IN VITRO GENERATION OF TRANSPLANTABLE INSULIN-PRODUCING CELLS FROM CANINE ADIPOSE-DERIVED MESENCHYMAL STEM CELLS

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การสร้างเซลล์สังเคราะห์อินซูลินที่พร้อมปลูกถ่ายจากเซลล์ต้นกำเนิดมีเซนไคม์ของไขมันสุนัข

น.ส.ชุนห์ ลี ดัง

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Thesis Title	IN VITRO GENERATION OF TRANSPLANTABLE INSULIN-PRODUCING CELLS FROM CANINE ADIPOSE-DERIVED MESENCHYMAL STEM CELLS
By	Miss Quynh Le Dang
Field of Study	Veterinary Science and technology
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ชุนห์ ลี ดัง : การสร้างเซลล์สังเคราะห์อินซูลินที่พร้อมปลูกถ่ายจากเซลล์ต้นกำเนิดมีเซนไคม์ของไขมันสุนัข. (IN VITRO GENERATION OF TRANSPLANTABLE INSULIN-PRODUCING CELLS FROM CANINE ADIPOSE-DERIVED MESENCHYMAL STEM CELLS) อ.ที่ปรึกษาหลัก : เจนภพ สว่างเมฆ

เซลล์ต้นกำเนิดมีเซนไคม์จากสุนัขมีศักยภาพในการพัฒนาและนำมาใช้ประโยชน์สำหรับแนวทางการรักษาแบบฟื้นฟู ซึ่งรวมไปถึงการสร้างเซลล์สังเคราะห์อินซูลินเพื่อใช้ในการศึกษาและรักษาเบาหวาน งานวิจัยนี้จึงมุ่งเน้นในการพัฒนากรรมวิธีการสร้างเซลล์สังเคราะห์อินซูลินจากเซลล์ต้นกำเนิดมีเซนไคม์ที่ได้จากไขมันสุนัขและกระบวนการเพาะเลี้ยงเพื่อเก็บรักษาคุณภาพและพัฒนาให้เป็นเซลล์สังเคราะห์อินซูลินจากเซลล์ต้นกำเนิดมีเซนไคม์ที่ได้จากไขมันสุนัขที่พร้อมสำหรับการนำมาใช้ในทางคลินิกด้วยกรรมวิธีห่อหุ้มเซลล์สังเคราะห์อินซูลินจากเซลล์ต้นกำเนิดมีเซนไคม์ที่ได้จากไขมันสุนัขด้วยสารก่อเจลอัลจิเนตร่วมกับพลูโรนิค เอฟ127 ผลการศึกษาพบว่าเซลล์ต้นกำเนิดมีเซนไคม์ที่ได้จากไขมันสุนัขสามารถเปลี่ยนแปลงเป็นเซลล์สังเคราะห์อินซูลินด้วยกรรมวิธีเหนี่ยวนำ 3 ขั้นตอน โดยขั้นตอนที่หนึ่ง พบว่าการใช้ Chir99021 ร่วมกับ Activin A สามารถผลักดันให้เซลล์ต้นกำเนิดเข้าสู่สภาวะเดฟฟินิทีฟเอนโดเดิร์ม ที่มีการแสดงออกของยีน *ซอกซ์-17* ได้อย่างมีประสิทธิภาพ ในขั้นตอนที่สอง พบว่าการเหนี่ยวนำเซลล์ในสภาวะเดฟฟินิทีฟเอนโดเดิร์ม ด้วย Taurine Retinoic acid FGF2 EGF TGFβ-inhibitor Dorsomorphin Nicotinamide และ DAPT สามารถเพิ่มการเหนี่ยวนำให้เข้าสู่สภาวะแพนครีเอทิกเอนโดเดิร์ม โดยการเพิ่มการแสดงออกของยีน *พีดีเอ็กซ์-1* และ *เอ็นจีเอ็น-3* ซึ่งเป็นเครื่องหมายพันธุกรรมในสภาวะแพนครีเอทิกเอนโดเดิร์มได้อย่างมีนัยสำคัญ และในขั้นตอนสุดท้าย พบว่า Taurine Nicotinamide Glp-1 Forskolin PI3K-inhibitor และ TGFβ-inhibitor สามารถเหนี่ยวนำเซลล์ในสภาวะแพนครีเอทิกเอนโดเดิร์มเข้าสู่สภาวะของเซลล์สังเคราะห์อินซูลินได้อย่างมีประสิทธิภาพ จากนั้น เมื่อทำการห่อหุ้มเซลล์สังเคราะห์อินซูลินจากขั้นตอนสุดท้ายและทำการเพาะเลี้ยงต่อ พบว่า เซลล์สังเคราะห์อินซูลินมีคุณสมบัติและประสิทธิภาพเพิ่มมากขึ้น เมื่อทำการเพาะเลี้ยงในอาหารเลี้ยงเซลล์ที่ถูกปรับปรุงขึ้นมาใหม่ VSCBIC-1 จากผลการทดลองดังกล่าวสามารถสรุปได้ว่ากรรมวิธีการเหนี่ยวนำ 3 ขั้นตอนนี้ มีศักยภาพในการเหนี่ยวนำเซลล์ต้นกำเนิดมีเซนไคม์ที่ได้จากไขมันสุนัขให้เป็นแปลงเป็นเซลล์สังเคราะห์อินซูลิน และการห่อหุ้มเซลล์สังเคราะห์อินซูลินด้วยสารก่อเจลอัลจิเนตร่วมกับพลูโรนิค เอฟ127 ร่วมกับการเพาะเลี้ยงในอาหารเลี้ยงเซลล์ VSCBIC-1 สามารถช่วยฟื้นฟูและเพิ่มความสามารถในการทำงานของเซลล์สังเคราะห์อินซูลินจากเซลล์ต้นกำเนิดมีเซนไคม์ที่ได้จากไขมันสุนัขในหลอดทดลองได้

สาขาวิชา	วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี	ลายมือชื่อนิสิต .....
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KEYWORD: canine adipose-derived mesenchymal stem cell (cAD-MSC), diabetes mellitus, encapsulation, insulin-producing cells (IPCs)

