รายงานโครงการวิจัยฉบับสมบูรณ์

เรื่อง

การพัฒนาวิธีการควบคุมด้านพันธุกรรมในห้องปฏิบัติการสำหรับการสร้างกลุ่มเซลล์ที่เหมือนไอเล็ทหรือเซลล์สังเคราะห์อินซูลินจากเซลล์ต้นกำเนิดมีเซนไคม์จากไขกระดูกสุนัข

Development of genetic manipulation approach for in vitro production of islet-like cell cluster (ILCCs) or insulin-producing cells (IPCs) from canine bone marrow-derived mesenchymal stem cells (cBM-MSCs).

ทุนอุดหนุนการวิจัยจากเงินรายได้ ประเภทเงินอุดหนุนการวิจัยจากรัฐบาล จุฬาลงกรณ์มหาวิทยาลัย ประจำปีงบประมาณ 2561 (ปีที่ 2)
(โครงการต่อเนื่อง ปีงบประมาณ 2560 - 2561)

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โครงการวิจัยนี้ได้รับทุนอุดหนุนการวิจัยจากจุฬาลงกรณ์มหาวิทยาลัย ทุนอุดหนุนการวิจัยจากเงินรายได้ ประเภทเงินอุดหนุนการวิจัยจากรัฐบาล จุฬาลงกรณ์มหาวิทยาลัย ประจำปีงบประมาณ 2561 (ปีที่ 2) (โครงการต่อเนื่อง ปีงบประมาณ 2560 - 2561) (This research is funded by Chulalongkorn University)

ABSTRACT

งานวิจัยนี้ เป็นการศึกษาเบื้องต้นในการพัฒนาแนวทางการรักษาโรคเบาหวานด้วยเซลล์ต้นกำเนิดในทางสัตวแพทย์ โดยมีจุดมุ่งหมายในการสร้างกลุ่มเซลล์ตับอ่อนจากเซลล์ต้นกำเนิดมีเซนไคม์จากไขกระดูกของสุนัขโดยการใช้วิธีการควบคุมด้านพันธุกรรม

ในการทดสอบประสิทธิภาพในการนำส่งชิ้นส่วนพันธุกรรมโดยเลนติไวรัสรุ่นที่ 2 สู่เซลล์อาศัยการแสดงออกของโปรตีนเรืองแสงสีเขียว (pLenti CMV GFP Puro (658-5) Addgene plasmid #17448) ผลการทดสอบพบว่าเซลล์ต้นกำเนิดมีเซนไคม์จากไขกระดูกของสุนัขสามารถรับการส่งชิ้นส่วนพันธุกรรมดังกล่าวได้ และสำหรับการทดสอบการส่งชิ้นส่วนพันธุกรรมของยีนพีดีเอ็กซ์ 1 นั้น อาศัยพลาสมิด pWPT-PDX1 plasmid (Addgene plasmid # 12256) ที่ MOI 20 30 และ 50 ผลการศึกษาพบว่าเซลล์มีการเปลี่ยนแปลงลักษณะคล้ายโคโลนี โดยเป็นการเปลี่ยนแปลงที่ขึ้นอยู่กับความแรงของ MOI และช่วงเวลาในการเหนี่ยวนำ และมีการเปลี่ยนแปลงของยีนที่จำเพาะต่อเบต้าเซลล์ นอกจากนี้การเหนี่ยวนำด้วยวิธีการควบคุมสิ่งแลดล้อมที่ได้ปรับปรุงขึ้นมาใหม่ พบว่าเทคนิคการเลี้ยงกลับหัว และการเลี้ยงในโดม สามารถเหนี่ยวนำให้เซลล์เปลี่ยนแปลงสู่เซลล์ตับอ่อนได้ การศึกษาต่อเนื่องพบว่าเทคนิคการเลี้ยงกลับหัวร่วมกับการดัดแปลงพันธุกรรม สามารถเหนี่ยวนำให้เกิดการเปลี่ยนแปลงสู่เซลล์ตับอ่อนและการแสดงออกของยีนที่จำเพาะต่อเซลล์ตับอ่อนในระดับสูง

ดังนั้น การศึกษานี้จึงแสดงให้เห็นถึงความเป็นไปได้ในการสร้างเซลล์ตับอ่อนจากเซลล์ต้นกำเนิดมีเซนไคม์จากไขกระดูกของสุนัขโดยการใช้วิธีการควบคุมด้านพันธุกรรมร่วมกับการวิธีการควบคุมสิ่งแลดล้อมที่ได้ปรับปรุงขึ้นมาใหม่

ABSTRACT

In this study, trend of stem cell-based treatment for diabetes type 1 in veterinary practice has been preliminarily investigated. Production of pancreatic lineages by canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) using genetic manipulation approach has been studied. Transfection efficiency of second-generation lentiviral vector on cBM-MSCs employing "pLenti CMV GFP Puro (658-5)" (Addgene plasmid #17448) was investigated and the results suggested the susceptibility of the cell to such transfection. Further study was performed to evaluate the efficiency of *PDX1* transfection on cBM-MSCs, focusing on cell fate after transfection. The lentiviral vector containing "pWPT-PDX1 plasmid" (Addgene plasmid # 12256) was used. The multiplicity of infection (MOI) 20, 30, and 50 were employed. The results illustrated that *PDX-1* transfection could enhance dose- and time-dependent cell morphological change toward colony-like structure. Some of pancreatic gene markers were also upregulated. Upon the induction by modified three-dimension (3D) micro-environmental manipulating protocol, "hanging drop-culture technique" and "hanging-drop culture technique with Matrigel-formed dome culture technique" could effectively enhance pancreatic differentiation by cBM-MSCs. Further study on integrating hanging drop-cell culture technique with genetic manipulation illustrated that cBM-MSCs could be differentiated toward pancreatic lineage with dramatic expression of pancreatic mRNA markers.

Thus, this study demonstrated that cBM-MSCs could be used as the source of pancreatic lineage derivation by using integration of genetic and microenvironmental manipulating protocol *in vitro.*

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Abbreviations	
ASCs	Adult stem cells
cBM-MSCs	Canine bone marrow-derived mesenchymal stem cells
ESCs	Embryonic stem cells
ILCs	Islet-like cell cluster
IPCs	Insulin-producing cells
iPSCs	Induced pluripotent stem cells

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การพัฒนาวิธีการควบคุมด้านพันธุกรรมในห้องปฏิบัติการ สำหรับการสร้างกลุ่มเซลล์ที่เหมือนไอเล็ทหรือเซลล์สังเคราะห์อินซูลิน จากเซลล์ต้นกำเนิดมีเซนไคม์จากไขกระดูกสุนัข

INTRODUCTION

Background and rationale

Diabetes mellitus, an intractable disease leading to hyperglycemia, is a leading cause of illness and serious complications of people around the world. Recently, American Diabetes Association (ADA) has announced the latest issue of "Standard of medical care in diabetes" which suggested the classification of diabetes into 4 categories comprising type I, type II, gestational diabetes mellitus (GDM), and specific types of diabetes (American Diabetes, 2017). Additionally, in 2011, the "Global burden of metabolic risk factors of chronic diseases collaborating group" has published an interesting systematic review in Lancet. The authors launched health examination surveys and epidemiological studies covering 2.7 million of participants form 370 country-year during 1980-2008 and found global increases of glycemia (fasting plasma glucose, FPG) and diabetes prevalence which were correlated with population growth and ageing (Danaei et al., 2011). Moreover, the global prevalence of diabetes is expected to be 366 million in all age-groups worldwide by 2030 (Wild et al., 2004). In animals, there were many of case reports regarding evidence of diabetes in companion animals especially dogs and cats. Most of diabetic dogs were mainly type I while type II was usually found in diabetic cats. These findings suggested an importance of diabetes as global issue in both human and animal.

Regarding diabetes treatment, several approaches have been clinically introduced to manage hyperglycemic conditions and consequence complications e.g. exogenous insulin and pharmacotherapeutic preparations (e.g. metformin, glucagon-like peptide (GLP) 1 receptor antagonists, sodium-glucose cotransporter (SGLT) 2 inhibitors, dipeptidyl peptidase (DPP) 4 inhibitors, thiazolidinediones, alpha-glucosidase inhibitors, colesevelam, bromocriptine quick release, sulfonylureas-glinide, and pramlintide etc.) (Handelsman et al., 2015). However, there were some reports

suggested an evidence of hypoglycemia and adverse events regarding therapeutic regimens varying from gastrointestinal signs to life-threatening symptoms (Amiel et al., 2015). From these reasons, many of research groups have been trying in finding novel approach to cope diabetes and its complications. In 2000, the first human islet transplantation for type I diabetes according to Edmonton protocol has been reported (Shapiro, 2012). Interestingly, the efficiency and reproducibility of the protocol have been clinically verified suggesting possibility of regenerative treatment for diabetes. However, there were some limitations regarding the protocol i.e. adverse events of immunosuppressants, limited availability of donors, and limited duration of insulin independent period (Jamiolkowski et al., 2012; Sekine et al., 2012; Vantyghem et al., 2014). By the limitations of Edmonton protocol, trend of stem cell-based therapy has been announced as a candidate and promising protocol for diabetes treatment aiming on production of transplantable insulin-producing cells (IPCs). With high capacity of cell differentiation, various types of pluripotent stem cells have been employed for generation of IPCs *in vitro*. Derivation of pancreatic progenitors and matured IPCs from mouse and human embryonic stem cells (ESCs) has been reported (Chmielowiec and Borowiak, 2014; Schiesser et al., 2014). Besides, several groups have announced their success in IPCs production from human induced pluripotent stem cells (iPSCs) (Drummond et al., 2011; Holditch et al., 2014). In contrary, there were some evidences suggested burdens regarding immunocompatibility between cell donors and recipients that caused by human leukocyte antigen (HLA), ABO blood group, and minor antigens (de Rham and Villard, 2014). Moreover, manipulation and differentiation protocols of pluripotent cells were very complicated and expensive.

To address the problems, adult stem cells (ASCs)-based autologous transplantation and HLA-based allogenic cell therapy have been widely introduced (de Rham and Villard, 2014). Various types of mesenchymal stem cells (MSCs) have been used for generation of IPCs *in vitro*. Human and mouse bone marrow-derived MSCs (BM-MSCs) have been reported as an *in vitro* IPCs inducible cells (Rahmati et al., 2013; Xie et al., 2009). In aspect of IPCs production from other MSCs for example dental tissue-derived MSCs, *in vitro* generation of IPCs was firstly achieved by using MSCs isolated from juvenile dental pulp tissue so-called stem cell from human exfoliated deciduous teeth (SHED) (Govindasamy et al., 2011). Recently, with modification of previous published protocol, our team has launched a publication suggesting an *in vitro* IPCs differentiation potential by dental pulp stem cells (DPSCs) and periodontal ligament stem cells (PDLSCs) harvested from adult premolars and molars (Sawangmake et al., 2014). In correlation with other published reports, the evidences suggested possibility of dental tissue-derived MSCs utilization in cell-based regenerative therapy

for diabetes (Carnevale et al., 2013; Ishkitiev et al., 2013; Kanafi et al., 2013). According to our published protocol regarding microenvironment manipulation by using active small molecules, the induction protocol was able to drive the IPCs differentiation. However, the amount of obtained IPCs colonies was still low. Approximately 10-15% of cells were undergone IPCs differentiation and maturation *in vitro* (Sawangmake et al., 2014). This led to an unmet gap of desired differentiation capacity. To enhance the protocol efficiency, genetic manipulating approach has been introduced and widely studied. Several crucial genes involving pancreatic commitment and differentiation, e.g. PDX1, MAFA, and NGN3 etc., were employed, and the significant success of *in vitro* IPCs production was revealed (Chen et al., 2014).

However, regarding stem cell-based therapy for diabetes in companion animals, there was no any evidence supporting trend of application. This led to unmet knowledge and opportunity for treating intractable disease. To address the limitation regarding regenerative treatment for diabetes in animals, *in vitro* IPCs differentiation by canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) using genetic manipulation approach utilizing key pancreatic commitment/development regulating genes focusing on PDX1 will be employed in IPCs differentiation protocol. The obtained results will provide an important information regarding enhancement of IPCs production *in vitro* by cBM-MSCs and will be a basic knowledge for establishment of pancreatic differentiation protocol of other canine stem cell types. Moreover, the success according to IPCs formation in term of cell differentiation and maturation will be an important step before starting an *in vivo* transplantation study which is critical for the clinical protocol establishment.

Reviewed literatures

According to an *in vitro* IPCs induction protocol, various methods have been studied and could be categorized into 2 approaches comprising microenvironment and genetic manipulation (Chmielowiec and Borowiak, 2014; Holditch et al., 2014; Pandian et al., 2014).

<u>IPCs induction by microenvironment manipulation approach</u>

By using active small molecules, the IPCs induction protocol could enhance phenotype changing of pluripotent or multipotent cells *in vitro*. However, to design the multi-step IPCs induction protocol, the process regarding *in vivo* pancreatic development should be clearly investigated and defied. The natural pancreatic development in both human and animal would suggest clues of molecule family that could enhance the success of each induction step (Kordowich et al., 2010). In

correlation with pancreatic development, the IPCs induction protocol could theoretically divided into 4 steps which include 1) definitive endoderm induction 2) pancreatic endoderm induction 3) pancreatic endocrine precursors induction and 4) pancreatic beta-cell or IPCs induction (Schiesser and Wells, 2014). Additionally, to identify the stage of differentiation, various criteria have been evaluated e.g. cell morphology, expression of stage-specific genes and proteins, and functional property of matured cells etc. Interestingly, morphology of differentiated IPCs closely resembled islet of Langerhans but differed in size and cell density. Besides, stage-specific genes and proteins comprised 4 sets of markers i.e. definitive endoderm (CXCR-4, GOOSECOID, FOXA2, SOX-17, and BMP-2 etc.), pancreatic endoderm (PDX-1, and HNF-6 etc.), pancreatic endocrine precursors (NGN-3, NEUROD1, MAF-B, NKX-2.2, and PAX-4 etc.), and pancreatic beta-cell or IPCs (INSULIN, NKX-6.1, MAF-A, and GLUT-2 etc.) (Kordowich et al., 2010; Schiesser and Wells, 2014). For identification of matured IPCs *in vitro*, functional property of differentiated cell was evaluated by analysis of insulin or C-peptide released upon glucose stimulation (Schiesser and Wells, 2014).

In term of *in vitro* IPCs induction protocol establishment, various sets of protocol regarding microenvironment manipulation have been tried and reported. However, the efficiency of protocol was varied and theoretically relied on numerous factors for example pluripotency/multipotency of cells, passage numbers, enhancing/signaling molecules used for each induction step, culture conditions etc. (Kordowich et al., 2010; Schiesser and Wells, 2014). In 2008, Vaca et al. published a report showing that treating undifferentiated mouse ESCs with nicotinamide, the poly (ADP-ribose) polymerase (PARP) inhibitor, could enhance expression of insulin and trigger normalization of blood glucose in diabetic mice. However, values of insulin and C-peptide released *in vitro* were relative to 10% of normal mouse values (Vaca et al., 2008). This suggested that only one key molecule could actively enhance differentiation of pluripotent cells toward pancreatic lineage but the levels of insulin expression and secretion were still very low.

