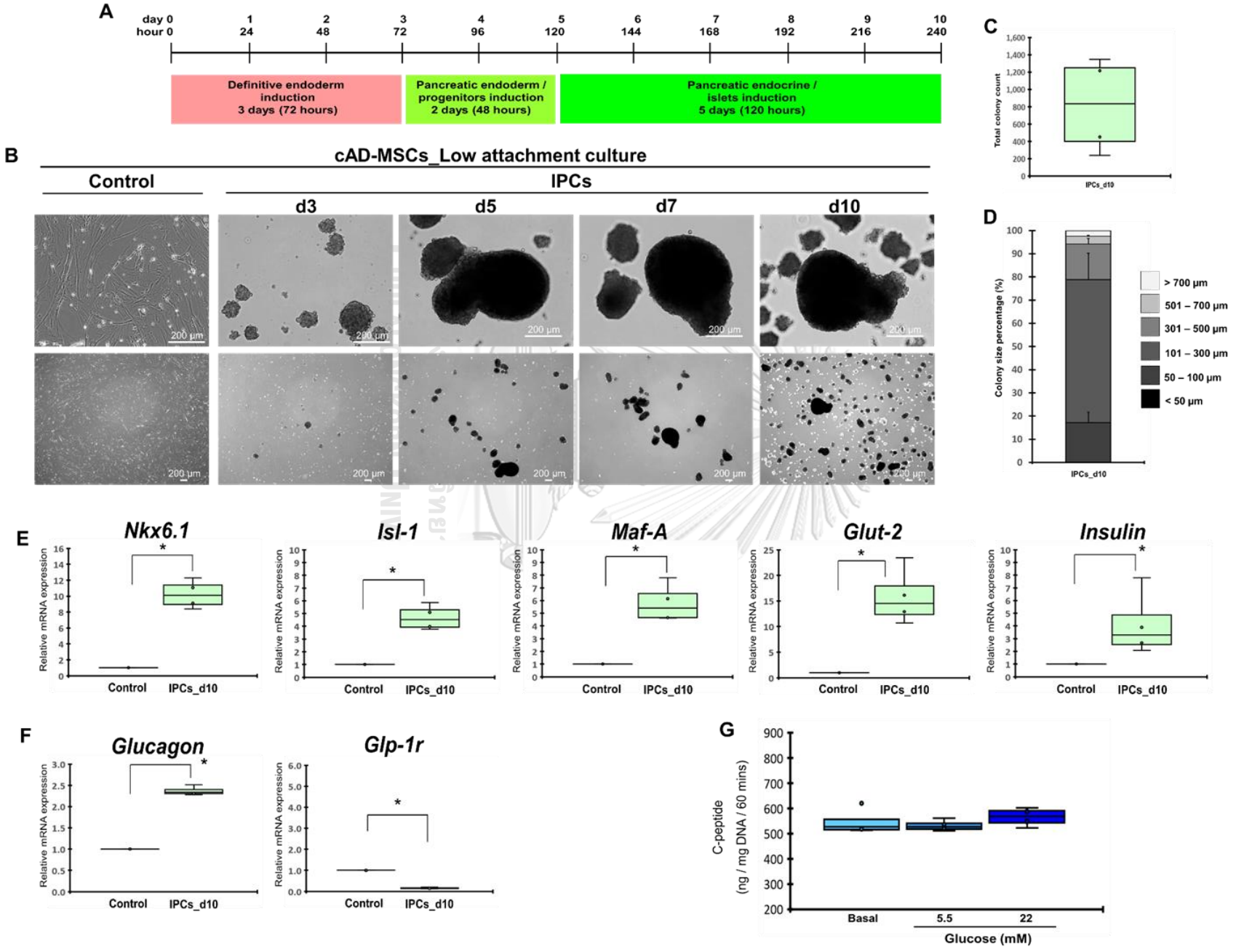

A
day 0 hour 0
1 24
2 48
3 72
4 96
5 120
6 144
7 168
8 192
9 216
10 240
Definitive endoderm induction 3 days (72 hours)
Pancreatic endoderm / progenitors induction 2 days (48 hours)
Pancreatic endocrine / islets induction 5 days (120 hours)
B
cAD-MSCs_Low attachment culture
Control
IPCs
d3
d5
d7
d10
200 µm
C
Total colony count
IPCs_d10
D
Colony size percentage (%)
> 700 µm
501 – 700 µm
301 – 500 µm
101 – 300 µm
50 – 100 µm
< 50 µm
IPCs_d10
E
Nkx6.1
Isl-1
Maf-A
Glut-2
Insulin
Relative mRNA expression
Control
IPCs_d10
F
Glucagon
Glp-1r
Relative mRNA expression
Control
IPCs_d10
G
C-peptide (ng / mg DNA / 60 mins)
Basal
5.5
22
Glucose (mM)

Figure 5 Generation of cAD-MSC-derived IPC-like cells by microenvironmental manipulation.

Diagram of culture technique used for the generation of cAD-MSC-derived IPC-like cells 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 (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 (G). Bars indicated significant difference (*, *p*-value < 0.05).

Notch signaling optimization generates potential cAD-MSC-derived IPC-like cells

According to the summary of IPC-like cell induction protocol efficiency illustrated in Table 2, it has been suggested that generation of cAD-MSC-derived IPC-like cells 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 (glucose-stimulated C-peptide secretion: 0, 5.5, and 22 mM glucose). In this regard, Notch signaling optimization was performed for generating the potential cAD-MSC-derived IPC-like cells using protocol mentioned in our previous report (66).