Quynh Le Dang : IN VITRO GENERATION OF TRANSPLANTABLE INSULIN-PRODUCING CELLS FROM CANINE ADIPOSE-DERIVED MESENCHYMAL STEM CELLS . Advisor: Asst. Prof. Dr. CHENPHOP SAWANGMAKE, D.V.M., M.Sc., Ph.D

Canine mesenchymal stem cells (cMSCs) have potential applications for regenerative therapy, including the generation of insulin-producing cells (IPCs) for studying and treating diabetes. In this study, we established a useful protocol for generating IPCs from canine adipose mesenchymal stem cells (cAD-MSCs). Subsequently, *in vitro* preservation of pluronic F127-coated alginate (ALGPA)-encapsulated cAD-MSC-derived IPCs was performed to verify ready-to-use (RTU) IPCs. IPCs were induced from cAD-MSCs with the modulated three-stepwise protocol. The first step of definitive endoderm (DE) induction showed that the cooperation of Chir99021 and Activin A created the effective production of *Sox17*-expressed DE cells. The second step for pancreatic endocrine (PE) progenitor induction from DE indicated that the treatment with taurine, retinoic acid (RA), FGF2, EGF, TGFβ inhibitor, dorsomorphin, nicotinamide (NIC), and DAPT show the significant upregulation of the PE precursor markers *Pdx1* and *Ngn3*. The last step of IPC production, the combination of taurine, NIC, GLP-1, forskolin, PI3K inhibitor, and TGFβ inhibitor, yielded efficiently functional IPCs from PE precursors. Afterward, the maintenance of ALGPA-encapsulated cAD-MSC-derived IPCs with VSCBIC-1, a specialized medium, enhanced IPC properties. Conclusion, the modulated three-stepwise protocol generates the functional IPCs. Together, the encapsulation of cAD-MSC-derived IPCs and the cultivation with VSCBIC-1 enrich the maturation of generated IPCs.

Field of Study:	Veterinary Science and technology	Student's Signature .....
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CHAPTER I

INTRODUCTION

Importance and Rationale

Diabetes mellitus (DM) is a chronic, metabolic disease characterized by hyperglycemia due to decreasing insulin function and secretion, which leads to serious damages to heart, blood vessels, eyes, kidneys and nerves. Diabetes is not only a popular disease affecting 442 million people in 2014 worldwide and predicted a notably increasing trend, but also detected and expanding in companion animals, mostly dogs and cats, considering as a major cause of euthanasia especially for well-uncontrolled animals (Roglic, 2016; Niessen et al., 2017).There are two main types of diabetes, type 1 (T1DM) and type 2 (T2DM). The most popular type of diabetes in human (around 90-95%) and cats (80-95%) is T2DM which occurs when pancreatic β-cells fail to produce enough insulin for the demand of body due to insulin resistance in the peripheral tissues (Shulman, 2000; Rand et al., 2004; Chang-Chen et al., 2008; Artasensi et al., 2020). By contrast, T1DM is characterized by islet β-cells destruction as a result of the body's autoimmune processes leading to absolute insulin deficiency, which occupies at least 50% of diabetic dogs diagnosed by the presence of antibodies against islet β-cells (Rand et al., 2004).

Currently, some therapies are being applied in diabetes treatment such as insulin therapy, pancreas or islet transplantation. Islet transplantation, also called "Edmonton protocol", is considered as the most ideal method for treating T1DM and advanced T2DM cases, however, this approach has limitations (Aigha et al., 2018). The first difficulty is foreign-tissue rejection of the recipient's body, although immunosuppressive drugs can be used to solve this situation but causing long-term consequences. The second challenge is the availability of islet source. Therefore, insulin-producing β-like cell-derived stem cell production and ready-to-use (RTU) insulin-producing cell (IPC) transplantation platform have become the main focused

research points. Nowadays, any protocols of IPC induction derived from both human and animal pluripotent stem cells have been established and approved benefits on veterinary clinical application and human pre-clinical trials (D'Amour et al., 2005; Chandra et al., 2009; Gibly et al., 2011; Wong, 2011; Lilly et al., 2016; Vanikar et al., 2016). Simultaneously, delivery platforms for islet β-cell transplantation are still being explored and developed (Calafiore et al., 2006; Hwang et al., 2016; Vegas et al., 2016; Kuncorojakti et al., 2020). Nonetheless, obstacles on optimizing the protocol's efficiency and safety are still concerned.

Mesenchymal stem cells (MSCs) are one of the stem cell types and specifically possess immune-privileged and highly plastic which help them to become a wonderful and safe choice to generate differentiated cells for transplantation therapy (Pavathuparambil Abdul Manaph et al., 2019). The capacity of different sources of human MSCs (hMSCs) differentiating into IPCs has been shown in many previous papers (D'Amour et al., 2006; Zanini et al., 2011; Xu et al., 2020), while that of canine mesenchymal stem cells (cMSCs), especially canine adipose-derived mesenchymal stem cells (cAD-MSCs), are still scarce. Mostly, the IPC induction protocols used for study on cMSCs are grabbed from the protocols on hMSCs and particular stages of differentiation procedure are not definitely separated and established (Teshima et al., 2020; Camara et al., 2021). Although IPCs can be produced from cAD-MSCs, their function is still limited. Thus, these restrictions of the present protocols have caused disadvantages for optimizing for each step of the cMSC-derived IPC induction protocol.

Therefore, this study is focused on establishing the induction protocol for cAD-MSCs toward mature IPCs *in vitro.* Besides, establishment of the enriched medium for preserving the double-encapsulated cAD-MSC-derived IPCs' function and viability is also considered to produce RTU cAD-MSC-derived IPCs for transplantation.