To improve the efficiency of protocol, multi-step induction/differentiation approach has been introduced. Nostro et al. published a 5-stage IPCs induction protocol so-called “Nostro protocol” which employed molecules regulating transforming growth factor (TGF)-beta and wingless-type MMTV integration site family (WNT) signaling (Nostro et al., 2011). In this protocol, fibroblast-derived human iPSCs (hiPSCs) and human ESCs (hESCs) were induced through 5 differentiation stages including embryoid bodies (EBs), endoderm progenitors, primitive gut tube, pancreatic endoderm, and endocrine cells. Regarding the protocol, numerous signaling and signal-controlling molecules have been used to drive the cell differentiation along pancreatic

lineage starting from EBs formation. The hiPSCs- or hESCs-derived EBs were seeded onto low-cluster plates and maintained in serum free medium (SFM) supplemented with bone morphogenic protein (BMP)-4, basic fibroblast growth factor (bFGF), activin A, and vascular endothelial growth factor (VEGF). For endoderm progenitor formation, EBs were trypsinized into single cell and plated on gelatin-coated dishes. SFM supplemented with fibroblast growth factor (FGF) 10, Wnt3A, dickkopf (DKK) 1, dorsomophin, and noggin was then used for cell induction. To commit the cell toward pancreatic lineage, step of primitive gut tube induction was performed by maintaining the cells as monolayer and cultured with Dulbecco modified Eagle's medium (DMEM) enriched with B27 supplement, activin A, KAAD-cyclopamine, all-trans retinoic acids, noggin, and FGF10. The pancreatic endoderm induction was then carried on by culturing the monolayer-cells with DMEM. B27 supplement, ascorbic acid, SB431542, and noggin were used as supplements. Finally, the endocrine cells were induced by SFM supplemented with D-glucose, SB431542, noggin, and gamma-secretase inhibitor-L685,458 (Nostro et al., 2011).

With the very complex protocol, the authors emphasized that nodal/activin A signaling played a vital role in definitive endoderm establishment while Wnt signaling enhanced posterior endoderm and pancreatic lineage development. In addition, BMP signaling was crucial for IPCs formation as optimal manipulation upon specific stage could enhance a 250-fold expression of insulin and 25% of C-peptide-positive cell population (Nostro et al., 2011).

Basford et al. also employed the "Nostro protocol" to enhance IPCs differentiation of green fluorescent protein (GFP)-reporter hESCs. The INS-targeted GFP homologous recombinant (INSGFP/w) provided a tool for insulin-positive cell identification and purification *in vitro*. The authors found that the GFP-reporter hESCs-derived IPCs were mutihormonal cells containing insulin and glucagon, and the GFP-positive cells contained similar expression patterns of genes regarding endocrine-specific transcription factors, glucose sensing, and microgranule exocytosis. The electrophysiology of cell revealed a proper function of glucagon secretion, but the glucose-stimulated insulin release was incompletely developed due to a reduced glucose-stimulated cytosolic Ca2+ influx (Basford et al., 2012). This report indicated that, to generate mature IPCs, cell function in term of electrophysical property was still of concern.

As we know that researches regarding stem cell biology and therapy are very competitive and many research groups have been launching publications regarding establishment of new IPCs formation protocol. Group of Professor Timothy Kieffer are actively working on IPCs generation and has published series of IPCs induction protocol

in various journals. In 2012, the team published 4-stage IPCs induction protocol for hESCs which included induction steps of definitive endoderm, primitive gut tube, posterior foregut, and pancreatic endoderm/endocrine precursors, respectively (Rezania et al., 2012). The obtained PDX1-positive pancreatic progenitors were then transplanted into immunodeficient diabetic mice receiving exogenous insulin for maintaining normoglycemic conditions. As the graft-derived pancreatic endocrine cells get matured, insulin and C-peptide secreted upon meal and glucose challenges were detected, and the exogenous insulin administration was eventually diminished. Interestingly, the pattern of hESCs-derived pancreatic endocrine maturation resembled the fetal pancreatic development path (Rezania et al., 2012). This work supported an idea regarding cell-based therapy for diabetes by transplanting of induced pancreatic progenitor cells.

In 2013, the team also published a report employing previous 4-stage IPCs induction protocol with a strategy for enhancing an *in vivo* maturation of grafted hESCs-derived pancreatic precursors (Rezania et al., 2013). By fractionation of NKX6.1-positive pancreatic progenitor cells before transplantation, two groups of pancreatic precursors, NKX6.1-high and NKX6.1-low, were obtained and transplanted into immunodeficient diabetic mice and found that mice grafted with NKX6.1-high cells were faster recovered from hyperglycemia and showed better responsive insulin secretion comparing with another. In addition, NKX6.1-high grafts exhibited MAFA nuclear expression along with higher proportion of insulin- and somatostatin-positive cells while NKX6.1-low grafts particularly contained glucagon-positive cells (Rezania et al., 2013). The study demonstrated strategy for enriching an *in vivo* induced pancreatic precursors and enhancing technique for *in vivo* pancreatic maturation of transplanted pancreatic endocrine precursors.

Recently, Professor Kieffer and colleagues have launched a publication in Nature Biotechnology reporting a 7-stage protocol for IPCs generation *in vitro* which comprised induction steps for definitive endoderm, primitive gut tube, posterior foregut, pancreatic endoderm, pancreatic endocrine precursors, immature beta cells, and maturing beta cells, respectively (Rezania et al., 2014). For this protocol, 2 culture techniques have been utilized starting by seeding hESCs or hiPSCs onto Matrigel-coated plates during the first 4 steps (step 1-4) then cell colonies were trypsinized into single cell and reseeded as micro-drops on filter inserts at an air-liquid interface for the last 3 steps (step 5-7). Interestingly, cells derived from the protocol remarkably expressed stage-specific pancreatic markers including pancreatic endoderm (stage 4; PDX1 and NKX6.1), pancreatic endocrine precursors (stage 5; PDX1, NKX6.1 and NEUROD1), immature beta cells (stage 6; NKX6.1 and INSULIN), and matured beta cells (stage 7;

NKX6.1, INSULIN, and MAFA). The authors proposed that the efficiency of protocol was approximately 50% since every two hESCs seeded could yield one NKX6.1/INSULIN-positive cell at stage 7. The obtained beta cell exhibited an *in vitro* glucose-stimulated insulin secretion as well as a normalization of glycemia in diabetic mice. Furthermore, the authors concluded that transplantation of induced beta cells or IPCs could be a promising tool for diabetes treatment alternative to progenitor cells or cadaveric islets (Rezania et al., 2014).

Regarding the details described above, it could be suggested that microenvironment manipulation approach was able to direct the differentiation of pluripotent/multipotent cells toward pancreatic lineage, and functional beta cells or IPCs could be obtained by *in vitro* or *in vivo* method. However, the protocols were very complex and time-consumed. From these reasons, IPCs generation by using genetic manipulation approach was introduced and has been investigated widely.

IPCs induction by genetic manipulation approach

IPCs formation using genetic manipulation approach has been studied and reported by various research teams. Different types of cells and protocols have been investigated in order to finding and optimizing therapeutic approach for diabetes. Kajiyama et al. published a report suggesting transplantation of pancreatic and duodenal homebox 1 (Pdx1)-transfected mouse adipose tissue-derived stem cells (ADSCs) could enhance IPCs differentiation *in vivo* and reduce blood glucose in streptozotocin-induced diabetic mice (Kajiyama et al., 2010). Besides, lentiviral vector-mediated transfection of PDX1 into human BM-MSCs (hBM-MSCs) resulted in IPCs formation *in vitro* as illustrated by expression of insulin mRNA and protein along with response in insulin and C-peptide secretion upon glucose stimulation *in vitro* (Allahverdi et al., 2015).

In addition to IPCs formation from stem cell resources, direct differentiation or transdifferentiation of various cell types toward pancreatic lineages and IPCs has been illustrated. In 2005, Sapir et al. published an article in Proceeding of the National Academy of Sciences (PNAS) showing that adenovirus-mediated transfection of PDX1 into human primary liver cells and co-culturing with soluble factors containing EGF and nicotinamide could enhance transdifferentiation of the cells toward IPCs *in vitro* (Sapir et al., 2005). The PDX1-transfected liver cells could produce and store insulin in form of defined granules and could release insulin in dose-dependent fashion when stimulated with different concentrations of glucose. In this work, replication-deficient recombinant adenovirus encoding rat PDX1 cDNA under cytomegalovirus promoter control was employed. Additionally, transplantation of IPCs derived from PDX1-

transfected liver cells into renal capsule of immunodeficient diabetic mice could regulate glycemic condition efficiently (Sapir et al., 2005). Moreover, Ham et al. have published a report supporting IPCs transdifferentiation potential of liver cells by genetic manipulation approach (Ham et al., 2013). Neonatal porcine liver cells were transfected with adenovirus containing three important pancreatic genes including Pdx1/VP16, Beta2/NeuroD, and MafA. The transfected cells were then grown in condition medium containing glucose in high concentration, EGF, and nicotinamide. The authors illustrated that cells were aggregated and differentiated toward pancreatic lineage assessing by expression of pancreatic markers and insulin secretion physiologically. Transplantation of differentiated IPCs into STZ-induced immunodeficient mice could reverse hyperglycemic condition, and the results illustrated that, at 6 weeks after transplantation, approximately 18% of grafted cells expressed insulin *in vivo* (Ham et al., 2013). The evidences supported IPCs transdifferentiation potential of liver cells by overexpression of pancreatic development regulating factors in vitro.

In aspect of IPCs transdifferentiation by genetic manipulation approach *in vivo*, liver cells and liver tissues have been used as model since liver-derived cells contained transdifferentiation potential toward pancreatic lineage *in vitro* as illustrated in various publications (Ham et al., 2013; Sapir et al., 2005). Moreover, pancreatic and hepatic tissues were developmentally related due to the same of original tissue, upper primitive foregut endoderm (Ham et al., 2013). Wang et al. published a work supporting this idea in 2007 by using adeno-associated virus (AAV) serotype 8 to deliver Pdx1 or Ngn3 into liver tissue of diabetic mice (Wang et al., 2007). The authors found that transfection of Pdx1 and Ngn3 with AAV vectors could not render hyperglycemia, but co-delivering of Pdx1 and Ngn3 expression cassettes in non-viral vectors could normalize glycemic condition in diabetic mice (Ham et al., 2013). In 2012, Banga et al. published a report in PNAS suggesting the success of direct reprogramming of liver toward pancreatic lineages by polycistronic-mediated overexpression of Pdx1, Ngn3, and MafA. After transfection, pancreatic genes and proteins were expressed by Sox9-positive liver cells, and glycemic condition of STZ-induced mice was normalized (Banga et al., 2012).

Besides, studies regarding direct differentiation toward IPCs from different tissue resources have been reported. Team of Professor Douglas Melton published report in Nature illustrating the transdifferentiation potential of adult pancreatic exocrine cells toward IPCs *in vivo* (Zhou et al., 2008). By delivering three key pancreatic developmental factors including Pdx1, Ngn3, and MafA to exocrine cells, the cells were undergone reprogramming toward pancreatic IPCs resembling beta-cells in size, shape,

and intracellular structures. Moreover, after reprogramming, normoglycemic condition of diabetic mice was rendered (Zhou et al., 2008). Lee et al. have also published work in eLife showing the supporting evidence for transdifferentiation potential of adult pancreatic cell toward IPCs. This work was done *in vitro* by reprogramming of human pancreatic ductal cells by delivering four pancreatic differentiation factors comprised PDX1, NGN3, MAFA, and PAX6 that could induce expressions of pancreatic markers as well as insulin synthesis, processing, storing, and secreting upon glucose and depolarizing stimuli (Lee et al., 2013). The evidences supported the potential of *in vivo* and *in vitro* direct reprogramming of adult pancreatic tissue toward IPCs.

There were other interesting models supporting efficiency of direct reprogramming/differentiation toward IPCs by genetic manipulation approach. In 2012, Professor Domenico Accili and colleagues reported evidence in Nature regarding IPCs differentiation of gut cells (Talchai et al., 2012). Generalized knockout of Foxo1 in mice resulted in autonomously occurring of gut insulin-positive cells, and the expression of gut cell-derived insulin could render normoglycemia in STZ-induced diabetic mice. The authors indicated that Foxo1 is required for Ngn3-positive enteroendocrine progenitors to prevent pancreatic beta cell differentiation (Talchai et al., 2012). Besides, in 2014, Professor Ben Stanger and colleagues published work in Cell reporting that transient expression of three pancreatic factors including Pdx1, Ngn3, and MafA in intestinal tissues resulted in conversion of intestinal crypts toward pancreatic lineage so-called "neoislets" which were able to express and secrete insulin and resembled to beta-cell ultrastructures. In addition, generation of neoislets in diabetic mice could normalize glycemic condition efficiently (Chen et al., 2014).

These findings supported a potential of genetic manipulation approach for conducting IPCs transdifferentiation/direct reprogramming of various cell and tissue resources *in vivo* and *in vitro*. By using one or set of pancreatic development regulating genes, fate of cells was efficiently changed. Reprogrammed IPCs expressed core pancreatic markers as well as illustrated their functional properties as shown in dose-dependent insulin secretion and ability to render normoglycemia in diabetic animal model.

Generation of IPCs from dental tissue-derived MSCs

In 2011, Govindasamy et al. reported evidence of *in vitro* IPCs formation by dental pulp-derived stem cell harvested from extracted primary teeth called stem cells from exfoliated deciduous teeth (SHED) (Govindasamy et al., 2011). By using 4-step induction protocol modified from previous published report (Chandra et al., 2009), SHED could be differentiated toward IPCs as shown by expression of pancreatic

markers and the formation of insulin *in vitro*. Upon stimulation of glucose, induced IPCs could secrete insulin and C-peptide in dose-dependent manner. For IPCs induction from other types of dental stem cells, in 2014, our group launched a publication in Biochemical and Biophysical research communication (BBRC) reporting a potential of IPCs generation from human dental pulp stem cells (hDPSCs) and human periodontal ligament stem cells (hPDLSCs) *in vitro* (Sawangmake et al., 2014). Employing published 4-step IPCs induction protocol with technical modification (Chandra et al., 2009; Govindasamy et al., 2011), we illustrated that DPSCs and PDLSCs were *in vitro* IPCs inducible cells supporting by islet-resembling morphology, expression of key pancreatic genes (PDX1, NGN3, NKX6.1, GLUT2, and INSULIN), expression of proinsulin protein, and secretion of C-peptide in dose-dependent fashion upon glucose stimulation. In addition, hDPSCs showed higher capacity of IPCs differentiation comparing with hPDLSCs in terms of IPCs colony number and pancreatic markers expression. Interestingly, we found that Notch signaling played roles in *in vitro* IPCs differentiation by hDPSCs as shown in upregulation of key Notch target genes, HES1 and HEY1, upon induction. However, due to limitation regarding cell multipotency and microenvironment manipulating protocol efficiency, approximate 10-15% of starting hDPSCs were differentiated and matured as functional IPCs *in vitro*. Manipulation the protocol by using Notch signaling inhibitor, DAPT, revealed an important finding that we were able to moderately enhance the protocol efficiency in regard of matured IPCs colony numbers and some of key pancreatic markers expression (Sawangmake et al., 2014). These suggested the possibility of using dental stem cells as resource for IPCs generation *in vitro*.

Merits, Significance and Implications

Since canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) contained crucial properties favoring the application for regenerative therapy comprising accessibility, availability, and plasticity. These suggested the potential of introducing cBM-MSCs for applying in stem cell-based regenerative therapy for diabetes in animal. Importantly, factors that crucial for the success of veterinary regenerative treatment of diabetes were efficiency and reliability of IPCs induction protocol to get high number of functional IPCs enough for further *in vivo* transplantation study.

For this regard, the objectives of study are aimed into two aspects which comprise Specific Aim 1) the *in vitro* generation of islet-like cell cluster (ILCC) or insulin-producing cells (IPCs) by canine bone marrow-derived mesenchymal stem cells (cBM-MSCs), employing genetic manipulation approach for delivering key pancreatic development regulating factor emphasizing on pancreatic and duodenal homebox 1

(PDX1), and Specific Aim 2) the elucidation of potential application and intracellular mechanism(s) relating Notch signaling in contribution to *in vitro* differentiation toward ILCC or IPCs by cBM-MSCs utilizing genetic manipulation approach for delivering key pancreatic development regulating factor, PDX1.

The obtained results will be employed as preliminary and integrative data suggesting the improvement and establishment of *in vitro* induction protocol to deliver ILCC or IPCs by using genetic manipulation approach especially for derivation of IPCs from cBM-MSCs and other sources of mesenchymal stem cells. In addition, the achievement of high performance IPCs formation *in vitro* will be important step forwarding to the success regarding *in vivo* transplantation study and application in veterinary clinical practice.