cAD-MSC-derived IPCs were generated using optimized three-step induction protocol (Fig 6A) with Notch signaling manipulation using gamma-secretase inhibitor, DAPT, during definitive endoderm induction (DAPT-A) (Fig 6B) or pancreatic endoderm/progenitor induction (DAPT-B) (Fig 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 (Fig 6D). Total colony counts (median) were 834, 691.5, and 504 colonies per batch ($1x10^6$ seeding cells) for control, DAPT-A, and DAPT-B, respectively (Fig 6E). It seemed that DAPT-B delivered more proportion of small-size colony (<50 μm and 50-100 μm), but statistical difference was not applied due to variation among groups (Fig 6F).

Pancreatic mRNA analysis illustrated that cAD-MSC-derived IPC-like cells 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 (Fig 7A and B). However, alpha-cell hormonal marker (*Glucagon*) of DAPT-B group was much lower than that in DAPT-A group. *Glp-1r* was downregulated in all conditions (Fig 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 (Fig 7D). Functional testing showed that cAD-MSC-derived IPC-like cells 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 (Fig 7E).

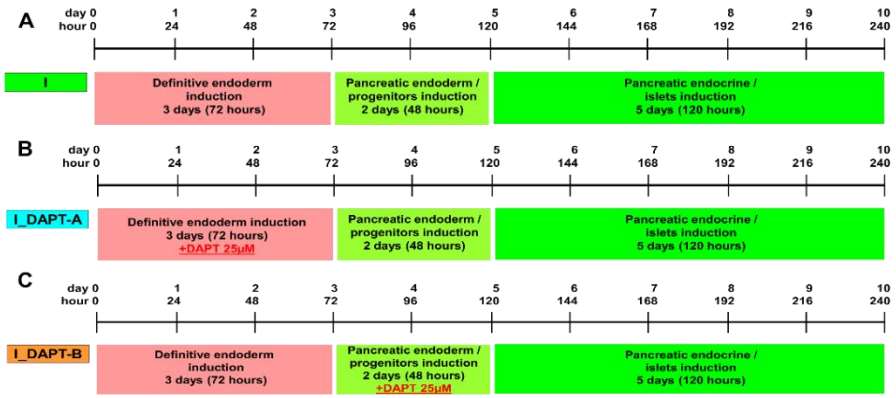
Taken together, the results suggested that cAD-MSC-derived IPC-like cells could be efficiently generated using microenvironmental manipulating approach with Notch optimization. The obtained IPC-like cells from Notch inhibition during pancreatic endoderm/progenitor induction showed pancreatic islet/beta-cell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