Objectives of Study

1. To establish the protocol for canine adipose-derived mesenchymal stem cell (cAD-MSC) induction toward mature insulin-producing cells (IPCs) *in vitro.*

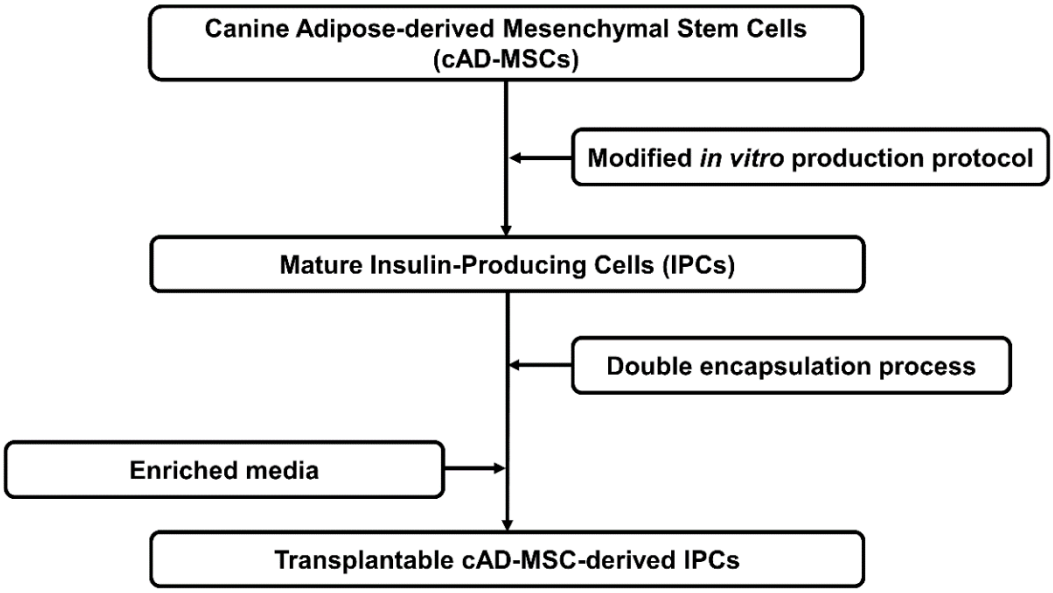
2. To study the enriched culture media for preserving the double-layer encapsulated cAD-MSC derived IPCs.

Hypothesis

1. cAD-MSCs can be induced toward mature IPCs *in vitro.*

2. The established enriched culture medium can preserve the double-layer encapsulated cAD-MSC derived IPCs.

Conceptual framework



CHPATER II

LITERATURE REVIEW

Pathophysiology of diabetic mellitus

DM is a complicated metabolic disease which is characterized by hyperglycosemia due to deficiency or impairment of the essential amount of endogenous insulin in blood. This is considered as a consequence of impairing islet β-cell mass or losing its function. Moreover, people with diabetes are living with apprehension, fear and worry about diabetic-related health problems because long-term hyperglycemia can cause various serious damages to heart, blood vessels, eyes, kidneys and nerves. According to the data from global reports of World Health Organization (WHO), a significant rise in the number of people with diabetes was from 108 million in 1980 up to 422 million in 2014 and a total of 1.5 million deaths were due to diabetes (Roglic, 2016). The newest study of International Diabetes Federation in regard to the global diabetes prevalence showed the increasing estimation between 2019 and 2045 is from 463 million to 700 million based on epidemiological data (Saeedi et al., 2019). Concerning the veterinary field, the percentage of canine dogs has increased by 79.7% since 2006, while feline diabetes has grown by 18.1% at the same time according to a database of Banfield Pet Hospital in the United States (Molly McAllister, 2016). Two studies on canine diabetes in the United Kingdom (UK), the factors including age (over 8 years of age), female entire dogs, male neutered dogs, dogs with obesity, and certain breeds have significant effects on the risk of dogs developing DM (Mattin et al., 2014; Heeley et al., 2020). In another study on feline diabetes in the UK, the higher risks of diabetic cats were reported being obesity and age (over 6 years old) (O'Neill et al., 2016).

Almost all cases of DM are classified into two broad etiopathogenetic categories being T1DM and T2DM. An absolute deficiency of insulin secretion caused islet β-cell destruction or dysfunction is characterized for T1DM. Genetics and environmental

factors that cause autoimmune destruction of islet β-cells are considered as main reasons for this type of DM. The risk of T1DM associated with the human leukocyte antigen (HLA) and non-HLA loci genetics has been identified (Noble, 2015). Besides, enterovirus infection, one of most common viruses all over the world, triggering the production of type I interferons (IFNs) and antiviral immune response relates to several candidate genes (*IFIH1, TLR7/TLR8, FUT2* and *GPR183*) which confer T1DM risks (Ferreira et al., 2014; Bergamin and Dib, 2015). Otherwise, toxins in food and water, and other environmental factors might also be the cause leading to T1DM-associated autoimmune mechanisms (Rewers and Ludvigsson, 2016; Blanter et al., 2019). T2DM gives reasons for 90% of all cases of DM which is known to be insulin resistance of the body's cells. In this case, the pancreatic β-cells initially produce enough insulin, but it has not been absorbed by the body's organs resulting in unusual blood sugar levels. Overtime, islet β-cells are exhausted due to a compensatory increase in insulin secretion. Some risk factors leading insulin resistance are obesity, physical inactivity, a poor diet, smoking, and generous consumption of alcohol (Hu et al., 2001; Eckel et al., 2011). The exact mechanisms are still being explored, for example, an excess of adipose tissue is thought to cause the release of free fatty acids, called adipokines, which are signaling molecules that can cause inflammation. The increase of inflammation cytokines such as Interleukin (IL)-1β, tumor necrosis factor (TNF)-α and IFN-γ has demonstrated involvement in islet β-cell destruction (Barlow et al., 2018). Besides, genome-wide association studies (GWAS) have pointed out over 400 genes related with the risk of T2DM, for examples, *Hnf1b*, *Pdx1*, *Ngn3*, *ABCC8*, *GCK, KCNJ11, Nkx6.1* (Krentz and Gloyn, 2020).

Adipose-derived mesenchymal stem cells

MSCs are adult stem cells derived from many various sources, such as bone marrow, adipose tissue, cartilage, dental pulp, umbilical cord and placenta. They have been widely studied over the past 30 years to obtain insights about their biological

nature, extended clinical potential, and application of tissue engineering. MSCs have self-renewal capacity and intrinsic differentiation potentials (Han et al., 2017). Importantly, MSCs also possess low immunogenicity and strong immunomodulation prospects through hampering the expression of proinflammatory cytokines (TNF-α and IFN-γ) while enhancing the expression of suppressive cytokines (IL-10) and prostaglandin E_2 (PGE_2) (Aggarwal and Pittenger, 2005). Upon these excellent features, MSCs are considered as a safe regenerative medicine and focused on clinical trial studies.