Objectives of Study

The feasibility, potential application, and intracellular mechanism(s) involving genetic manipulation approach to enhance islet-like cell cluster (ILCC) or insulin-producing cells (IPCs) differentiation by canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) will be elucidated.

Specific Aim 1) "*In vitro* generation of islet-like cell cluster (ILCC) or insulin-producing cells (IPCs) by canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) employing genetic manipulation approach for delivering key pancreatic development regulating factor emphasizing on pancreatic and duodenal homebox 1 (*PDX1*)." Strategies: 1a) To explore the feasibility regarding *in vitro* pancreatic lineages or IPCs differentiation by cBM-MSCs using genetic manipulation to deliver key pancreatic development regulator (*PDX1*). **1b)** To explore efficiency of *in vitro* pancreatic lineages or IPCs induction protocol employing genetic manipulation for introducing *Pdx1* gene.

Specific Aim 2) "Elucidation of potential application and intracellular mechanism(s) relating Notch signaling in contribution to *in vitro* differentiation toward ILCC or IPCs by cBM-MSCs utilizing genetic manipulation approach for delivering key pancreatic development regulating factor emphasizing on pancreatic and duodenal home box 1 (*PDX1*)." Strategies: 1a) To explore potential application of cBM-MSCs-derived ILCC or IPCs generated by genetic manipulation for introducing *Pdx1* gene. **1b)** To explore intracellular mechanism(s) relating Notch signaling contributing to *in vitro* pancreatic lineages or IPCs differentiation utilizing genetic manipulation for introducing *Pdx1* gene.

Scope of the study

Scope of the study is to explore the feasibility of an *in vitro* production of islet-like cell cluster (ILCC) or insulin-producing cells (IPCs) by canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) employing genetic manipulation approach delivering pancreatic development regulating factors focusing on *PDX1*. Regarding confirmation of optimized induction protocol, potential differentiation (cell morphology, pancreatic markers expression in mRNA and protein levels) and potential application / functional property (insulin or C-peptide secretion upon glucose stimulation *in vitro*) will be evaluated. In addition, relevance of Notch signaling upon differentiation will be examined.

Keywords (Thai):
เซลล์ต้นกำเนิดมีเซ็นไคม์จากไขกระดูกของสุนัข เซลล์สังเคราะห์อินซูลิน กลุ่มเซลล์ที่เหมือนไอเล็ท การเปลี่ยนแปลงสู่เนื้อเยื่อตับอ่อน

Keywords (English):
canine bone marrow-derived mesenchymal stem cells (cBM-MSCs), insulin-producing cells (IPCs), islet-like cell cluster (ILCC), pancreatic differentiation

Hypothesis

Islet-like cell cluster (ILCC) or insulin-producing cells (IPCs) will be able to generate *in vitro* by canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) employing genetic manipulation approach delivering pancreatic development regulating factors focusing on *PDX1*. Potential differentiation (cell morphology, pancreatic markers expression in mRNA and protein levels) and potential application / functional property (insulin or C-peptide secretion upon glucose stimulation *in vitro*) will be confirmed along with the relevance of Notch signaling upon differentiation process.

MATERIALS AND METHODS

According to the objectives of the study, the experimental approach is described in regard of proposed specific aims. All experimental protocols were submitted for an approval from the Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Science, Chulalongkorn University.

Specific Aim 1) "*In vitro* generation of islet-like cell cluster (ILCC) or insulin-producing cells (IPCs) by canine bone marrow-derived mesenchymal stem cells (cBM-MSCs), employing genetic manipulation approach for delivering key pancreatic development regulating factor emphasizing on pancreatic and duodenal homebox 1 (*PDX1*)."

Strategies:

1a) To explore the feasibility regarding *in vitro* ILCC or IPCs differentiation by cBM-MSCs using genetic manipulation to deliver key pancreatic development regulator (*PDX1*).

The aims of this part are to verify the success and screen for the optimized condition for *Pdx1* gene delivery.

cBM-MSCs were isolated by marrow aspiration technique. After identification of stem cells properties and multipotentiality, the isolated cBM-MSCs in passage 2-5 were employed for conducting ILCC or IPCs induction using genetic manipulation delivering *Pdx1* gene.

Sets of investigating procedures were used to identify the success of differentiation protocol. On day 2 and 7 after transfection, morphology of cells were observed and captured to evaluate the progress of cell differentiation. On day 2 and 7 after transfection, mRNA was collected to verify the differentiation stages by reverse transcription, real time-polymerase chain reaction (qRT-PCR) as followed; pancreatic islet; *NKX6.1, NKX2.2, PAX6, ISL-1, INSULIN* and *GLUT2*.

Potential differentiation toward ILCC or IPCs of *PDX1*-transfected cells was assessed.

1b) To explore efficiency of *in vitro* ILCC or IPCs induction protocol employing genetic manipulation for introducing *Pdx1* gene.

The aim of this part is to intensively explore the potential differentiation toward ILCC or IPCs by *PDX1*-transfected cells after verification and optimization of induction protocol.

Sets of intensive investigating protocols will be used to identify the differentiation path. On day 3, 5, and 10, ten pictures of induced cells will be captures

to evaluate size and obtained colony/aggregate number. mRNA will be harvested for verifying differentiation path by qRT-PCR as followed; definitive endoderm; *SOX17, GATA4* and *HNF3 beta*, pancreatic endoderm; *PDX1, NGN3, NEUROD* and *PAX4,* and pancreatic islet; *NKX6.1, NKX2.2, PAX6, ISL-1, INSULIN* and *GLUT2*.

On day 10, immunocytochemistry (ICC) staining of specific pancreatic protein markers i.e. proinsulin, c-peptide investigated by flow cytometer and fluorescent microscope will be performed to confirm the pancreatic islet differentiation.

Intracellular quantification of produced and stored insulin will be evaluated to confirm the differentiation path.

Specific Aim 2) "Elucidation of potential application and intracellular mechanism(s) relating Notch signaling in contribution to *in vitro* differentiation toward ILCC or IPCs by cBM-MSCs utilizing genetic manipulation approach for delivering key pancreatic development regulating factor emphasizing on pancreatic and duodenal home box 1 (*PDX1)*."

Strategies:

1a) To explore potential application of cBM-MSCs-derived ILCC or IPCs generated by genetic manipulation for introducing *Pdx1* gene.

This part is set for evaluation of maturation and functional property of induced ILCC or IPCs.

On day 10, ILCC or IPCs colonies will be harvested and processed to analyze the functional property regarding insulin or C-peptide secretion upon various concentration of glucose stimulation *in vitro*. ELISA detection of secreted molecules will be employed.

In addition, induced pancreatic lineages or IPCs will be tested for sustainability of differentiation by culturing in normal culturing medium for another ten days to see whether the expression of pancreatic markers and functional property will be changed overtime.

1b) To explore intracellular mechanism(s) relating Notch signaling contributing to *in vitro* ILCC or IPCs differentiation utilizing genetic manipulation for introducing *Pdx1* gene.

To investigate the relevance of Notch signaling on cell differentiation, 25 uM LY-374973 gamma-secretase inhibitor (DAPT) will be added into induction media throughout the protocol.

On day 3, 5, and 10, ten pictures of induced cells will be captured to evaluate size and number of obtained colonies, and mRNA will be collected to verify the

differentiation stages and Notch signaling genes i.e. Notch receptors (Notch receptor 1, 2, 3 and 4), Notch ligands (Jagged-1, Jagged-2, Delta-1, Delta-3 and Delta-4) and Notch target genes involving in organogenesis or differentiation (Hes-1 and Hey-1).

On day 10, immunocytochemistry (ICC) staining of specific pancreatic protein markers i.e. proinsulin, c-peptide investigated by flow cytometer and fluorescent microscope will be performed to confirm the pancreatic islet differentiation. Pancreatic lineages or IPCs colonies will be harvested and processed to analyze the functional property regarding insulin or C-peptide secretion upon various concentration of glucose stimulation *in vitro* by ELISA detection.

Statistical analysis

Results will be showed as mean ± standard deviation (SD) and analyzed using two-independent Student t-test for comparison of differences between two sample groups. One-way analysis of variance (ANOVA) and Dunnett test will be used to compare the differences among three or more groups. Three subjects and biological replicates (n=3) will be used in the study. Statistical significant difference will be recognized when p-value < 0.05.

Detailed materials and methods

cBM-MSCs isolation and expansion

The protocols were approved by the Institutional Animal Care and Use Committee, Faculty of Veterinary Science, Chulalongkorn University. cBM-MSCs were isolated from bone marrow aspirate according to protocol modified from previous published reports (Chung et al., 2012; Screven et al., 2014). In summary, 15-20 mL of bone marrow was collected from iliac crest using 18-guage Jamshidi® bone marrow biopsy aspiration needles (BD, USA). Bone marrow was aspirated into sterile plastic syringe containing heparin in ratio of 5,000 IU heparin/2 mL bone marrow aspirate. With aseptic technique, bone marrow aspirate was then transferred to a 50 mL sterile conical tube and processed to harvest cBM-MSCs. Cells were washed by adding equal volume of Hank's balanced salt solution (HBSS) (Invitrogen, USA), then gently mixed and centrifuged at 300 g for 15 minutes. Pellet was resuspended and washed again with 20 mL HBSS, then centrifuged at 1,000 g for 5 minutes. After that, pellet was resuspended with standard culture medium and seeded into T-75 culture flasks (Corning, USA). High glucose-Dulbecco's Modified Eagle Medium (HG-DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 1% antibiotic-antimycotic (Invitrogen), and L-glutamine (Invitrogen) were used as standard culture medium. Cells were maintained at 37 °c, humidified atmosphere with 5% CO_2

aeration. After incubation for 48 hours, the cultures were gently washed with HBSS to remove non-adherent cells. Culture medium was routinely substituted every 48 hours. At 90% confluence, the cells were trypsinized and subcultured using 0.25% trypsin-ethylenediaminetetraacetic acid (Invitrogen). The cells in passage 2-5 were used in the study.

In vitro differentiation of cBM-MSCs toward insulin-producing cells (IPCs) and exploration of Notch signaling intervention

cBM-MSCs were differentiated toward IPCs using genetic manipulation approach employing lentiviral vector for delivering *Pdx1* gene. 5 x 10^5 cBM-MSCs were seeded onto 24-well plate. When the confluence reach 80%, induction medium containing *Pdx1*-carrying lentiviral particles were added and maintained at 37 °C for 24 hours by the multiplicity of infection (MOI) of 20-150 depending on susceptibility of cells. Normal culture medium was then changed and replaced every 48 hours.

In further experiment, to investigate the relevance of Notch signaling in transdifferentiation of cBM-MSCs toward IPCs, the LY-374973 gamma-secretase inhibitor (DAPT) (Sigma) will be added in induction media at concentration of 25 μM in order to inhibit the Notch signaling cascade throughout the induction protocol.

Reverse transcription-polymerase chain reaction (RT-PCR) for IPCs (further study)

Gene markers detection will be performed in order to confirm and clarify the stages of cell differentiation. Reverse transcription, real time-polymerase chain reaction (qRT-PCR) will be employed as a detective method for mRNA transcripts. Sox-17, GATA-4 and Hnf-3 beta will be used as the definitive endoderm gene markers, while pancreatic endoderm gene markers consist of PDX-1, Ngn-3, NeuroD and Pax-4. Differentiation of the cells toward IPCs will be then checked by the expression of pancreatic islet gene markers including NKx 6.1, NKx 2.2, Pax-6, Isl-1, insulin and GLUT2.

The important signaling pathways involving in differentiation of cells toward IPCs, Notch signaling cascade, will be examined. qRT-PCR will be used to investigate the expression of genes involving in Notch signaling including Notch receptors, Notch ligands and Notch target genes.

qRT-PCR will be performed by cellular RNA extract with TRIzol® RNA isolation reagent (Invitrogen), then 1 ug of RNA samples will be converted to cDNA using reverse transcriptase enzyme kit (Promega, USA). For quantitative real-time PCR (qPCR), gene expression will be detected by FastStart-Essential DNA Green Master (Roche Diagnostics) using Applied Biosystems 7300 real-time PCR system (ThermoFisher

Scientific). The mRNA expression will be illustrated as relative mRNA expression normalized to 18S ribosomal RNA and the control.

Immunocytochemistry (ICC) staining for ILCs cluster / IPCs (further study)

Induced-pancreatic islet cells derived from cBM-MSCs will be then confirmed by immunocytochemistry (ICC) staining of specific pancreatic protein markers such as proinsulin and/or c-peptide. The results will be investigated by flow cytometer and fluorescent microscope incorporated with Carl Zeiss™ Apotome.2 apparatus (Carl Zeiss, Germany).

For ICC staining protocol, the ILCs cluster or IPCs will be fixed in cold methanol for 15 mins, then permeabilized with 0.1% Triton®-X100 (Sigma) in PBS for 1 min, and incubated with 10% donkey serum in PBS for 1 hour. Primary antibody, mouse anti-human pro-insulin, c-peptide (Millipore, USA), will be used to incubate at 1:100 dilution for 24 hours. After that, 1:500 goat anti-mouse antibody-biotin (Chemicon, USA) and 1:500 streptavidin-FITC (Sigma) will be used to incubate for 1 hour as the secondary antibody and reporter, respectively. DAPI (0.1 ug/mL) will be used for nuclei counterstaining. All of procedures will be performed at 4 °c and washed with PBS in every step.

Functional tests for IPCs (further study)

Glucose-stimulated insulin and c-peptide secretion will be performed to assess the function of induced ILCs cluster or IPCs. Various glucose anhydrous (Sigma) concentrations (5.55 mM (100 mg/dL), 10 mM (180 mg/dL) and 20 mM (360 mg/dL)) will be used. Levels of insulin and c-peptide secreted from induced ILCs cluster or IPCs will be detected by enzyme-linked immunosorbent assay (ELISA) test kits.

Table 1 Primer sequences

Gene	Accession number	Sequences	5' to 3'	Length (bp)	Tm (°C)
Zinc finger protein 42	XM_003639567.1	Forward	AGGTTCTCACAGCAAGCTCA	199	59.24
(ZFP42 or *Rex1)*		Reverse	CCAGCAAATTCTGCGCACTG		60.73
Octamer-binding transcription factor 4	XM_538830.1	Forward	AGGAGAAGCTGGAGCAAAACC	100	60.55
(*Oct4*)		Reverse	GTGATCCTCTTCTGCTTCAGGA		59.50
Pancreatic and duodenal homeobox 1	NM_001284471.2	Forward	AAGTCTACCAAGGCTCACGC	201	60.04
(*Pdx1*)		Reverse	GTGCCTCTCGGTCAAGTTCA		59.97
Neurogenin 3 (to be redesigned)	XM_546140.1	Forward	GAGCAAGCAGCGACG	177	60.43
(*Neurog3*)		Reverse	AGCGCCCAGATGTAGTTGTG		60.39
NK6 homeobox 1	XM_544960.5	Forward	CAGGAGTTATGCAGAGCCCG	111	60.53
(*Nkx6-1*)		Reverse	ACGTGGGTCTCGTGTGTTTT		60.11
ISL LIM homeobox 1	XM_848628.4	Forward	TGGCTTACAGGCAAACCCAG	171	60.54
(*Isl1*)		Reverse	GACATCGACGCCACTTCACT		60.39
MAF bZIP transcription factor A	XM_003431814.3	Forward	GCTTCAGCAAGGAGGAGGTC	136	60.39
(*MafA*)		Reverse	CTCTGGAGCTGGCACTTCTC		60.11
Solute carrier family 2 member 2	XM_545289.5	Forward	ACTCATCACAGGACGTGGAG	108	59.11
(*Slc2A2, Glut2*)		Reverse	AGCTGAGTGTAGCGGTGAAG		59.76
Insulin	NM_001130093.1	Forward	TGGTAGAGGCTCTGTACCTGG	235	60.34
(*Ins*)		Reverse	CGCCCCTAGTTGCAGTAATTC		59.06
Glucagon	NM_001003044.1	Forward	TCCAATCGCGGTGTCAGAAG	197	60.39
(*Gcg*)		Reverse	ACCCTGAGAATGACGCTTGT		59.31
Glucagon like peptide 1 receptor	XM_014118246.1	Forward	CACGGTGGGCTATACACTCTC	116	59.93
(*Glp1r*)		Reverse	AGGACGCAAACAGGTTCAGG		60.54
Glyceraldehyde 3-phosphate dehydrogenase	NM_001003142.1	Forward	CCAACTGCTTGGCTCCTCTA	100	59.38
(*GAPDH*)		Reverse	GTCTTCTGGGTGGCAGTGAT		59.67

Table 1 Primer sequences

RESULTS

cBM-MSCs isolation, characterization, and differentiation potential determination

cBM-MSCs were isolated from bone marrow aspirate, and then cultured and expanded *in vitro*. Cells adhered to culture plate and showed fibroblast-like morphology (Fig. 1A and B). mRNA expression of stemness markers were examined, and the expressions of *zinc finger protein 42 (ZFN42 or Rex1)* and octamer-binding transcription factor 4 *(Oct4)* were found (Fig. 1C). mRNA expression of surface markers referring to mesenchymal stem cells were also determined. The results illustrated the expression of *CD44, CD73, CD90*, and *CD105*, while the expression of hematopoietic stem cell surface marker, *CD45*, was absent (Fig. 1D). For stemness and surface markers mRNA analysis, mRNA expression of target genes normalized to reference gene, *GAPDH*, were used. The results suggested that cBM-MSCs could be isolated and expanded *in vitro*. The expression of mRNA markers referring cell lineage and potentiality were also found.