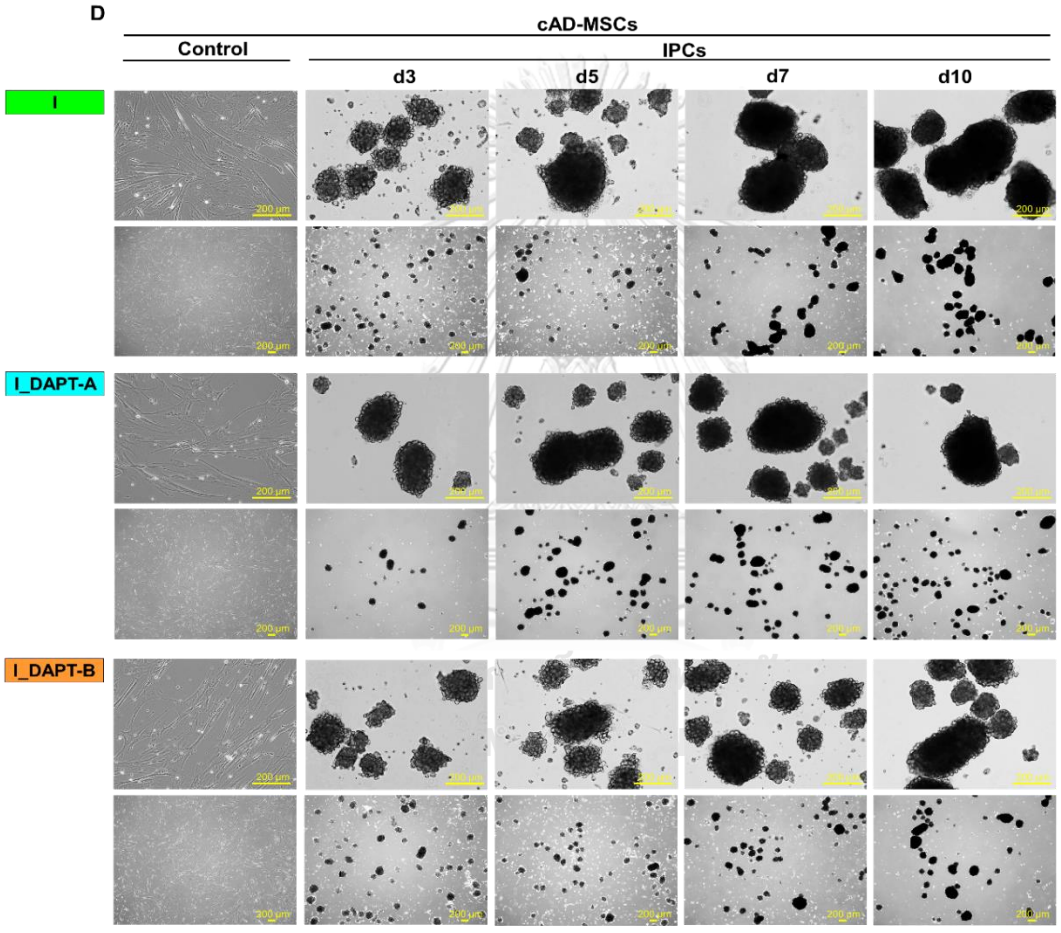
Table 2 Summary of IPC-like cell generation from cBM-MSCs and cAD-MSCs