AD-MSCs which is one of MSCs isolated from the stromal vascular fraction (SFV). Human AD-MSCs (hAD-MSCs) were clarified and characterized the self-renewal, proliferation, and differentiation properties (Zuk et al., 2002). The MSC surface markers express in AD-MSCs as CD73, CD44, CD90, and CD29 but not express markers of hematopoietic lineage cells such as CD45 (Huang et al., 2013). Beside MSC surface markers, stemness markers such as such as *Oct4*, and *Rex1* and proliferation marker *(Ki67)* are also characterized by hAD-MSCs (Baer et al., 2010; Paula et al., 2015; González-Garza et al., 2018). Moreover, previous studies published that hAD-MSCs could be promoted to express the specific pancreatic transcription factors such as *Pdx1*, *Isl1* and *Pax6*, this indicated AD-MSCs to be an ideal candidate for alternative cell therapy of DM because *Pdx1* and *Pax6* are found during pancreas development while *Isl1* is known as an enhancer binding to the promoter insulin gene (Timper et al., 2006; Dave et al., 2014). Thus, differentiation potential of AD-MSCs to IPCs have been demonstrated in many studies (D'Amour et al., 2006; Zanini et al., 2011; Xu et al., 2020).

For cAD-MSCs, several previous reports were showed that expression of CD73 and CD90 of cAD-MSCs just achieved at moderate levels compared to hAD-MSCs (Takemitsu et al., 2012; Chow et al., 2017; Ivanovska et al., 2017). In addition, expression of *Nanog* in the population of cAD-MSCs is fairly blurred as well as their differentiation

prospects into osteogenesis, chondrogenesis, and adipogenesis are illustrated (Neupane et al., 2008; Guercio et al., 2012; Kisiel et al., 2012). *In vitro* induction of cAD-MSCs to IPCs have performed and achieved a few of certain results, but this IPCs' function is still restricted (Teshima et al., 2020; Camara et al., 2021).

Pancreatic endocrine development and novel strategies of islet β-cell production

The pancreas contains two principal components comprising exocrine and endocrine compartments (Márquez-Aguirre et al., 2015). The exocrine pancreas involves acinar and duct cells, while endocrine pancreas is organized in clusters containing five cell types called islet of Langerhans. The five islet cell types including alpha, beta, delta, epsilon, and upsilon are secreting five typical hormones composed of glucagon, insulin, somatostatin, ghrelin, and polypeptide, respectively (Márquez-Aguirre et al., 2015).

For embryonic pancreas development, multiple signaling pathways and various cascades of transcription factors are tremendously essential to keep the correctly differentiation process of embryonic cells toward the appropriate pancreatic cells (Arda et al., 2013). The pancreatic development has been described in many previous reviews (Caronna et al., 2013; Jennings et al., 2015; Márquez-Aguirre et al., 2015). Primitive streak (PS)/mesendoderm (ME) specification from ESCs characterized by *Mixl1, Eomes,* and *Goosecoid (GSC)* genes is the first step of pancreatic development. Definitive endoderm (DE) formation is the next stage with the expression of *Gata4, Gata6, Cxcr4, Sox17*, and *Foxa2*. Then, DE develops into a gut tube which is divided into foregut, midgut, and hindgut. Posterior and anterior foregut develops into ventral and dorsal pancreatic buds which are formed by multipotent pancreatic progenitors (MPP). Exocrine cells and endocrine cells will be given rise from these MPPs. The endocrine cell specification requires the expression of pro-endocrine gene *Ngn3*, and then followed by several transcription factors such as *Nkx2.2, NeuroD1, Nkx6.1, Pax4, Pax6* and *Isl1* which controls pancreatic endocrine (PE) differentiation. Finally, the

endocrine progenitors differentiate into specific hormone secreting cells which aggregate together to form Langerhans islets.

Obviously, the recent strategies of IPC production are mimicking aspects of pancreatic development (Jacobson and Tzanakakis, 2017). In other words, stem cells are induced to IPCs by microenvironment manipulations which allows cells to be exposed with appropriate signals or molecules during the induction route corresponding to the stages of pancreatic development (Gheibi et al., 2020). Although several signaling pathways and small molecules have proved the roles in differentiation into IPCs, a lot of mystery is still undisclosed.

In DE differentiation stage, several findings have shown that the absence of PS formation in mouse embryos with the knockout of Nodal or Wnt signal (Zhou et al., 1993; Conlon et al., 1994; Liu et al., 1999; Barrow et al., 2007), while PS is known as a vital first differentiation step for the development of endoderm, mesoderm and ectoderm organogenesis development (Márquez-Aguirre et al., 2015). Activin A is a member of the TGF-β superfamily which has demonstrated and applied for many studies of IPC induction (D'Amour et al., 2005; D'Amour et al., 2006; Shim et al., 2007; Sawangmake et al., 2014a; Sawangmake et al., 2014b). Activin A induces Nodal signaling at high concentration (50-100 ng/mL) which stimulates the signaling event cascades regulating pancreatic development process during DE differentiation (Kubo et al., 2004; Shim et al., 2007). Alongside Activin A, the activation of Wnt pathway via Wnt3a or an inhibitor of glycogen synthase kinase-3β (GSK-3β) also enhances DE specification (Naujok et al., 2014; Teo et al., 2014; Huang et al., 2017). Chir99021 is known as GSK-3β inhibitor helping in Canonical Wnt signaling pathway activation which enhances the releasing and accumulation of β-catenin inside the nucleus (Huang et al., 2017). In addition, these events allow the expression of *Mixl1, Eomes* and *GSC* genes to promote PS status (Naujok et al., 2014; Teo et al., 2014). After that, Activin A is used to suppress

expression of *Mixl1* as well as enhance the appearance of *GSC* and *Eomes* which regulates cells to express the DE-defined markers (Naujok et al., 2014).