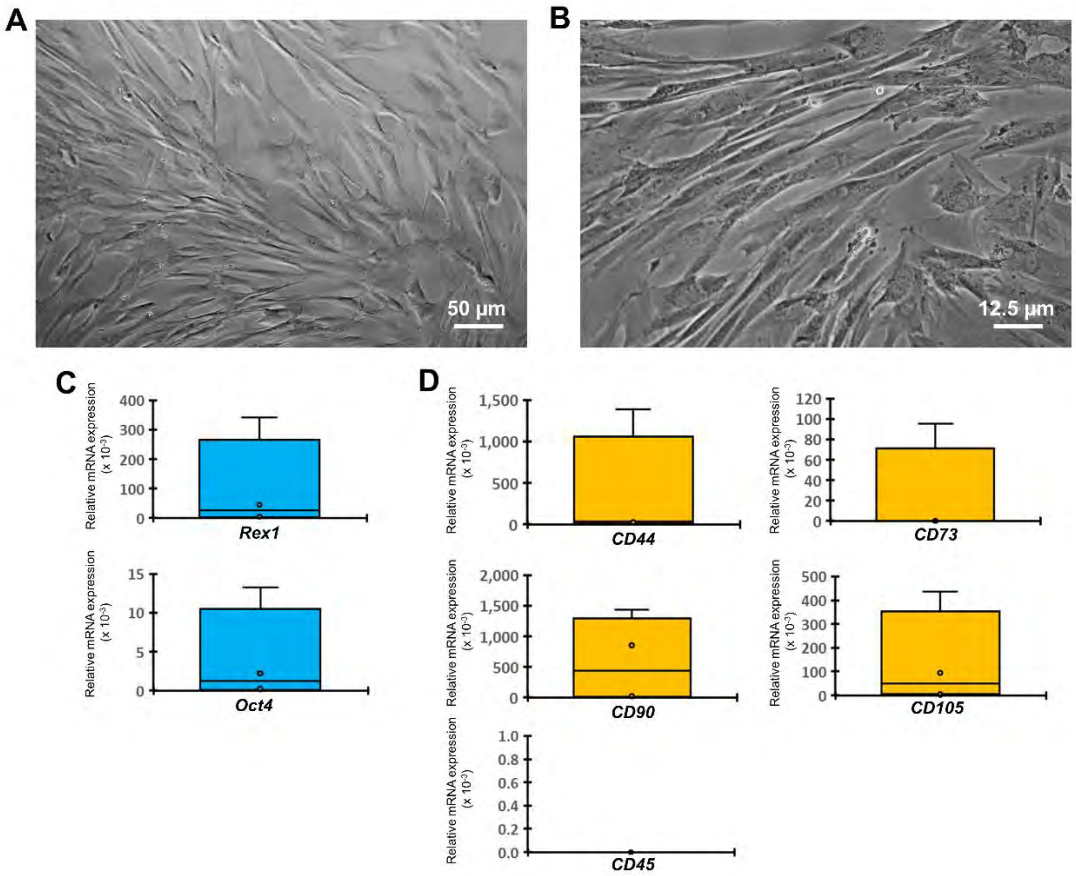

Figure 1 Characterizations of isolated canine bone marrow-derived mesenchymal stem cells (cBM-MSCs). Morphological appearances of the isolated cBM-MSCs at 100X (A) and 400X (B) magnifications were observed. The mRNA expression of stemness markers, *Rex1* and *Oct4*, was analyzed (C). The mRNA expression of surface markers referring mesenchymal stem cells (*CD44, CD73, CD90,* and *CD105*) and hematopoietic stem cells (*CD45*) were analyzed (D). The mRNA expression of the genes was normalized to reference gene, *GAPDH*.

To determine differentiation potential of the isolated cells, an *in vitro* osteogenic differentiation was employed. In this study, a 14-day osteogenic induction protocol was used. Set of protocols including alkaline phosphatase activity assay, Alizarin Red S staining, and exploration of osteogenic gene expression were used to confirm the differentiation. At day 14 post-induction, alkaline phosphatase activity of osteogenic induced cells was significantly higher than undifferentiated control group (Fig. 2B). Detection of matrix mineralization by Alizarin Red S staining showed that osteogenic group was positively stained with Crimson red color of the dye suggesting an accumulation of minerals on culture surface (Fig. 2A). qRT-PCR was employed for an analysis of a representative osteogenic gene expression. An upregulation of *Ocn* and *Collagen I A1*, markers referring mineralization stage, was found upon osteogenic differentiation (Fig. 2C). The results illustrated a differentiation potential toward osteogenic lineage of the isolated cBM-MSCs after an induction *in vitro*.

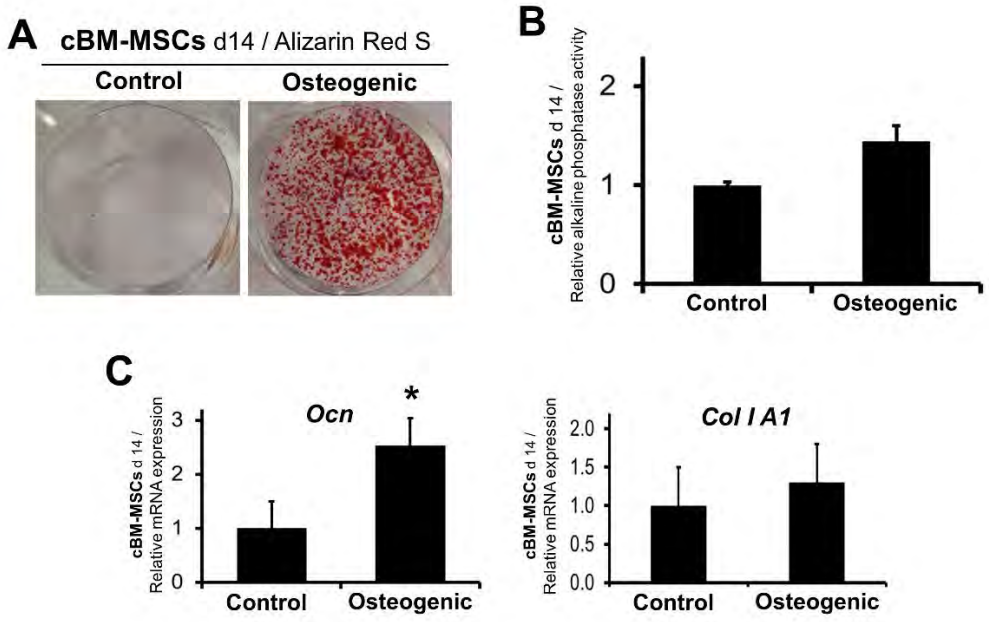

Figure 2 An *in vitro* osteogenic differentiation of canine bone marrow-derived mesenchymal stem cells (cBM-MSCs). An *in vitro* osteogenic differentiation potential of cBM-MSCs was confirmed by set of protocols. At day 14 post-induction, an alkaline phosphatase activity assay (B) along with Alizarin Red S staining for matrix mineralization (A) were performed. An mRNA expression of *osteocalcin (Ocn)* and *Collagen I A1*, markers referring mature osteoblastic differentiation, was analyzed using qRT-PCR (C). An mRNA expression of the gene was normalized to reference gene, *GAPDH*, and the undifferentiated control. The asterisks indicated the statistical significance comparing with control.

Transfection efficiency of lentiviral vector for canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) transfection

For inducing cBM-MSCs toward pancreatic lineages using genetic manipulating approach, lentiviral vector was employed due to its previous reported efficiency on mammalian primary cells. In this study second-generation lentiviral vector was used. According to lentiviral transfection protocol, the "pLenti CMV GFP Puro (658-5)" plasmid, gift from Eric Campeau (Addgene plasmid #17448), was used to determine the transfection efficiency of viral vector and susceptibility of cBM-MSCs *in vitro* (Fig. 3). This plasmid was kindly provided by Assistant Prof Dr. Theerawat Tharasanit.

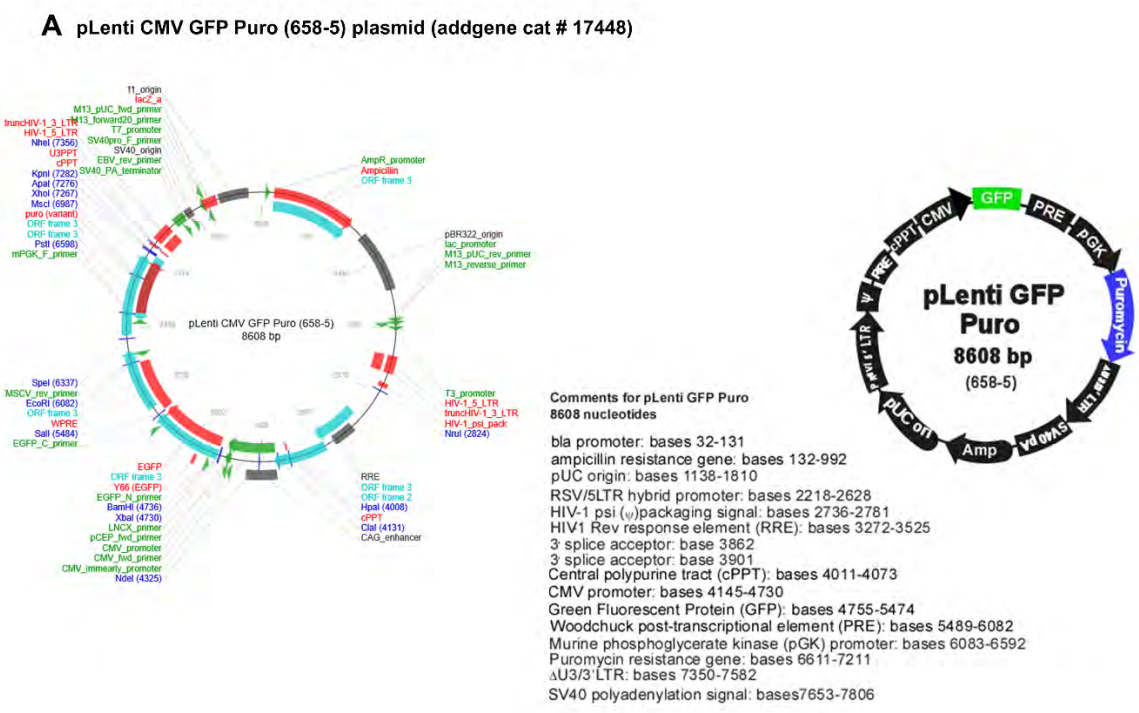

Figure 3 pLenti CMV GFP Puro (658-5) plasmid (Addgene plasmid #17448). The "pLenti CMV GFP Puro (658-5)" plasmid, gift from Eric Campeau (Addgene plasmid #17448), was used to determine the transfection efficiency of viral vector and susceptibility of cBM-MSCs *in vitro* (kindly provided by Assistant Prof Dr. Theerawat Tharasanit).

The plasmid preparation, amplification, and purification were performed according to manufacturer's protocols. HEK293FT cell line was used for an *in vitro* viral packaging. Second-generation lentiviral system was used in this regard. Plasmid-packed lentivirus was prepared by using pMD2.G and psPAX2 viral construct plasmids. In each batch of virus preparation, 3 of T-75 culture flasks with 90% confluence of HEK293FT cells were used for packaging. In each flask, 20 µg of target plasmid, 10 µg of pMD2.G, and 10 µg of psPAX2 were used. Culture medium was changed every 24 hours. Virus-containing medium was collected at 48 and 72 hours post-packaging, then pooled and filtered through 0.45 µm low protein-binding syringe filters. The viral filtrate was concentrated by ultracentrifugation. After resuspension of the viral aggregate, the stock viral solution was ready for use.

The transfection efficiency and cell susceptibility were tested by 6 different concentrations of "pLenti CMV GFP Puro (658-5)"-containing viral solution, ranging from 10^{0} to 10^{-5} folds. The transfection course was a 24-hour period, then transfection medium was substituted with growth medium. Fluorescent microscopy and flow cytometry were used for analyzing the transfection efficiency at 48 hours post-transfection course.

The results showed that cBM-MSCs were susceptible to lentiviral transfection as shown in green fluorescent color according to GFP expression (Fig. 4). The transfection efficiency was varied in dose-dependent manner as analyzed by flow cytometry (Fig. 5). Calculated transduction unit (TU) of viral solution in this production batch was 23.7×10^{3} TU/µL. This Lentiviral titer was then used for further calculation of desired multiplicity of infection (MOI).

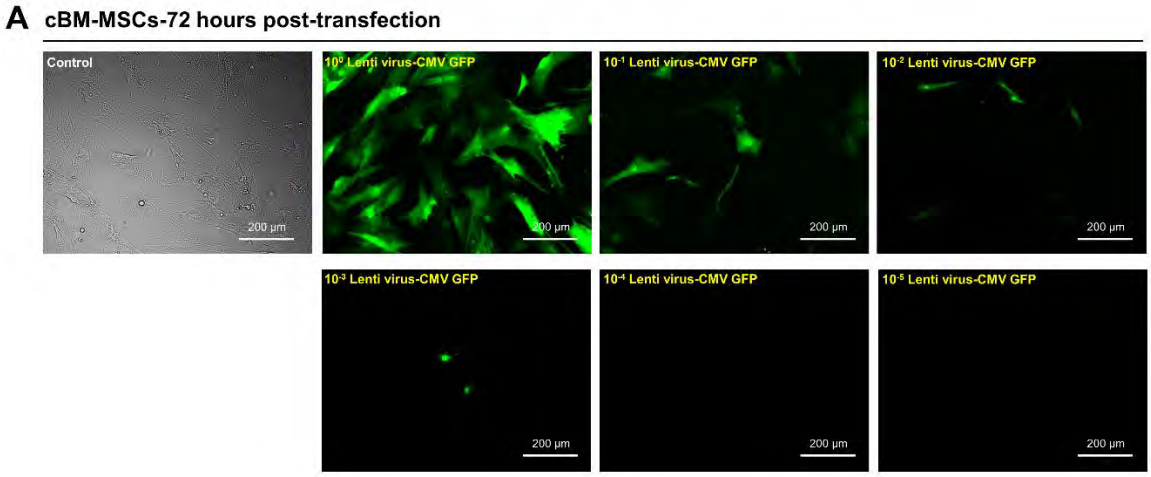

Figure 4 Transfection efficiency of second-generation lentiviral vector in canine bone marrow-derived mesenchymal stem cells (cBM-MSCs). Transfection efficiency of second-generation lentiviral vector containing "pLenti CMV GFP Puro (658-5) plasmid (Addgene #17448)" in cBM-MSCs was evaluated by fluorescent inverted microscope at 72 hours post-transfection. (scale bar, 200 µm)

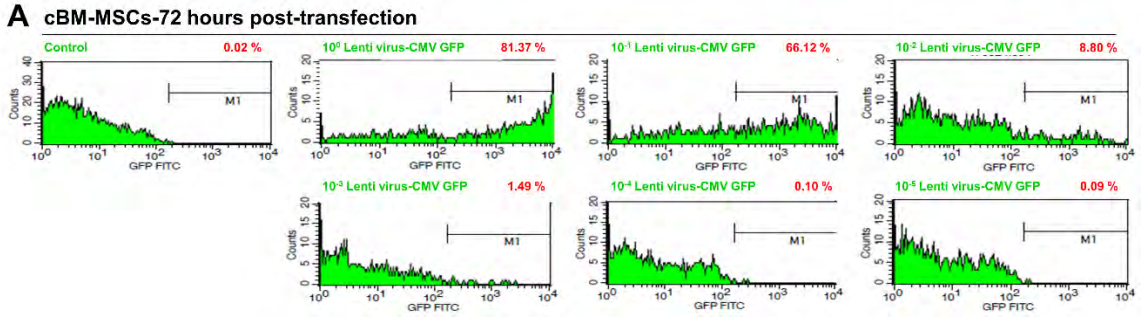

Figure 5 Transfection efficiency of second-generation lentiviral vector in canine bone marrow-derived mesenchymal stem cells (cBM-MSCs). Transfection efficiency of second-generation lentiviral vector containing "pLenti CMV GFP Puro (658-5) plasmid (Addgene #17448)" in cBM-MSCs was analyzed by flow cytometry at 72 hours post-transfection.