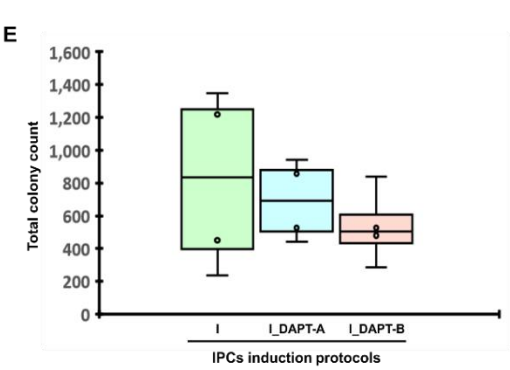
Criteria	cBM-MSC-derived IPC-like cells							cAD-MSC-derived IPC-like cells
	Microenvironmental manipulation			Genetic manipulation			Integration	Microenvironmental manipulation (Low attachment culture)
	Low attachment culture	Hanging-drop culture	Hanging-drop + Matrigel[2]-embedded culture	*PDX1* (MOI20)	*PDX1* (MOI30)	*PDX1* (MOI50)	*PDX1* (MOI20) + Hanging-drop culture	
(1) Morphological appearance and colony number								
1.1) **Floating (3D) colony-like structure**	-	√ (50-200 µm)	√ (50-200 µm)	-	-	-	√ (100-200 µm)	√ (<50 µm to > 700 µm)
1.2) **Colony number (per batch)**	-	96 colonies	< 96 colonies	-	-	-	96 colonies	834 colonies*
(2) Pancreatic mRNA marker expression								
2.1) **Pancreatic endoderm marker** (*Pdx1*)	n/a	√	√	√	√	√	√	-
2.2) **Pancreatic beta-cell markers** (*Nkx6.1*, *Isl-1*, *Maf-A*, *Glut-2*, *Insulin*)	n/a	√	√	√ -Not detect: *Nkx6.1*	√ -Not detect: *Nkx6.1*, *Insulin*	√ -Not detect: *Nkx6.1*, *Insulin*	√ - Not detect: *Nkx6.1*	√
2.3) **Pancreatic-relating marker** (*Glucagon*)	n/a	√	-	√	-	-	√	√ (Upregulation)
2.3) **Pancreatic-relating marker** (*Glp-1r*)	n/a	√	-	-	-	-	-	√ (Downregulation)
(3) Functional property (glucose-stimulated C-peptide secretion: 0, 5.5, and 22 mM glucose) (ng C-peptide/mg DNA/60 mins)								
Basal C-peptide secretion (0 mM glucose)	n/a	1,092.40*	n/a	n/a	n/a	n/a	564.22*	526.67*
Low glucose stimulation (5.5 mM glucose)	n/a	1,176.15*	n/a	n/a	n/a	n/a	1070.30*	527.78*
High glucose stimulation (22 mM glucose)	n/a	1,174.10*	n/a	n/a	n/a	n/a	1078.85*	568.38*

*Median value

n/a (not applicable)


A
day 0
hour 0
1
24
2
48
3
72
4
96
5
120
6
144
7
168
8
192
9
216
10
240
I
Definitive endoderm induction
3 days (72 hours)
Pancreatic endoderm / progenitors induction
2 days (48 hours)
Pancreatic endocrine / islets induction
5 days (120 hours)
B
I_DAPT-A
Definitive endoderm induction
3 days (72 hours)
+DAPT 25µM
Pancreatic endoderm / progenitors induction
2 days (48 hours)
Pancreatic endocrine / islets induction
5 days (120 hours)
C
I_DAPT-B
Definitive endoderm induction
3 days (72 hours)
Pancreatic endoderm / progenitors induction
2 days (48 hours)
+DAPT 25µM
Pancreatic endocrine / islets induction
5 days (120 hours)


D
cAD-MSCs
Control
IPCs
d3
d5
d7
d10
I
I_DAPT-A
I_DAPT-B
200 µm


E
Total colony count
1,600
1,400
1,200
1,000
800
600
400
200
0
I
I_DAPT-A
I_DAPT-B
IPCs induction protocols