Before pancreatic endocrine (PE) progenitor specification, MPPs are an important intermediate milestone. In MPP status, the expression of *Hnf1*b, *Hnf4a*, *Hnf6* (*Onecut1*), *Ptf1a*, *Pdx1*, *Hes1*, *Sox9*, and *Nkx6.1* will be initiating and regulating expression of transcription factors to decide pancreatic cell fates (Arda et al., 2013). For example, *Ptf1a* which is commonly found in acinar cells has ability to control the expression of essential genes of other cells such as *Pdx1*, *Nkx6.1*, *Ngn3* in PE progenitors, however, *Nkx6.1* has the negative feedback by suppressing the presence of *Ptf1a* (Aigha and Abdelalim, 2020). Retinoid acid (RA) is a metabolite of vitamin A that can upregulate the expression of *Pdx1* and *Ptf1a* through promoting the expression of *Hnf1b* and *Fzd4* (Gere-Becker et al., 2018). Notochord factors including Activin-βB and basic fibroblast growth factor (bFGF or FGF2) repress endodermal sonic hedgehog (Shh) to permit the upstream of *Pdx1* and *Insulin* (Hebrok et al., 1998). According to previous studies, intermediate concentration of FGF2 induces pancreatic progenitor differentiation (Ameri et al., 2010). In addition, *Gli1,* a member of Shh pathway, inhibits the expression of *Sox9* that is known a regulator of *Nng3* while *Gli2,* another of Shh pathway, upregulates the expression of *Hes1* that is showed an inhibitor of *Ngn3* (Bien-Willner et al., 2007; Wall et al., 2009). Then, PE progenitors will express a load of genes such as *Nkx2.2*, *Nkx6.1*, *Sox9*, *Pax4*, *NeuroD1*, *Isl1*, *MafB*, *Glut2*, and especially *Ngn3*. Upregulation of *Ngn3* is extremely important for driving cells in endocrine cell fates while co-expression of *Nkx6.1* and *Sox9* are a vital crucial for generation of $Ngn3^+$ PE progenitors and maturation of IPCs (Aigha and Abdelalim, 2020). Therefore, the evidences shown that dorsomorphin as BMP inhibitor, SB431542 as TGF-β inhibitor, nicotinamide (NIC), and RA can promote differentiation of $Nkx6.1^+$ progenitors and then upregulate *Ngn3* expression for supporting wonderful insights of endocrine induction protocol establishment (Öström et al., 2008; Zhu et al., 2011; Kunisada et al., 2012;

Shahjalal et al., 2014; Nostro et al., 2015). Furthermore, a γ-secretase inhibitor, DATP, is used to suppress Notch signaling pathway which downregulates the expression of *Hes1*, and consequently enhances the expression of *Ngn3* is significantly enhanced (Champeris Tsaniras and Jones, 2010; McCracken and Wells, 2012; Sawangmake et al., 2014a). In addition, epidermal growth factor (EGF) has evaluated its ability in expanding $Pdx1^{+}/Sox9^{+}$ expressed-pancreatic progenitors (Konagaya and Iwata, 2019).

The final step is IPCs' induction and maturation, matured IPCs are recognized by the expression of genes such as *Nkx6.1*, *Isl1*, *MafA, Glut2*, *Glp1r*, *Rfx6*, *NeuroD1, Glis3*, *Insulin*, and *Glucagon.* Forskolin is a mediate-factor activating protein kinase (PKA) and protein kinase C (PKC) signaling pathway by converting Adenosine triphosphate (ATP) to dibutyryl adenosine 3',5'-cyclic AMP (cAMP) via adenylate cyclase receptor, which rises the pancreatic hormone secretion (Hermansen, 1985; Wan et al., 2004). Maintaining the inhibition of TGF-β signaling pathway via ALK5 receptor during PE progenitor differentiation toward IPCs enhances the increasing of *NeuroD1*, *Insulin*, and *Glucagon* and promotes the maturation of endocrine progenitors (Lee et al., 2020). Moreover, the signals which are activated by the ALK5 receptor can suppress the expression of *MafA* and *NeuroD1* because of the down-regulation of Foxo1 protein (Toren-Haritan and Efrat, 2015). Another factor is phosphoinositide 3-kinases (PI3K) inhibitor, LY294002, which also contributes the functional maturation of IPCs (Hori et al., 2002), but the mechanism detail of this pathway is still limited.

Encapsulation for regenerative medicines

Currently, the establishment of a suitable delivery platform for IPCs still remains. Encapsulation is an expectant strategy for cell-based therapy due to its advantages and applied potentials. The aims of encapsulation are to immobilize the implants as well as build a wall from the body's immune system. Hydrogel materials are special suitable for encapsulation of biological elements due to their biocompatibility and permeability as well as the ability of oxygen, nutrient and metabolic waste diffusion

between inside and outside of membrane (Pérez-Luna and González-Reynoso, 2018). In a previous study, the formed membrane from 0.1-1% alginate has the pores with diameter range from 7.2-8.0 nm, thus the formed capsules will be restricted to 21-25 kDa of dextran and 78-103 of proteins, which is sufficient to prevent antibodies across the membrane (Dembczynski and Jankowski, 2001). However, the size of pores depends on concentration of alginate (Wang et al., 2009). According to these benefits, alginate has widely used and applied in biomedical science as well as a regenerative medicine. For whole islet or islet β-cell transplantation, alginate is an ideal material for creating the protective membrane. A trial on dogs as an animal model, the grafts of alginate-encapsulated islets can maintain their function during 174 days without using immunosuppression administration (Hwang et al., 2016). Moreover, the transplantation of alginate-encapsulation islets in mice models also showed a similar result, these implants can stabilize the blood glucose levels until their removal for 174 days with no immunosuppressive treatment before their removal (Vegas et al., 2016). The efficiency of alginate encapsulation against immunological reaction was also demonstrated *in vitro*, when alginate-encapsulated rat MSCs were cultured within media containing lymphocytes, the level of IL-2 which produced by the immune cells is low (Ramezanzadeh Andevari et al., 2018). Surprisingly, a previous report shown alginate encapsulation for human embryonic stem cells (hESCs) promotes differentiation into pancreatic islet cells, and alginate-encapsulated hESCs also shown the stronger differentiation toward pancreatic islet cells by increasing C-peptide protein synthesis about 20-fold more than the two-dimensional cultures (Richardson et al., 2014). However, a greatest obstacle is still existing as the protrusion of cells from monolayer capsules, which may cause the body's rejection or fibrotic response leading to the necrosis of cells inside capsules.