Efficiency testing of *PDX1* transfection on target cells focusing on cell fate after transfection

For PDX1 transfection, pWPT-PDX1 plasmid which was a gift from Didier Trono (Addgene plasmid # 12256) was used (Figure 6). This plasmid was kindly provided by Assistant Prof Dr. Theerawat Tharasanit. The protocols for plasmid preparation, amplification, purification, and viral packaging were the same as described above.

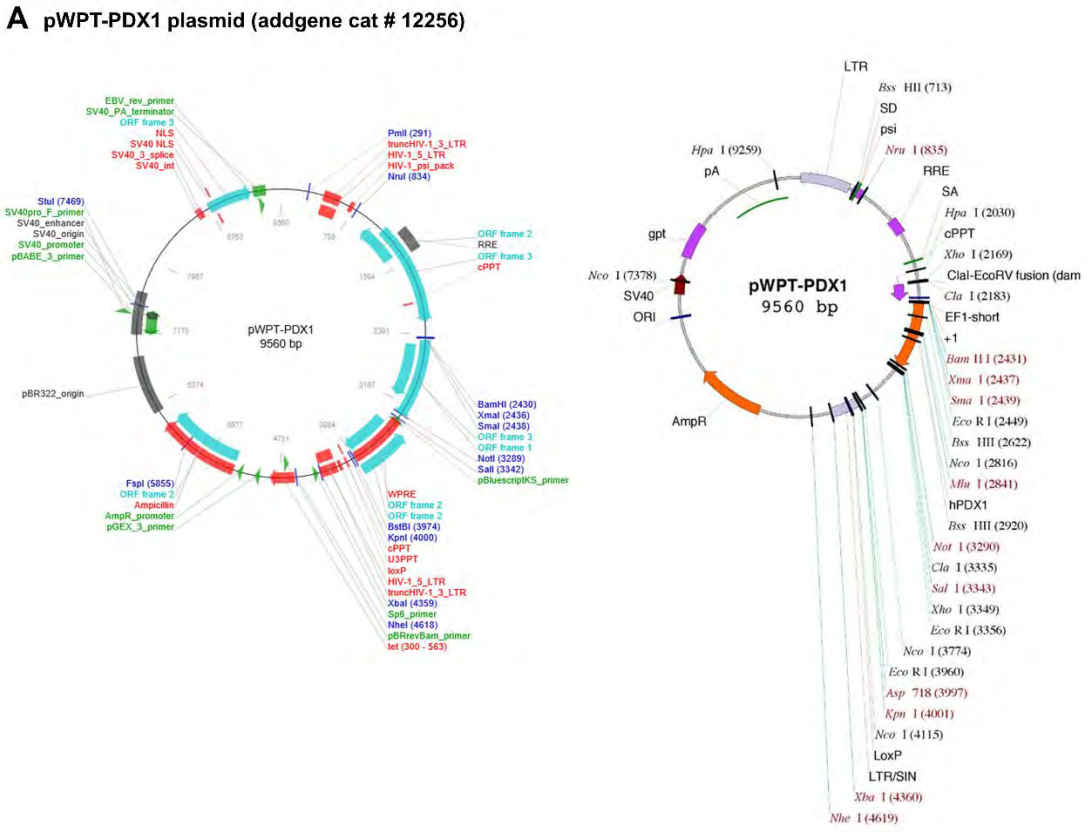

Figure 6 pWPT-PDX1 plasmid (Addgene plasmid #12256**).** The "pWPT-PDX1 plasmid", a gift from Didier Trono (Addgene plasmid # 12256), was used to determine the efficiency of *PDX1* transfection on differentiation path of cBM-MSCs *in vitro*.

The 20 MOI was used for the experiment. According to the testing timeline, a 24-hour transfection course was used, and the transfected cells were then maintained in serum free medium (SFM) for another 168 hours (7 days) for observing differentiation path without any influences from growth factors or molecules contained in fetal bovine serum (FBS) (Figure 7). At 48 and 168 hours post-transfection, phenotypic phase of the cells was evaluated by cell and colony morphology. The results showed that transfection of cBM-MSCs with lentiviral vector carrying pWPT-PDX1 plasmid could trigger cell morphological change since 48-hour post-transfection. The colony-like changing was observed at 168-hour post-transfection in MOI 20 transfected group comparing with SFM-control and DMEM/F12-control groups (Figure 8). However, only some population of the cells underwent morphological changing. This might suggest an insufficiency of viral transduction unit used. Moreover, according to viral exposure, dead cells were observed and quite correlated with the viral dosage. It seemed that the number of living cells in MOI 20 transfection group was enough for colony forming during the period of SFM maintenance.

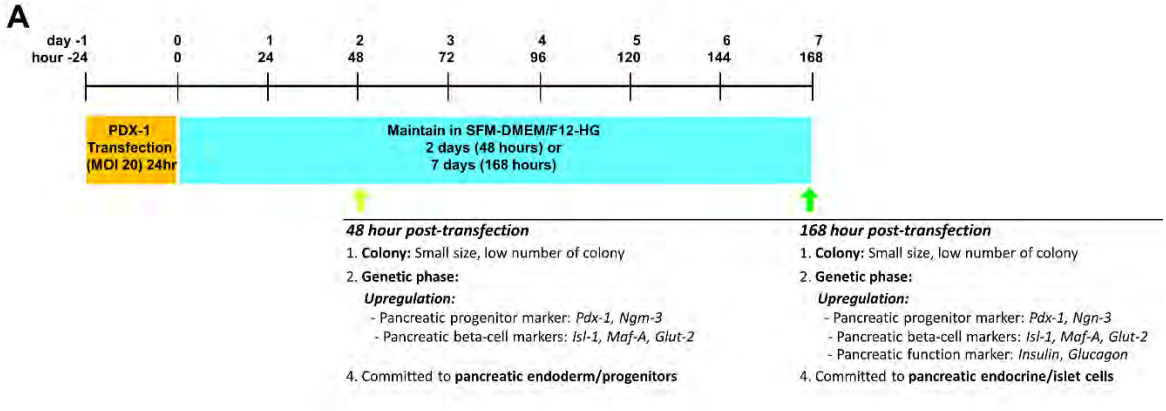

Figure 7 Experimental timeline for efficiency testing of *PDX1* transfection in canine bone marrow-derived mesenchymal stem cells (cBM-MSCs). The experiment timeline and harvesting plan for efficiency testing of *PDX1* transfection in cBM-MSCs were summarized.

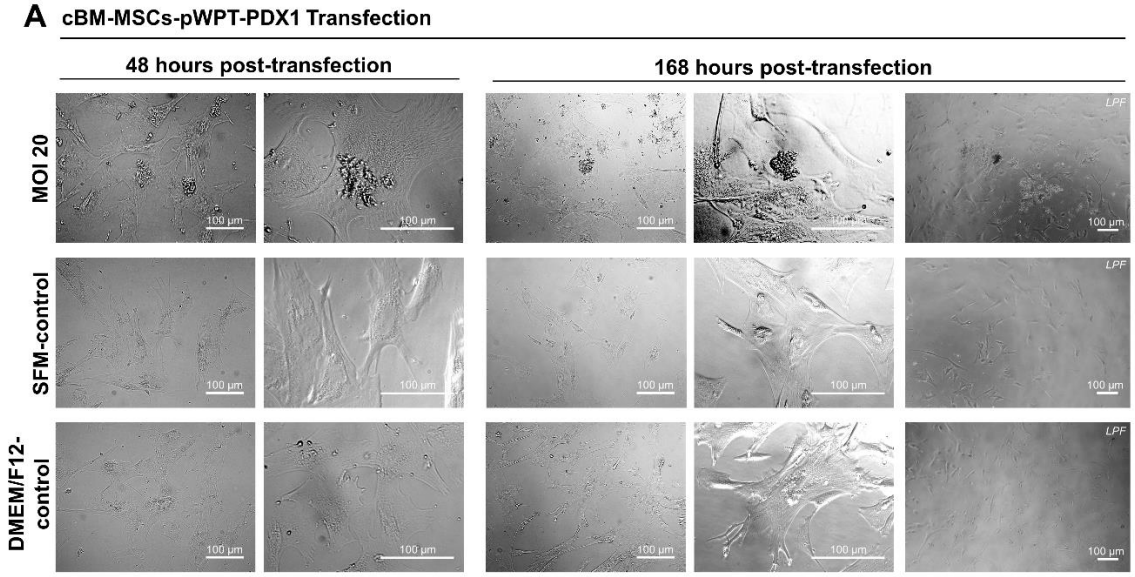

Figure 8 Efficiency testing of *PDX1* transfection in canine bone marrow-derived mesenchymal stem cells (cBM-MSCs). Morphological appearance of cBM-MSCs undergone *PDX1* transfection at 48 and 168 hours post-transfection were observed. Lentiviral vector containing pWPT-PDX1 plasmid at MOI 20 was used. (scale bar, 100 µm)

Further gene expression analysis was performed using qRT-PCR. Set of pancreatic mRNA markers were analyzed. The results illustrated the upregulating trend

of pancreatic progenitor markers (*cPdx-1* and *cNgn-3*) (Figure 9A), pancreatic islet markers (*cIsl-1, cMaf-A,* and *cGlut-2*) (Figure 9B), and pancreatic-related marker (*cGlucagon*) (Figure 9C). Most of the genes were upregulated in time-dependent manner. However, the upregulation of *cInsulin* was not observed upon the induction. These suggested that transfection with lentiviral vector carrying pWPT-PDX1 plasmid at MOI20 could enhance set of pancreatic gene marker expression in time-dependent manner, but not for *cInsulin* gene.

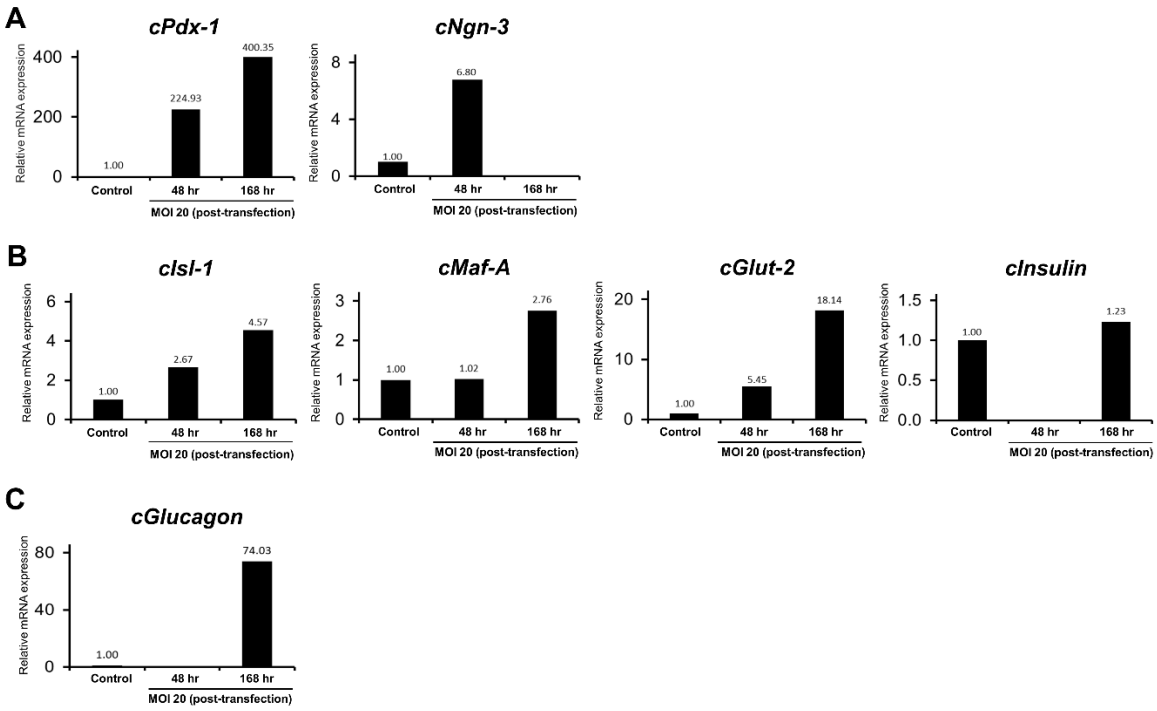

Figure 9 Efficiency testing of *PDX1* transfection in canine bone marrow-derived mesenchymal stem cells (cBM-MSCs). Efficiency of *PDX1* overexpression on pancreatic gene marker expression was determined using qRT-PCR. Sets of gene included pancreatic progenitor markers (*cPdx-1* and *cNgn-3*) (A), pancreatic islet markers (*cIsl-1, cMaf-A, cGlut-2,* and *cInsulin*) (B), and pancreatic-related marker (*cGlucagon*) (C). An mRNA expression of the gene was normalized to reference gene, *GAPDH*, and the undifferentiated control.

Efficiency of *PDX1* overexpression on pancreatic differentiation by cBM-MSCs.

According to the preliminary study, overexpression of *PDX1* by using lentiviral vector containing pWPT-PDX1 plasmid at MOI 20 could trigger morphological change and upregulation of pancreatic markers by cBM-MSCs. However, changing of cell morphology was not obvious. To enhance the pancreatic differentiation potential,

overexpression of *PDX1* by using lentiviral vector containing pWPT-PDX1 plasmid at MOI 30 and 50 was performed. The transfection period was 24 hours, then cells were maintained in SFM-DMEM/F12-HG medium for another 7-day period (Figure 10A and B).