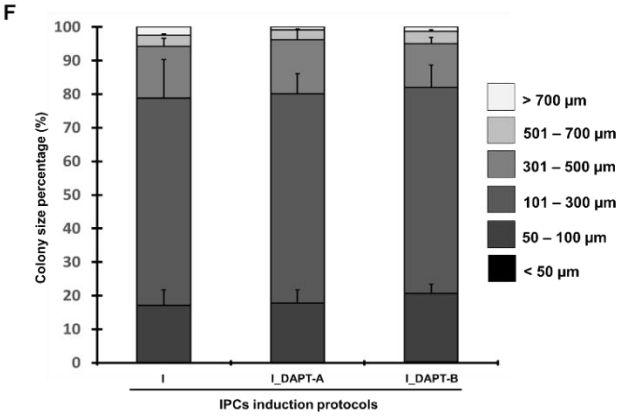

F
Colony size percentage (%)
100
90
80
70
60
50
40
30
20
10
0
I
I_DAPT-A
I_DAPT-B
IPCs induction protocols
> 700 µm
501 – 700 µm
301 – 500 µm
101 – 300 µm
50 – 100 µm
< 50 µm

Figure 6 Generation of cAD-MSC-derived IPC-like cells with Notch signaling manipulation.

Diagrams of Notch signaling manipulation used for the generation of cAD-MSC-derived IPC-like cells 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.