To prevent cell protrusion and improve the encapsulation technique, multilayer encapsulation is proposed. In 2006, a report showed that islets encapsulated with a

poly-L-ornithine (PLO) and alginate-double layer can improve the blood glucose levels and maintain insulin response up to 1 year in 2 of 10 DM volunteers (Calafiore et al., 2006). Therefore, multilayer encapsulation is able to prevent cell's protrusion of inside cells and provide the second barrier for protecting transplanted grafts from the host immune system (Ramezanzadeh Andevari et al., 2018). Pluronic F127 is a hydrogel material with biocompatible potential, therefore, it is suitable for promising biomedical applications (Lippens et al., 2013). Importantly, our previous study was demonstrated that alginate and pluronic F127 encapsulation for human dental pulp stem cells (hDPSCs) could maintain cells' viability, in addition the double encapsulation for hDPSC-derived IPCs could preserve both IPCs' viability and function (Kuncorojakti et al., 2020).

CHAPTER III

MATERIALS AND METHODS

According to the main objectives, the methodology of the study was performed as described below.

Specific aim 1: To establish the protocol for canine adipose-derived mesenchymal stem cells (cAD-MSC) induction toward mature insulin-producing cells (IPCs) *in vitro*

Strategy 1a) Isolation and characterization of cAD-MSCs

All experiment procedures were approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Science, Chulalongkorn University. cAD-MSCs used in all experiments were derived from our cell bank. cAD-MSCs were harvested from biopsied adipose tissues. The surgery procedures were performed by veterinarians at the Department of Surgery, Small Animal Hospital, Chulalongkorn University with the owner's consent. The isolation, expansion and characterization of cAD-MSCs were modified from the previous report (Rodprasert et al., 2021). Cells in passage 2-4 were used for the experiments.

The isolated cells were further characterized by identifying mesenchymal cell surface markers (CD29, CD90, CD44 and CD73) and hematopoietic cell surface marker (CD45) with flow cytometry as well as defining the morphological appearance and mRNA expression of stemness markers (*Oct4*, *and Rex1*) and of proliferation marker (*Ki67*) using reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Additionally, *in vitro* differentiation potential properties of isolated cells were investigated including osteogenic, chondrogenic and adipogenic differentiation. These protocols are based on our previous reports (Nantavisai et al., 2020). For osteogenic differentiation, cells were seeded onto a 24-well plate and maintained in osteogenic induction medium for 14 days, and osteogenic cells were evaluated using Alizarin Red S staining. For adipogenic differentiation, cells were cultured in adipogenic induction media after seeding in 24-well plate for 28 days, then the lipid droplets of adipocytes

were stained with Oil Red O. For chondrogenic differentiation, cells also were seeded onto 24-well plate and maintained in chondrogenic induction media for 21 days. The assessment of chondrogenic differentiation was examined by Alcian blue staining. In addition, osteogenic mRNA markers (*Runx2,* and *Ocn*), adipogenic mRNA markers (*Leptin*, and *LPL*), and chondrogenic mRNA markers (*Sox9*, and *Col2a1*) were measured by RT-qPCR. Undifferentiated cells were considered as a control.

The isolated cells with clarified MSC characterization were stored in a cell bank and used for the experiments.

Strategy 1b) Modifications of cAD-MSC-derived IPC production protocol *in vitro*

For production of cAD-MSC-derived IPC *in vitro*, the protocol was innovated from previous published studies (D'Amour et al., 2006; Chandra et al., 2009; Naujok et al., 2014; Sawangmake et al., 2014a; Sawangmake et al., 2020). The induction procedure of cAD-MSC-derived IPC *in vitro* from these reports are classified into 3 main stages (Figure 1A) including 1) definitive endoderm (DE), 2) pancreatic endocrine (PE) progenitors and 3) insulin-producing β-like cells (IPCs) whilst two stages consisting of mesendoderm (ME) and multipotent pancreatic progenitors (MPPs) were additionally established in this induction protocol (Figure 1B). Low attachment surface culture was used as a special technique allowing colony formation. After each stage of induction, related mRNA expression, morphological appearance, a total number of colonies and size of colony were assessed and chosen for verifying the next step of the induction procedure.

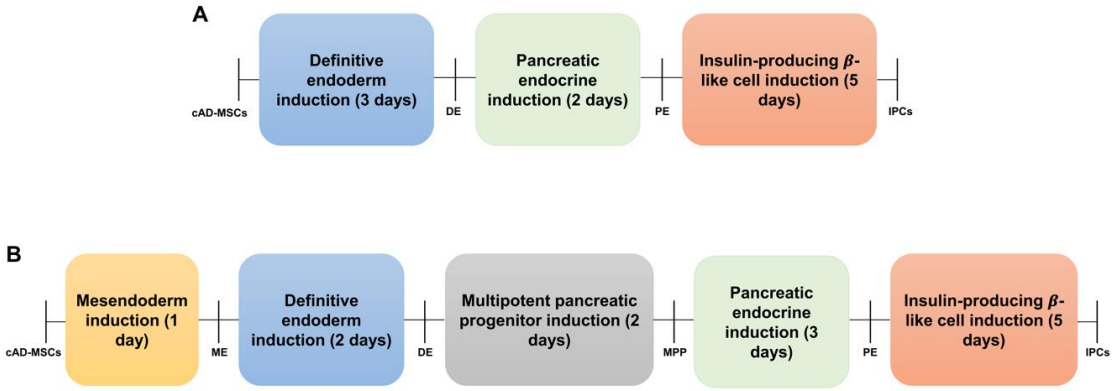


Figure 1 **Insulin-producing differentiation protocols**

Scheme of IPC differentiation protocol by 3 stages (A). Scheme of IPC different differentiation protocol by 5 stages (B).