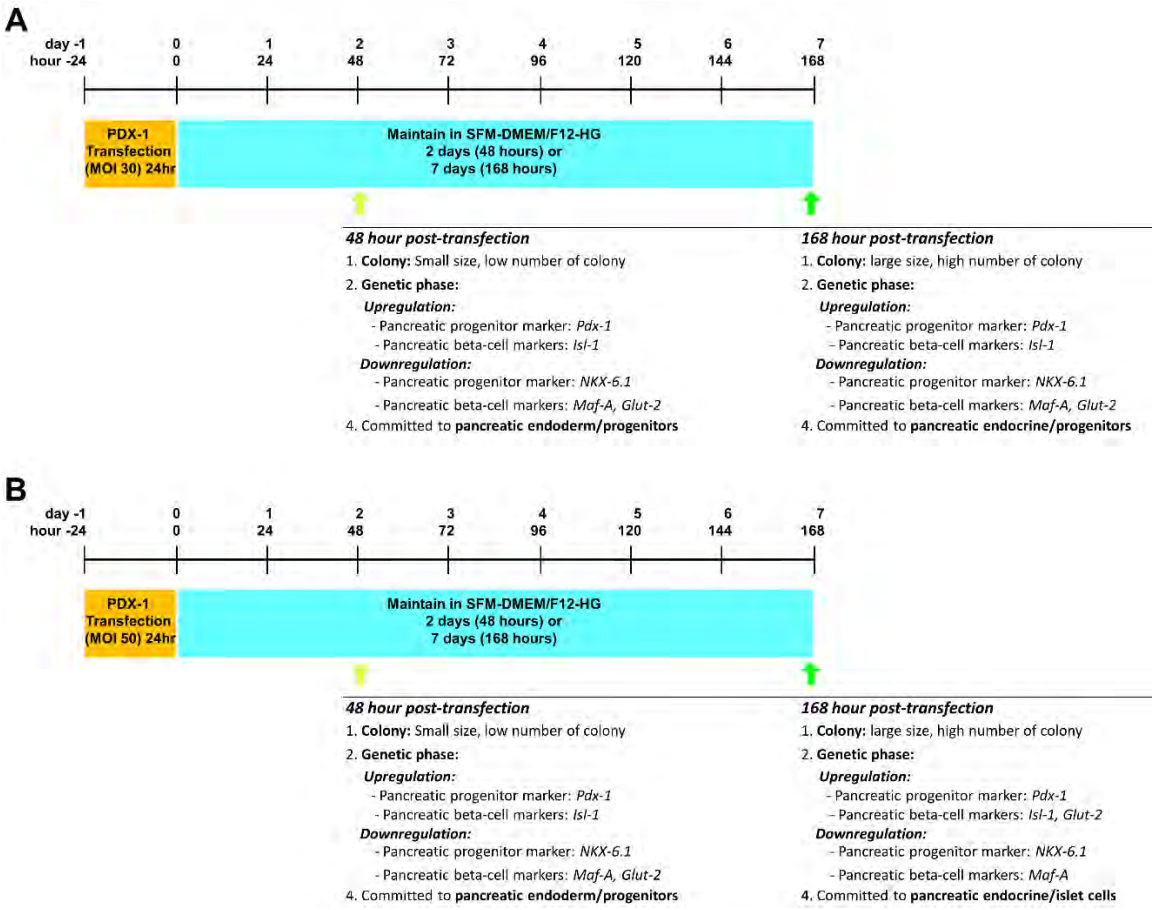

Figure 10 Experimental timeline for efficiency testing of *PDX1* transfection in canine bone marrow-derived mesenchymal stem cells (cBM-MSCs). The experiment timeline and harvesting plan for efficiency testing of *PDX1* transfection in cBM-MSCs were summarized.

The results illustrated that *PDX1* overexpression at MOI 30 and 50 could enhance cell morphology and cell aggregation since 48-hour post-transfection (Figure 11). Cell aggregation was clearly observed in both MOI 30 and 50 transfection groups. However, at 168 hours post-transfection, MOI 50 transfection group showed dense colony-like structure as well as more colony number compared to MOI 30 transfection and SFM-control groups (Figure 11). Some population of cells in both transfection groups showed fibroblast-like structure.

To investigate the differentiation path, pancreatic marker expression was analyzed using qRT-PCR at 168-hour post-transfection. The results illustrated the trend of *cPdx-1* upregulation in dose- and time-dependent patterns (Figure 12A). There was a trend of *cIsl-1* upregulation upon an induction. However, other pancreatic progenitor marker (*cNkx-6.1*) (Figure 12A) and pancreatic islet markers (*cMaf-A* and *cGlut-2*) (Figure 12B) were not upregulated. Expressions of *cInsulin* and *cGlucagon* were not detected.

The results suggested that overexpression of *PDX1* by using lentiviral vector containing pWPT-PDX1 plasmid at MOI 30 and 50 was sufficient to trigger cell morphological change and upregulation of some key pancreatic gene markers. However, only *PDX1* overexpression alone could not efficiently trigger pancreatic islet or beta-cell maturation by cBM-MSCs (Figure 10A and B).

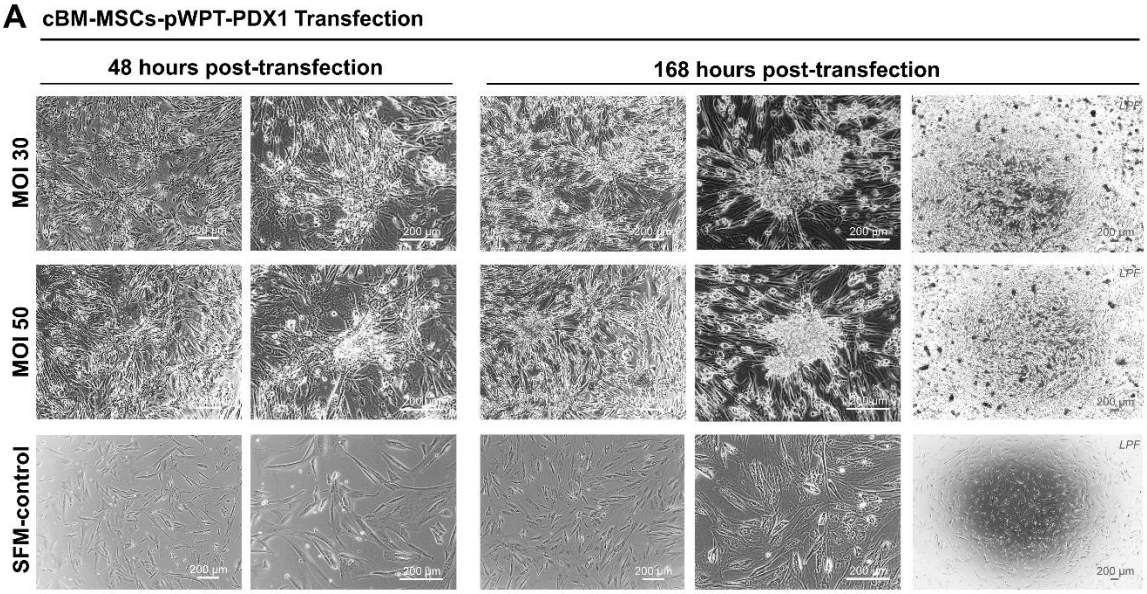

Figure 11 Efficiency testing of *PDX1* transfection in canine bone marrow-derived mesenchymal stem cells (cBM-MSCs). Morphological appearance of cBM-MSCs undergone *PDX1* transfection at 48 and 168 hours post-transfection were observed. Lentiviral vector containing pWPT-PDX1 plasmid at MOI 30 and 50 was used. (scale bar, 200 µm)

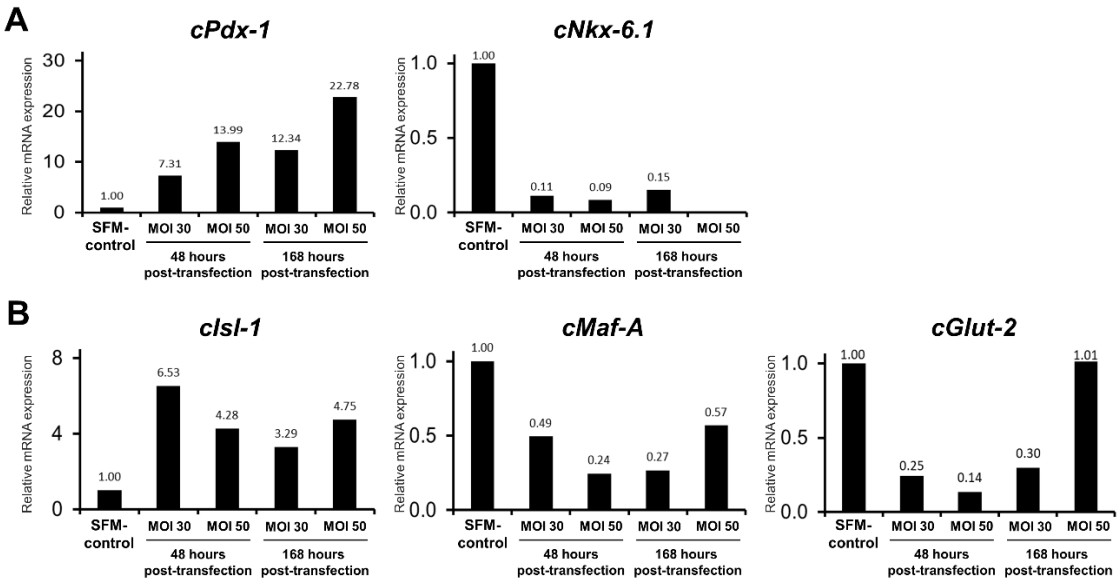

Figure 12 Efficiency testing of *PDX1* transfection in canine bone marrow-derived mesenchymal stem cells (cBM-MSCs). Efficiency of *PDX1* overexpression on pancreatic gene marker expression was determined using qRT-PCR. Sets of gene included pancreatic progenitor markers (*cPdx-1* and *cNkx-6.1*) (A) and pancreatic islet markers (*cIsl-1, cMaf-A,* and *cGlut-2*) (B). An mRNA expression of the gene was normalized to reference gene, *GAPDH*, and the undifferentiated control.

Induction of pancreatic differentiation by cBM-MSCs using an integration of genetic and micro-environmental manipulating protocol *in vitro*.

According to our preliminary results, an integration of pancreatic induction protocol using genetic and micro-environmental manipulating approach could enhance pancreatic differentiation potential by human dental pulp stem cells (hDPSCs) *in vitro* (data not shown). This part of the study was conducted to determine an *in vitro* pancreatic differentiation potential by cBM-MSCs employing an integration of genetic and micro-environmental manipulating approach. Pancreatic differentiation potential by cBM-MSCs using three different induction protocols was determined and compared – 1) micro-environmental manipulation, 2) genetic manipulation, and integrating protocol.

The initial part of this study was the establishment of "micro-environmental manipulation" to produce insulin-producing cells or pancreatic lineages from cBM-MSCs. This protocol was used in an integration between genetic and micro-environmental manipulating protocols as well.

The protocol comprised three steps of differentiation—1) definitive endoderm induction, 2) pancreatic endoderm/progenitor induction, and 3) pancreatic endocrine/islets induction (Figure 13). Upon the induction, cBM-MSCs were unable to

form cell aggregates or colony-like structure. At day 3, 5, 7, and 10 post-induction, most of the cells maintained their fibroblast-like structure and attached to culture plate. Some cells were appeared as floating cells but looked not healthy (Figure 14). This suggested that cBM-MSCs could not efficiently enhanced toward pancreatic lineages by using previous published three-step micro-environmental manipulating protocol.

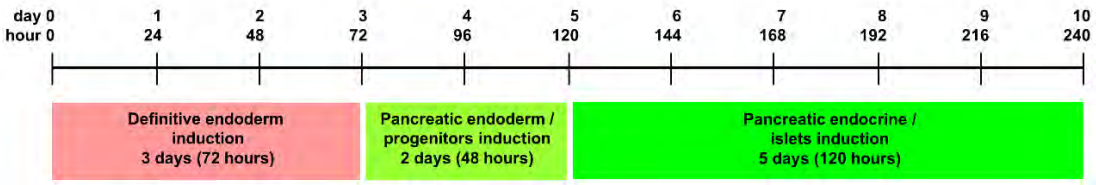

Figure 13 Experimental timeline for efficiency testing of pancreatic induction approaches in canine bone marrow-derived mesenchymal stem cells (cBM-MSCs). The experiment timeline and harvesting plan for efficiency testing of pancreatic induction approaches in cBM-MSCs were summarized.

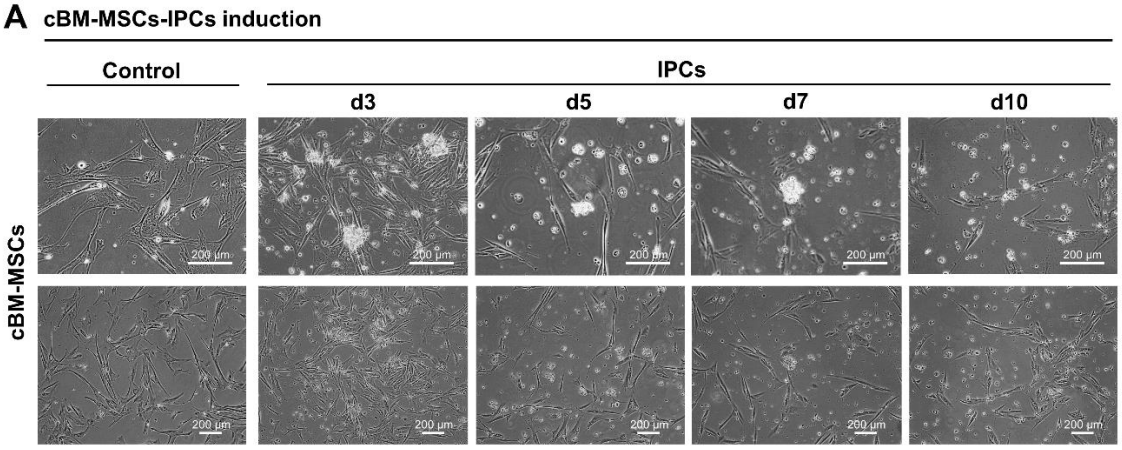

Figure 14 Pancreatic induction of canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) using previous published three-step micro-environmental manipulating protocol *in vitro*. Morphological appearances of cBM-MSCs undergone pancreatic induction by using previous published three-step micro-environmental manipulating protocol were observed. (scale bar, 200 µm)

Establishment of the modified micro-environmental manipulating protocols for pancreatic lineages derivation from cBM-MSCs.

To address the problems regarding the low efficiency of pancreatic lineage differentiation using previous published three-step induction protocol. Modification of three-dimensional (3-D) micro-environmental manipulating assay was performed by using two additional strategies—1) hanging-drop culture technique, and 2) hanging-drop culture technique with Matrigel-formed dome culture technique. Protocols were summarized in Figure 15A and B.

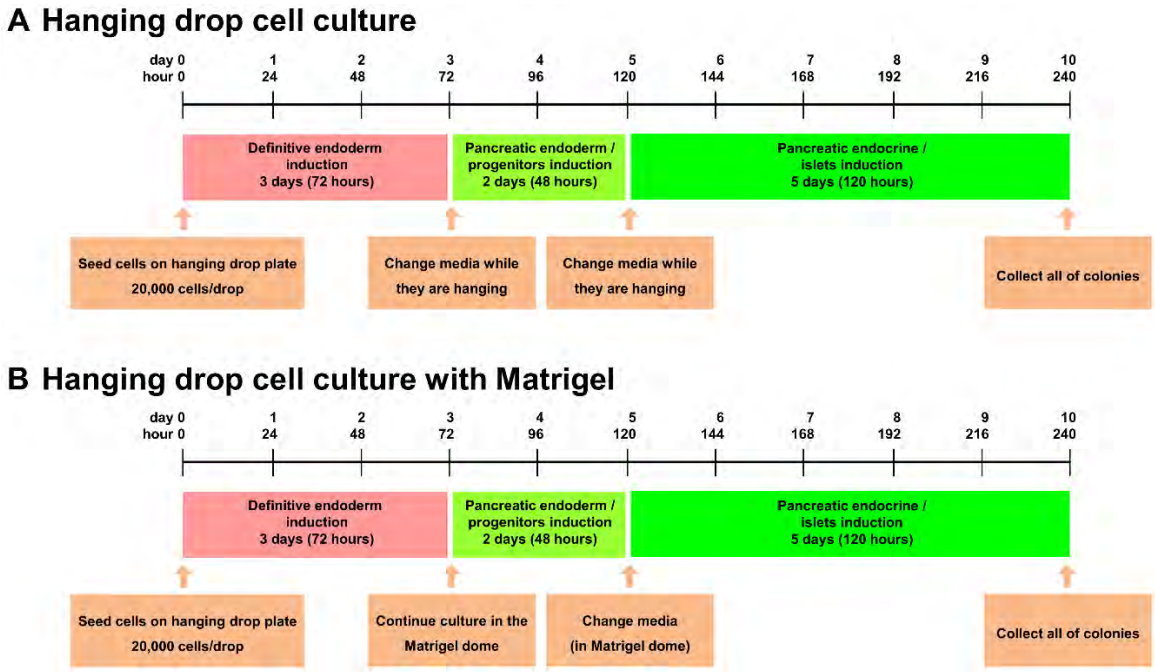

Figure 15 Establishment of modified three-dimension (3-D) micro-environmental manipulating protocols for derivation of pancreatic lineages from canine bone marrow-derived mesenchymal stem cells (cBM-MSCs). The experiment timeline and harvesting plan for efficiency testing of pancreatic induction approaches in cBM-MSCs were summarized. Two strategies were set—1) "hanging-drop culture technique" (A), and 2) "hanging-drop culture technique with Matrigel-formed dome culture technique" (B).