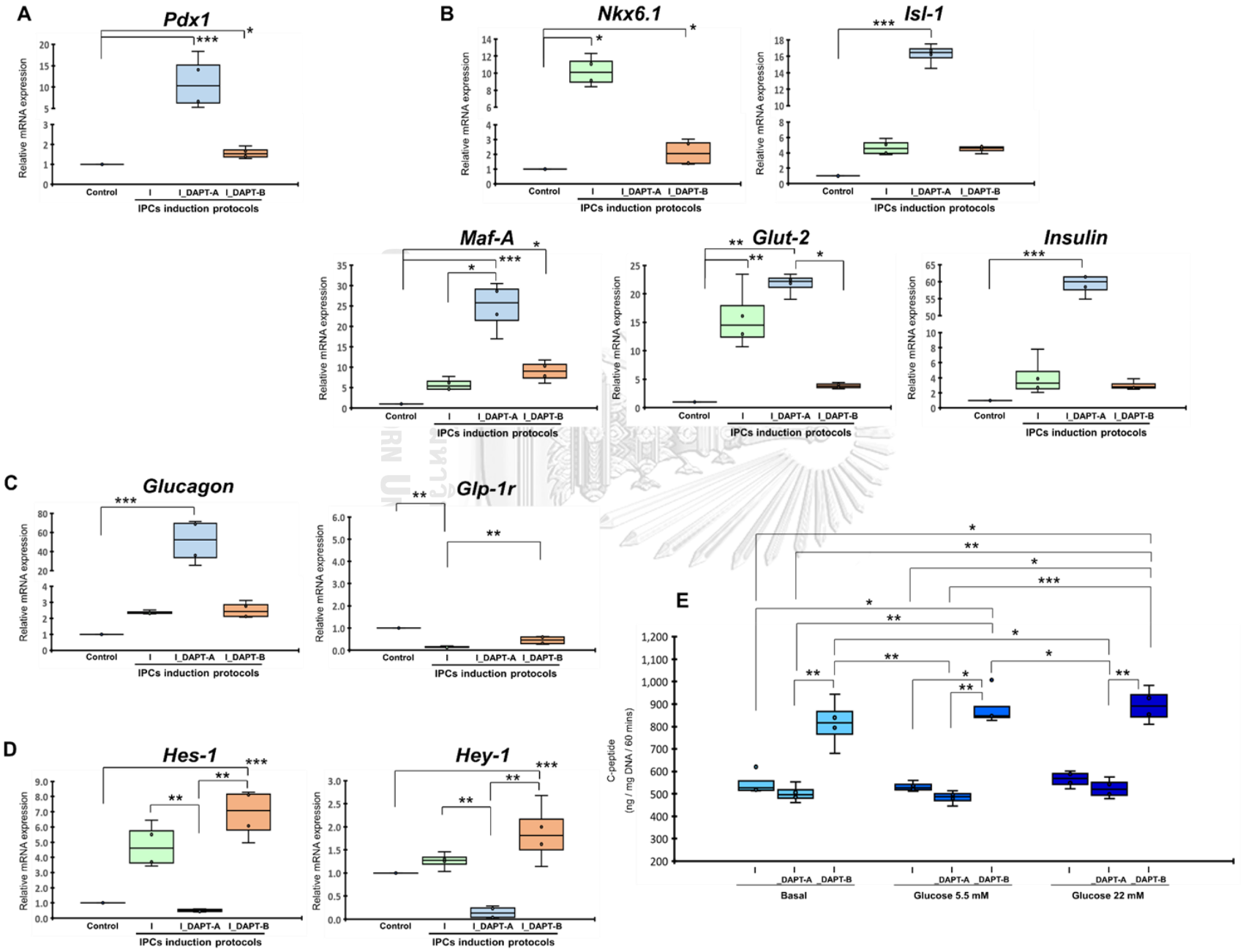

A
Pdx1
B
Nkx6.1
Isl-1
Maf-A
Glut-2
Insulin
C
Glucagon
Glp-1r
D
Hes-1
Hey-1
E
Relative mRNA expression
C-peptide (ng / mg DNA / 60 mins)
Control
I
I_DAPT-A
I_DAPT-B
IPCs induction protocols
Basal
Glucose 5.5 mM
Glucose 22 mM

Figure 7 Generation of cAD-MSC-derived IPC-like cells 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; **, $p < 0.01$; ***, $p < 0.001$).

DISCUSSION

As the proof-of-concept evidences for treating diabetes by regenerative therapy have been reported in human and animal models (15, 17, 18, 32, 51, 68), MSCs have been proposed as one of the promising resources for generating clinical applicable IPCs (48, 51, 54, 88-92). 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 (83, 92-94). Their characteristics were similar as described in previous reports including fibroblast-like structure; mRNA expression related to stemness, proliferation, and MSC markers; and osteogenic differentiation potential (83, 93-97). These evidences supported the consistency of the cMSCs' properties used in this report. It should be suggested that comparing the expression of stemness-, proliferation-, and MSC-related markers of the isolated cMSCs with the initial tissues (bone marrow aspirate and adipose tissue) might reflect the homogeneity of the isolated MSC population.

In term of IPC formation *in vitro*, various protocols employing either microenvironmental manipulation or genetic manipulation have been reported (21, 24, 26, 33, 34). The strategies used in these studies usually relied on origin and pluripotency/multipotency of the cells (43-46, 98). It should be noted that pluripotent SCs, embryonic SCs (ESCs) and induced pluripotent SCs (iPSCs),

contained high capability of pancreatogenesis *in vitro* (99-104). However, due to their ethical and safety concerns, MSCs have been proposed as an alternative source for IPC generation (20, 21, 25, 26, 28-32, 100).

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 IPC-like cells. 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 (20, 66, 103, 105-110). 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 (54, 111). 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 (24, 54, 57, 58, 66, 112-116). It was quite interesting that maintaining the 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 (24, 58, 66, 104, 117-119). Therefore, the 3D culture condition using hanging-drop and Matrigel®-embedded culture techniques were used for generating the cBM-MSC-derived IPC-like colony. It has been shown that hanging-drop culture was an efficient technique for embryoid body/cell colony formation *in vitro* (120-123), and natural/synthetic hydrogel-embedded culture was one of effective culture techniques used for organoid formation and expansion (124-128). In this study we demonstrated the successful IPC-like colony formation by these two culture techniques. However, it was quite difficult to collect and expand the IPC-like colonies since colony maintaining and medium changing for hanging-drop culture were time-consumed, and treating the Matrigel®-embedded colony with hydrogel digesting solution (Cell Recovery Solution®, Corning, USA) caused colony dissociation. Further functional assay could only be performed for IPC-like colonies derived from hanging-drop culture and found that the obtained IPC-like 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-like colony. Expression of *Glucagon*, a hormonal gene of alpha-cell (33, 49), was also found.

At MOI 30 and MOI 50, cell cluster formation was apparently found, correlating with previous report (129). However, the expression of pancreatic mRNA markers was very much lower comparing with MOI 20 transfection. This might due to a decreased cell viability after high viral titer transfection as mentioned in previous study (130).