For DE induction, Activin A through TGF-β signaling pathway plays an important role in directing stem cells to DE (D'Amour et al., 2005; Tada et al., 2005). Moreover, much evidence from previous research showed that the Wnt-canonical signaling pathway also plays a significant role in DE induction (Naujok et al., 2014; Teo et al., 2014). In this regard, the protocol of DE induction from cAD-MSC was carried out by treating with Wnt activator for 24 hours and then handled with Activin A for 48 hours. cAD-MSCs were induced to DE by only treating with Activin A as a control protocol. Expression trend of ME-related markers (*Mixl1, Eomes,* and *GSC*) and DE-specialized (*Gata4, Gata6, Cxcr4,* and *Sox17*) mRNAs were analyzed by RT-qPCR after 1 and 3 days.

As described in previous protocols, the removal of Activin A induces the progression from DE to PE (Guo and Hebrok, 2009). However, inhibition of TGF-β, Notch and BMP signaling pathways as well as the assistance of RA, NIC, FGF2 and EGF signals make a promotion for the induction to PE (Öström et al., 2008; Ameri et al., 2010; Kunisada et al., 2012; Sawangmake et al., 2014a; Jiang et al., 2017; Konagaya and Iwata, 2019; Tan et al., 2019). Therefore, DE clusters were induced to PE through the removal of Activin A and then the supplement of taurine as a control protocol, while new cocktail media consisted of SB431542, RA, DAPT, dorsomorphin, NIC, FGF2 and EGF was

manipulated for enhancing induction to PE progenitors. Expression tendency of pancreatic endoderm, primitive gut and posterior foregut-specialized markers (*Pdx1*, *Hnf1b, Hnf6, Hnf4a),* MPP-specialized markers (*Ptf1a, Sox9*, and *Nkx6.1*) and PE-specialized markers (*Nkx2.2, Pax4, Ngn3, NeuroD1, Isl1,* and *Glut2*), pre-mature marker (*MafB*), caNotch pathway-related marker (*Hes1),* a cell cycle regulator (*Cdkn1a*) were determined by RT-qPCR after 5 days and 8 days since the first day of DE induction.

Small molecules such as Glp-1 agonists, nicotinamide, taurine, forskolin, PI3K inhibitor, and TGF-β inhibitor have been demonstrated to make positive influences in the functional maturation of IPCs (Hori et al., 2002; Kunisada et al., 2012; Ohmine et al., 2012; Lee et al., 2020). Accordingly, a combination of all these reagents were utilized as an improved media to specify PE clusters into IPCs whilst a mixture only comprising Glp-1 agonists, NIC, taurine was considered as a control medium. Expression of pancreatic multipotent, pancreatic endoderm, and PE-specialized markers (*Nkx6.1*, *Pdx1, Ngn3, NeuroD1, Isl1,* and *Glut2)*, mature endocrine-specialized markers (*Glis3, Glp1r, MafA, Insulin,* and *Glucagon*), and hormone release-related markers (*Epac2*, and *Rfx6*) were measured by RT-qPCR after 13 days since the first day of DE induction.

Strategy 1c) Efficiency determination of modified cAD-MSC-derived IPC production protocol *in vitro*

To assess the maturation and functional capacity of cAD-MSC-derived IPCs, glucose-stimulated C-peptide secretion (GSCS) assay was done (Sawangmake et al., 2014a), C-peptide production will be investigated by enzyme-linked immunosorbent assay (ELISA). In addition, the expression of intracellular insulin was evaluated by immunocytochemistry (ICC) staining.

Specific aim 2: To study the enriched culture media for preserving the double-layer encapsulated cAD-MSC derived IPCs

Strategy 2a) Application of encapsulation technique

As described in the previous report, alginate/pluronic F127-based encapsulation can be applied for IPCs (Kuncorojakti et al., 2020). In this regard, cAD-MSC-derived PE

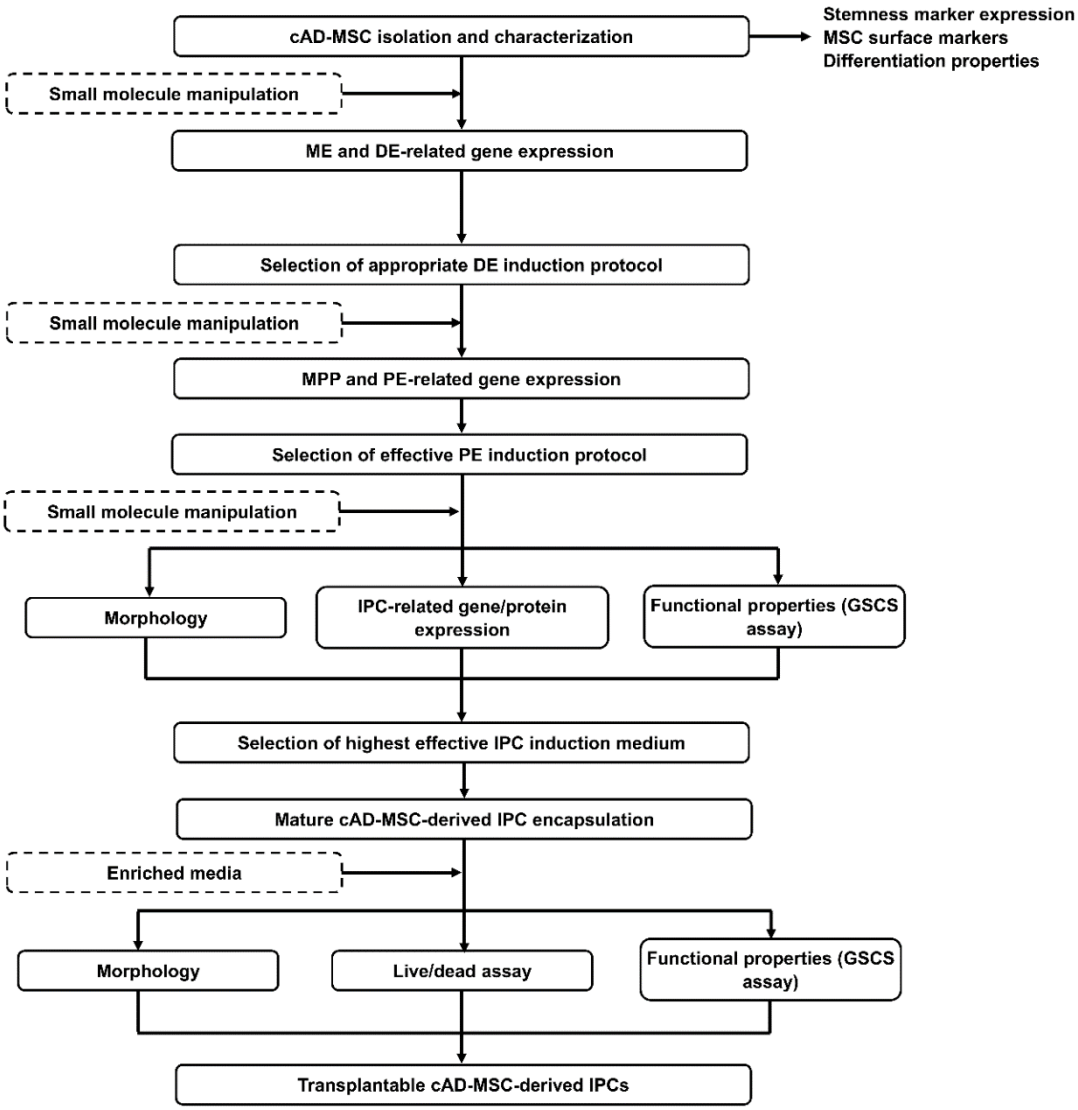
at day 8 was wrapped in alginate beads upon alginate gelling formation in a 10mM $CaCl_2$ solution. cAD-MCS-derived IPC- contained alginate beads were covered with a layer of pluronic F127, these beads were put in pluronic acid solution at 4°C and then polymerization would occur at room temperature (RT). Then, double-encapsulated PE colonies were induced to mature IPCs and then were culture to maintain their function and viability within 3 different media.