Briefly, the same condition media were used in two strategies. Different cell manipulation techniques were employed. For “hanging-drop culture technique” (Figure 16), 2 x 10^4 cBM-MSCs were seeded into each 40-50 µL hanging drop using hanging-drop cell culture plate (PerkinElmer, USA). Cells were always maintained in hanging-drop plate. Three conditioned media were substituted according to the induction plan. At day 10 post-induction, colonies were harvested and analyzed.

The results illustrated that upon the hanging-drop culture technique, cells stated gathering and form aggregates since 24-hour post-induction (data not shown). At day 3 post-induction, colonies and cell clumps were observed. Number and size of colonies were gradually increased along the induction period (Figure 17). Size of colony was approximately 50-200 µm, at day 10 post-induction.

For representative pancreatic mRNA marker expression, there were dramatic upregulation of *cIsl-1, cMaf-A,* and *cGlut-2,* at day 10 post-induction (Figure 18). The results suggested that cBM-MSCs could effectively induced toward pancreatic lineages by using “hanging-drop culture technique”.

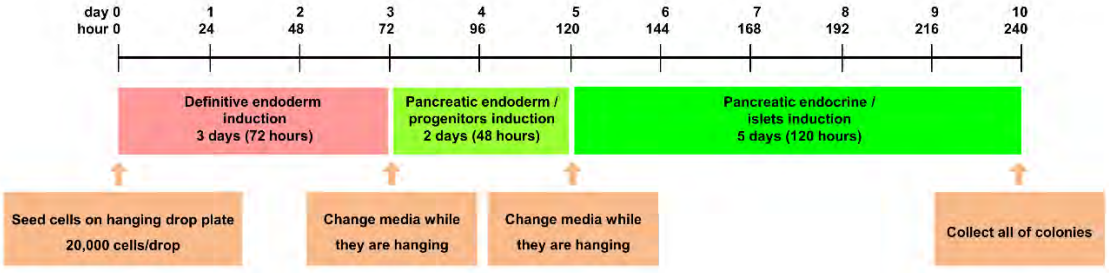

Figure 16 Establishment of modified three-dimension (3-D) micro-environmental manipulating protocols for derivation of pancreatic lineages from canine bone marrow-derived mesenchymal stem cells (cBM-MSCs). The experiment timeline and harvesting plan for efficiency testing of pancreatic induction approaches in cBM-MSCs were summarized. This figure represented “hanging-drop culture technique”.

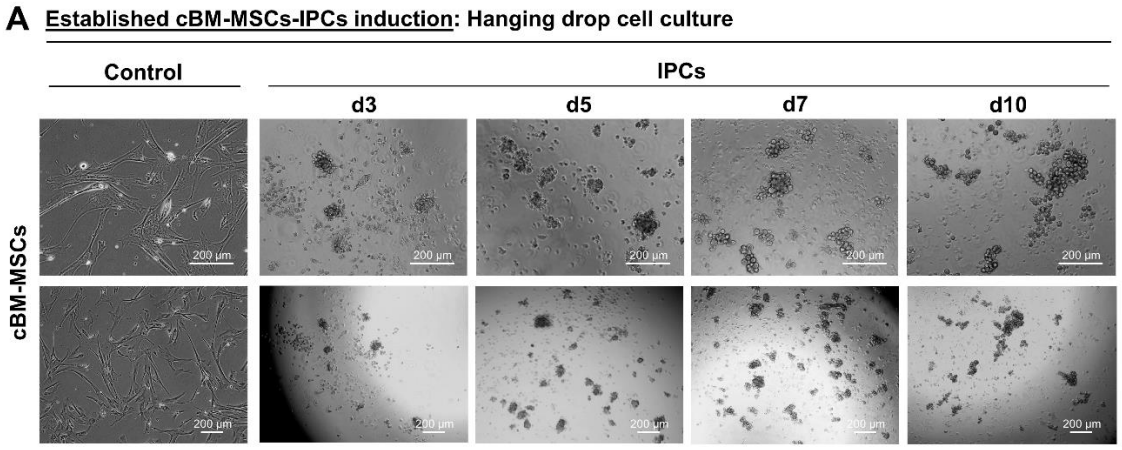

Figure 17 Pancreatic induction of canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) using modified three-dimension (3-D) micro-environmental manipulating protocol *in vitro*. Morphological appearances of cBM-MSCs undergone pancreatic induction by using modified 3-D micro-environmental manipulating protocol were observed. This figure represented "hanging-drop culture technique". (scale bar, 200 µm)

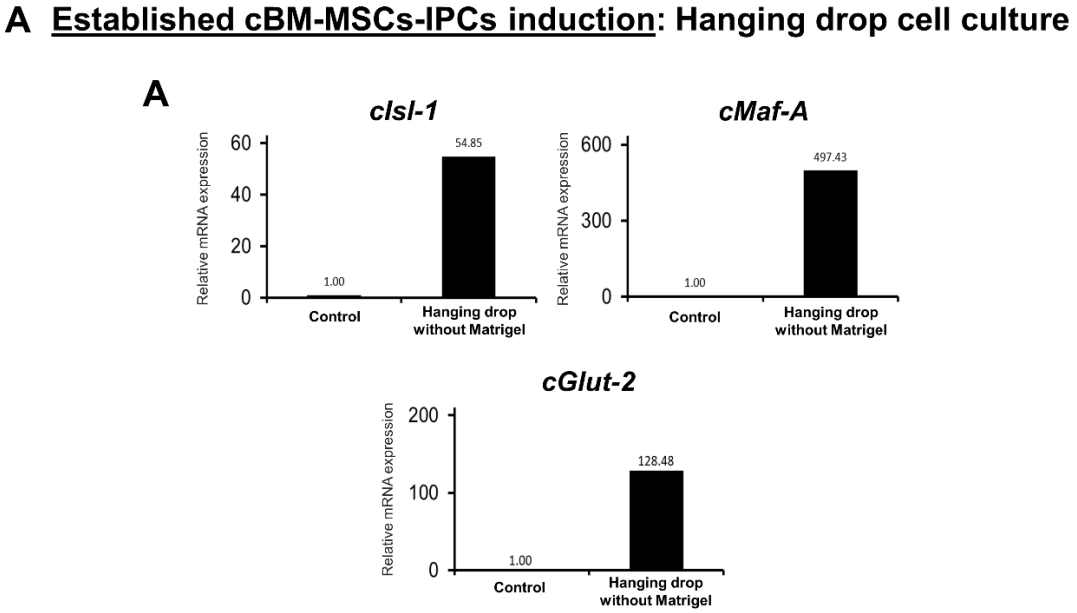

Figure 18 Pancreatic induction of canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) using modified three-dimension (3-D) micro-environmental manipulating protocol *in vitro*. This figure represented "hanging-drop culture technique". Pancreatic gene marker expression was determined using qRT-PCR. An mRNA expression of the gene was normalized to reference gene, *GAPDH*, and the undifferentiated control.

For “hanging-drop culture technique with Matrigel-formed dome culture technique” (Figure 19), cells were seeded onto hanging-drop plate according to the protocol described above. At day 3 post-induction, colonies were collected and continue cultured in Matrigel-formed dome. Conditioned media were substituted according to the induction plan. Colonies were maintained within dome until day 10 of the protocol, then cells were harvested using Cell Recovery Solution (Corning, USA) and analyzed.

The results showed that cells were able to form aggregates since 24-hour post-induction (data not shown). At day 3 post-induction, colonies and cell aggregates were clearly observed. After colonies harvest and dome formation, colonies structures were effectively maintained within Matrigel-formed dome (Figure 20). Number of colony was not clearly increased, but some colony showed dramatic size at day 10 post-induction.

There was also dramatic upregulation of representative pancreatic mRNA markers, *cMaf-A* and *cGlut-2* (Figure 21). The results suggested that “hanging-drop culture technique with Matrigel-formed dome culture technique” was able to drive pancreatic differentiation by cBM-MSCs effectively. However, some colonies were lost during colony harvest and dome formation.

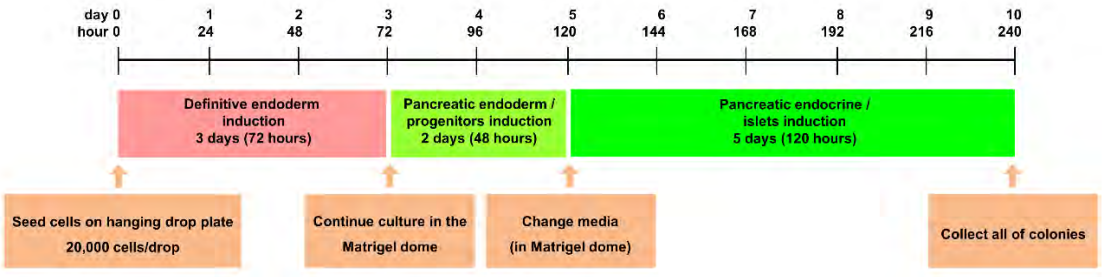

Figure 19 Establishment of modified three-dimension (3-D) micro-environmental manipulating protocols for derivation of pancreatic lineages from canine bone marrow-derived mesenchymal stem cells (cBM-MSCs). The experiment timeline and harvesting plan for efficiency testing of pancreatic induction approaches in cBM-MSCs were summarized. This figure represented “hanging-drop culture technique with Matrigel-formed dome culture technique”.

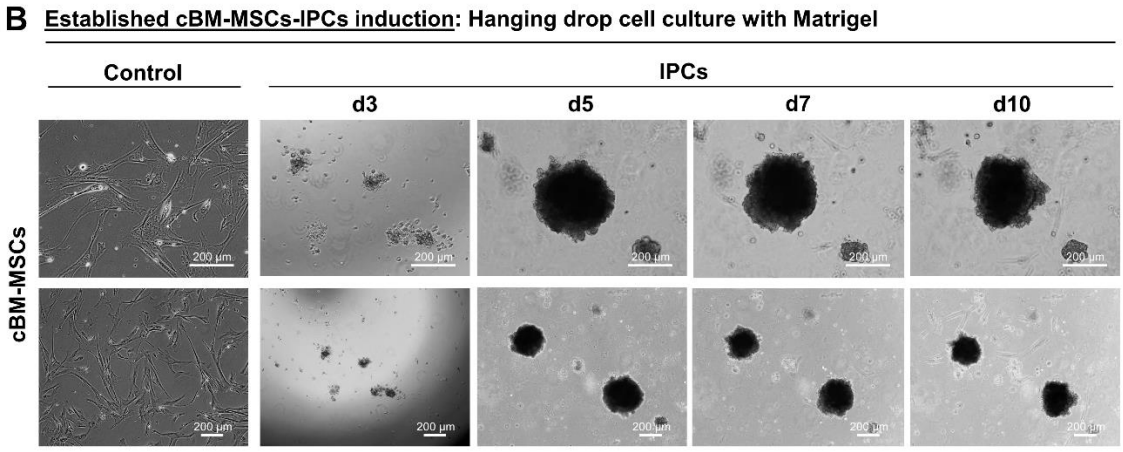

Figure 20 Pancreatic induction of canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) using modified three-dimension (3-D) micro-environmental manipulating protocol *in vitro*. Morphological appearances of cBM-MSCs undergone pancreatic induction by using modified 3-D micro-environmental manipulating protocol were observed. This figure represented "hanging-drop culture technique with Matrigel-formed dome culture technique". (scale bar, 200 µm)

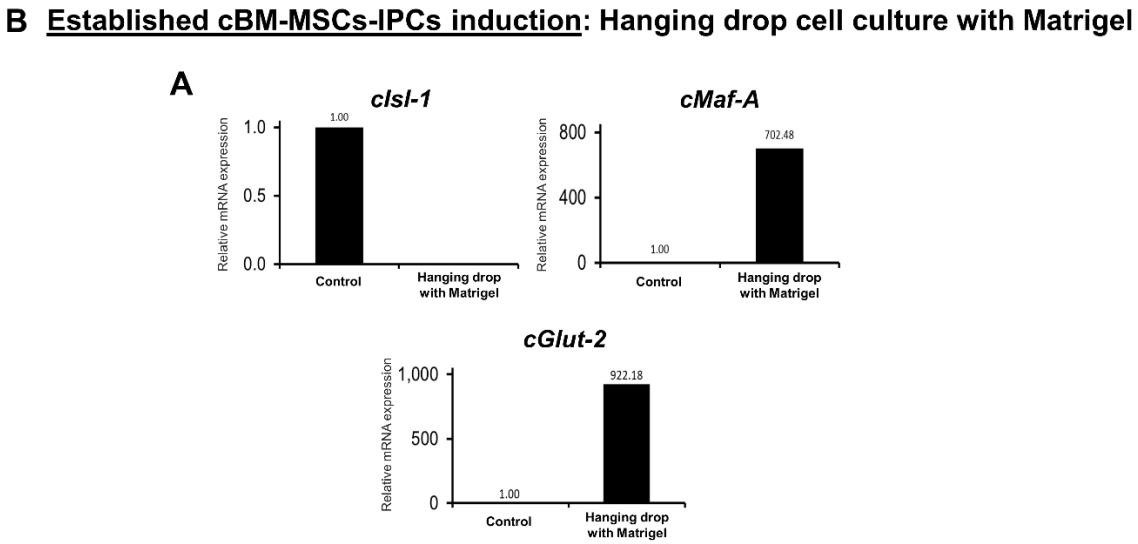

Figure 21 Pancreatic induction of canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) using modified three-dimension (3-D) micro-environmental manipulating protocol *in vitro*. This figure represented "hanging-drop culture technique with Matrigel-formed dome culture technique". Pancreatic gene marker expression was determined using qRT-PCR. An mRNA expression of the gene was normalized to reference gene, *GAPDH*, and the undifferentiated control.

Optimization of the hanging-drop culture technique for pancreatic lineages derivation from cBM-MSCs.

Further optimization of hanging-drop technique for pancreatic lineage derivation was performed by integrating genetic manipulation (*PDX1* overexpression) with the previous established protocols (Figure 22). Briefly, cells were transfected with *PDX1* at MOI 50, then underwent pancreatic induction using hanging-drop culture technique at 24 hours later. *PDX1*-transfected cBM-MSCs (2×10^4 cells) were seeded into each 40-50 µL hanging drop using hanging-drop cell culture plate (PerkinElmer, USA). Cells were always maintained in hanging-drop plate with new manipulation method. Three conditioned media were substituted according to the induction protocol. At day 10 post-induction, colonies were harvested and analyzed.

For pancreatic derivation from cBM-MSCs by hanging-drop cell culture technique with new manipulation method, the results illustrated that cells started gathering and formed aggregates since 24-hour post-induction (data not shown). At day 3 post-induction, colonies and cell clumps were observed. The obtained colonies were bigger than previous hanging-drop protocol, and the size of colonies were gradually increased along the induction period (Figure 23). Size of colony was approximately 100-300 µm, at day 10 post-induction. However, most of hanging-drop contained 1-2 colonies, which was lower than the previous protocol. For representative pancreatic mRNA marker expression, there were dramatic upregulation of *cPdx1, cNgn3, cIsl-1, cMaf-A, cGlut-2,* and *cGlucagon* at day 10 post-induction (Figure 24). The results suggested that cBM-MSCs could effectively induced toward pancreatic lineages by using "hanging-drop culture technique" with new manipulation method.