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 successful formation of 3D IPC colonies with significant pancreatic marker expression. Functional assay also confirmed the glucose-responsive C-peptide secretion of the obtained colonies. These findings were correlated to previous reports. *PDX1* is an essential gene in the first hierarchy of pancreatic organogenesis progressing toward beta-cell maturation (53, 54). PDX1-positive cells were considered as the pancreatic progenitors for three pancreatic lineages, comprising endocrine, exocrine, and ductal cells (20). It has been shown that 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 a ball-like cell colonies (26, 50). For cBM-MSCs, we found that 3D culture condition was required to form the IPC-like colony which was considered as the native pancreatic islet morphology (69, 117, 119).

Thus, cBM-MSC-derived IPC-like cells were able to obtain from the integrating protocol of genetic and microenvironmental manipulation. However, hanging-drop 3D culture technique was time- and labor-consumed, making it less clinical applicable.

To find an alternative way, cAD-MSCs has been proposed as a potential MSC candidate for regenerative diabetes therapy as mentioned in previous reports (49-51, 108, 131). We showed in this study that cAD-MSC-derived IPC-like colonies could efficiently be generated from low attachment culture with the expression of crucial pancreatic mRNA markers. Functional assay showed a basally C-peptide secretion with trend of glucose-responsive c-peptide secretion in high glucose (22 mM) stimulation. In order to compare the potential for further clinical application, it seemed that cBM-MSC-derived IPC-like cells showed less clinical application potential due to the complicated and time/labor-consumed induction protocol, so cAD-MSC-derived IPC-like cells were further optimized.

Various factors and signaling have been studied and shown 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* (55, 72, 73, 77, 79, 132-134, 135 , 136). cAD-MSC-derived IPC-like cells were generated using optimized three-step induction protocol with Notch signaling manipulation using gamma-secretase inhibitor, DAPT, during definitive endoderm induction or pancreatic endoderm/progenitor induction. We found that Notch inhibition during pancreatic

endoderm/progenitor induction yielded a significant functional benefit for cAD-MSC-derived IPC-like cells as seen in significant higher basal C-peptide secretion and positive trend of glucose-responsive C-peptide secretion stimulated by low (5.5 mM) and high (22 mM) glucose.

These findings were correlated with previous studies that Notch signaling played a major role in embryonic development during pancreatic organogenesis by the activation on the early stage of development, enhancing *Pdx1*-postive pancreatic precursors and inhibiting the latterly stage driving on pancreatic endocrine differentiation (73, 77, 134, 135 , 136). Inhibition of Notch during pancreatic endoderm induction in human dental pulp stem cells (hDPSCs) resulted in high number of IPC colony production with high expression of *PDX1,* while *NKX6.1*, an essential pancreatic beta-cell marker, was downregulated (55). It has been suggested that Notch signaling was required during late state IPC production and maturation (66, 134, 137). *Hes*-1 play and important role in preventing premature differentiation. Therefore, the *Hes-1*-defficiency showed the acceleration on all pancreatic endocrine cell differentiation which might affect the property of generated endocrine cells (82, 135). It was quite crucial that the IPC generated by the *in vitro* protocol should be fully matured as shown by an expression of crucial pancreatic beta-cell marker, an insulin production along with a significant C-peptide/insulin secretion in both of basal condition and glucose stimulation. To address this concern, various components should be further optimized. In addition, the positive control for pancreatic mRNA marker expression by using canine pancreas,

the negative control for glucose-stimulated C-peptide secretion (GSCS) study using undifferentiated cMSCs, and the ideal control using pre- and post-prandial levels of secreted C-peptide/insulin from canine model should also be considered.

CHAPTER V

CONCLUSION

In conclusion, we illustrated that cMSC-derived IPC-like cells 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. Thus, to make the clinical applicable cMSC-derived IPCs, further studies should be focused on IPC maturation, glucose-responsive function, and transplantation platform.

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