Additional optimization was done by integrating *PDX1* overexpression with hanging-drop technique. After *PDX1* transfection and pancreatic induction by series of condition media on hanging-drop, cells were aggregated as the medium size colony at day 5 (Figure 25). There was a difference in density of colony compared with non-transfected cBM-MSCs induction. Size of colony was gradually increased, and cells were packed. At day 12, the colonies were tightly packed and clearly seen in the hanging-drops. For representative pancreatic mRNA marker expression, there were dramatic upregulation of *cPdx1, cNgn3, cIsl-1, cMaf-A,* and *cGlut-2* at day 12 post-induction (Figure 26).

It seemed that the *PDX1* overexpression was able to enhance the pancreatic lineages derivation of the hanging-drop culture technique by getting more packed colonies and dramatic expression of representative pancreatic markers.

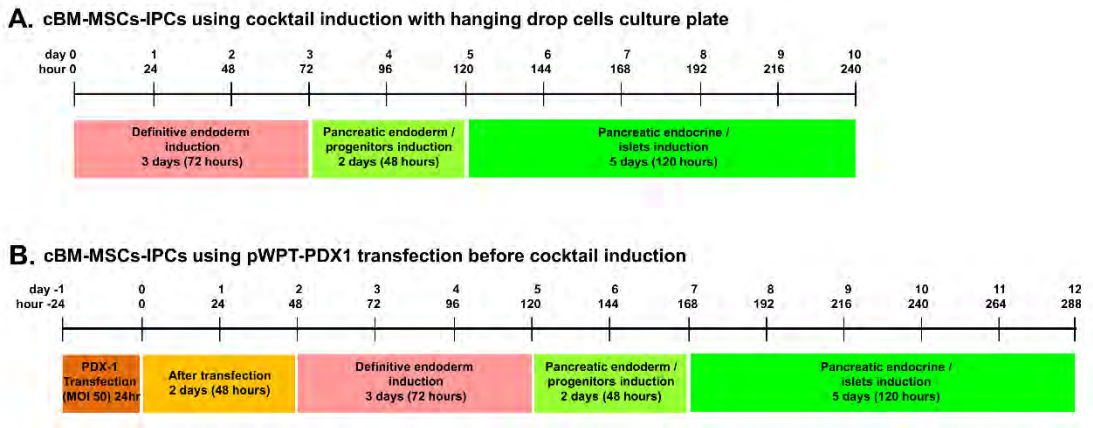

Figure 22 Experimental timeline for optimization of the hanging-drop culture technique for pancreatic lineages derivation from cBM-MSCs.

The experiment timeline and harvesting plan for optimization of the hanging-drop culture technique for pancreatic lineages derivation from cBM-MSCs were summarized.

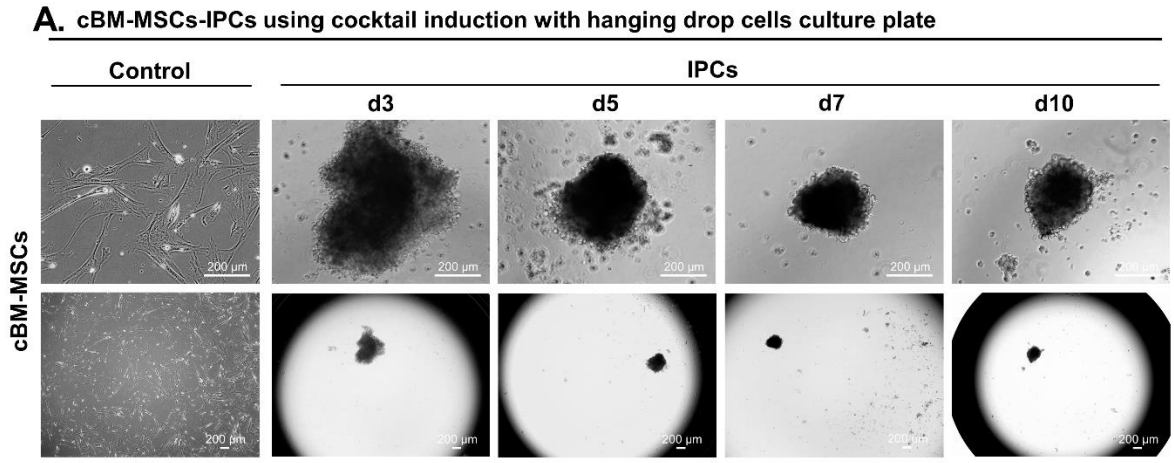

Figure 23 Pancreatic induction of canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) using modified three-dimension (3-D) micro-environmental manipulating protocol *in vitro*. Morphological appearances of cBM-MSCs undergone pancreatic induction by using modified 3-D micro-environmental manipulating protocol were observed. This figure represented "optimized hanging-drop culture technique with new manipulation method". (scale bar, 200 µm)

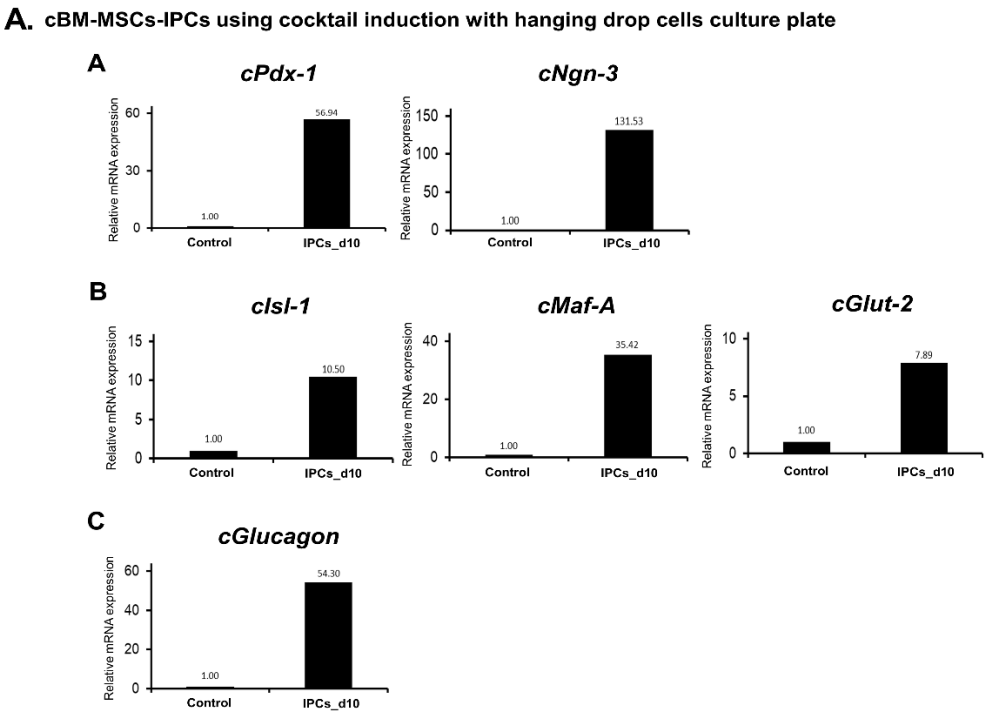

Figure 24 Pancreatic induction of canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) using modified three-dimension (3-D) micro-environmental manipulating protocol *in vitro*. This figure represented "optimized hanging-drop culture technique with new manipulation method". Pancreatic gene marker expression was determined using qRT-PCR. An mRNA expression of the gene was normalized to reference gene, *GAPDH*, and the undifferentiated control.

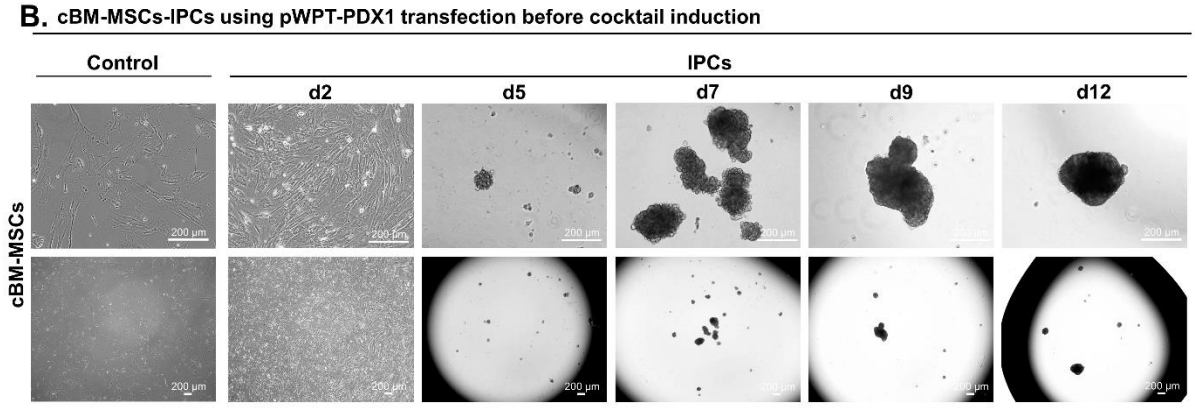

Figure 25 Pancreatic induction of canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) using modified three-dimension (3-D) micro-environmental manipulating protocol *in vitro*. Morphological appearances of cBM-MSCs undergone pancreatic induction by using modified 3-D micro-environmental manipulating protocol were observed. This figure represented "optimized hanging-drop culture technique integrating with genetic manipulation". (scale bar, 200 µm)

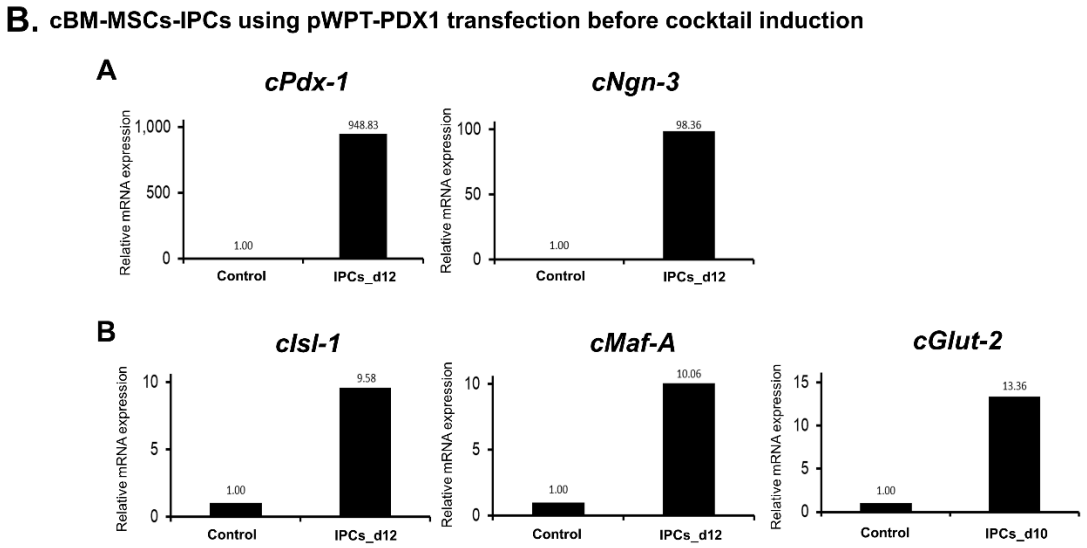

Figure 26 Pancreatic induction of canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) using modified three-dimension (3-D) micro-environmental manipulating protocol *in vitro*. This figure represented "optimized hanging-drop culture technique integrating with genetic manipulation". Pancreatic gene marker expression was determined using qRT-PCR. An mRNA expression of the gene was normalized to reference gene, *GAPDH*, and the undifferentiated control.

DISCUSSION AND CONCLUSION

During the first period of the study, the canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) were isolated and characterized according to our previous published protocols. Transfection efficiency of second-generation lentiviral vector for cBM-MSCs transfection was determined by transfecting "pLenti CMV GFP Puro (658-5)" plasmid (Addgene plasmid #17448). The transfected cells were able to detect by observing the green fluorescent color under fluorescent microscope or flow cytometer. Six different concentration of "pLenti CMV GFP Puro (658-5)"-containing viral solution, ranging from 10^0 to 10^{-5} folds were used. At 24-hour post-transfection, the results showed that cBM-MSCs were dose-dependently susceptible to lentiviral transfection as illustrated in GFP expression under fluorescent microscopy and flow cytometry analyses, and the calculated TU of this viral batch was 23.7×10^3 TU/μL.

The efficiency testing of *PDX1* transfection on cBM-MSCs was performed by the transfection of lentiviral vector-containing "pWPT-PDX1 plasmid" (Addgene plasmid # 12256). According to our previous preliminary testing in human dental pulp stem cells (hDPSCs), viral concentration at MOI 20 was employed. In this experiment, a 24-hour transfection period followed by the maintenance in SFM-culture medium was used. The main objectives of this experiment were to define the differentiation path of the cells after *PDX1* transfection and to optimize the MOI value that sufficient for triggering the significant changes in phenotypic and genotypic. The proposed plans of cell fate analysis were consisted of the exploration of morphological appearances and mRNA markers involving pancreatic differentiation and Notch signaling.

After MOI 20-*PDX1* transfection, the morphology of cBM-MSCs was a little bit changed at 48-hour post-transfection, and a small-size cell aggregates were observed at 168-hour post-transfection. However, only some population of cell underwent morphological changing when compared with SFM-control and DMEM/F12-control groups. The results suggested that viral concentration at MOI 20 could trigger cell morphological changes but was not able to induce a significant change toward colony-like appearance of all cell population. Determination of pancreatic gene marker expression revealed that only some pancreatic gene markers were upregulated, but not for *cInsulin*. In addition, according to viral exposure, dead cells were observed and quite correlated with the exposed viral dosage. The results suggested that *PDX1* transfection at MOI 20 was not efficient in driving cBM-MSCs differentiation toward pancreatic lineages.

Further studies were conducted by increasing *PDX1* viral dosage. In this regard MOI 30 and 50 were tested. The results illustrated that transfection of *PDX1* at MOI 30

and 50 could significantly trigger cell morphological change toward colony-like cell structure in dose-dependent manner. However, pancreatic gene expression of the transfected cell was not correlate with cell morphological changing. Only dose-and time-dependent *cPDX-1* upregulation was observed.

Another approach for cell differentiation toward pancreatic lineages was planned. According to our preliminary studies in human dental pulp stem cells (hDPSCs), integration of genetic and micro-environmental manipulating approach could enhance differentiation potential toward pancreatic lineages. In this regard, further studies were conducted to compare pancreatic differentiation potential by cBM-MSCs employing three different cell induction approaches—1) micro-environmental approach, 2) genetic manipulation approach, and 3) integration approach.

During protocol establishment, the previous published three-step micro-environmental manipulating protocol could not effectively enhance pancreatic differentiation by cBM-MSCs. Cells were not respond to the induction protocol. To address the problem, establishment of modified three-dimension micro-environmental manipulating protocol was performed. Two strategies were employed—1) “hanging-drop culture technique”, and 2) “hanging-drop culture technique with Matrigel-formed dome culture technique”. The results illustrated that both strategies could effectively enhance pancreatic differentiation by cBM-MSCs as shown by colony formation and dramatic upregulation of representative pancreatic mRNA markers. However, the latter method caused the lost of some colonies during cell harvest and dome formation.

Further optimization of hanging-drop culture technique was performed by integrating genetic manipulation approach with new manipulation method. *PDX1* transfection at MOI 50 was utilized for cell priming, then the *PDX1*-trasfecetd cBM-MSCs were undergone hanging-drop culture technique with series of pancreatic induction cocktails. The results showed the success on aggregation and colony formation inside the drops. Interestingly, *PDX1* overexpression was able to enhance a dramatic upregulation of key pancreatic lineage like *cPdx1, cNgn3, cIsl-1, cMaf-A,* and *cGlut-2* at day 12 post-induction.

Taken together, the present study illustrated the feasibility of pancreatic lineage derivation from cBM-MSCs using variety of genetic and/or microenvironmental manipulation which suggests the possibility of further development of stem cell-based diabetes therapy especially for type 1 diabetes in veterinary practice.

ACKNOWLEDGEMENT

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