Strategy 2b) Efficiency determination of enriched media on ALGPA-encapsulated cAD-MSC-derived IPCs' function and viability

To evaluate the efficiency of the enriched media for preserving the encapsulated cAD-MSC-derived IPCs, functional and viability assay were carried out during 2 weeks after the IPC induction protocol while cAD-MSC derived IPCs were used as a control. C-peptide production from the encapsulated cAD-MSC-derived IPCs was determined by ELISA after GSCS assay was done. To appraise cell viability, live/dead staining was performed before the results were observed under fluorescent microscope. Moreover, expression of pancreatic multipotent, pancreatic endoderm, and PE-specialized markers (*Nkx6.1*, *Pdx1, Ngn3, NeuroD1, Isl1,* and *Glut2)*, mature endocrine-specialized markers (*Glis3, Glp1r, MafA, Insulin,* and *Glucagon*), hormone release-related markers (*Epac2*, and *Rfx6*), proliferation (*Ki67*) and cell cycle regulator (*Cdkn1a*) mRNA markers were measured by RT-qPCR after 27 days since the first day of DE induction.

Briefly, the experiments in this study are described as the following flowchart.

Experimental plan



Detailed material and methods

Cell isolation, culture, and expansion

The biopsied adipose tissues were collected and kept in 10 mL of Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific Corporation, USA) from various species of dog at Department of Surgery, Small Animal Hospital, Chulalongkorn University. These samples were washed with Phosphate buffered saline (PBS) (Thermo Fisher Scientific) supplemented with 2% Antibiotics-Antimycotics (Thermo Fisher Scientific) twice. The tissues were minced into small species and then incubated in cell recovery solution (Corning, USA) overnight at 37°C in a humidified atmosphere with 5% CO_2. The mixture was filtered through 70 µm cell strainer (Corning) before centrifuging at 2000 g for 5 minutes. Cells were culture in DMEM supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific), 1% GlutaMax (Thermo Fisher Scientific), and 1% Antibiotics-Antimycotics (Thermo Fisher Scientific) at 37°C in humidified air with 5% CO_2. Culture medium was changed every 2 days. When cells reached 70-80% confluence, they were trypsinized with 0.25% trypsin-EDTA (Thermo Fisher Scientific) for subculture and freezing. Cells in passage 2-5 were utilized for the experiments.

Flow cytometry

MSC-related surface markers were analyzed by flow cytometry. In particular, the cells were stained with FITC-conjugated mouse anti-human CD45 antibody (BioLegend, USA), mouse anti-human CD73 monoclonal antibody (Thermo Fisher Scientific) and FITC-conjugated goat anti-mouse immunoglobulin G (IgG) secondary antibody (BioRad, USA), PE-conjugated rat anti-dog CD90 monoclonal antibody (eBioscience, USA), Alexa Fluor 488-conjugated rat anti-dog CD44 antibody (BioRad), PE-conjugated mouse anti-human CD29 monoclonal antibody (Bio Legend). FITC-conjugated mouse IgG1 kappa Isotype (BioLegend), mouse IgG2a kappa Isotype (Thermo Fisher Scientific), PE-conjugated rat IgG2b kappa Isotype (eBioscience), Alexa Fluor 488-conjugated rat IgG2a

Isotype (BioRad), PE-conjugated mouse IgG1 kappa Isotype (BioLegend) were used as isotype controls. The results were analyzed using a FACScalibur flow cytometer with CellQuest software (BD Bioscience, USA).

In vitro Multi-lineage differentiation

For adipogenic differentiation, 5x 10^4 cells were seeded in a 24-well culture plate and cultured in adipogenic differentiation medium for 28 days. The adipogenic differentiation medium was be contained with 0.1 mg/ml insulin (Sigma-Aldrich, USA), 1μM dexamethasone, 1 mM 1-Methyl-3-isobutylxanthine (IBMX) (Sigma-Aldrich), and 0.2 mM indomethacin (Sigma-Aldrich) in the growth medium (Sawangmake et al., 2014b; Nantavisai et al., 2020).

For osteogenic differentiation, the protocol was carried out following our previous reports (Sawangmake et al., 2014a; Nantavisai et al., 2020). cAD-MSCs were seeded into a 24-well culture plate (Corning) at concentration of 2.5x10^5 cells/well and induced to osteogenic cells for 14 days. Basal medium was used for preparing osteogenic induction medium by supplemented with 50 mg/mL ascorbic acid, 100 mM dexamethasone (Sigma-Aldrich, USA), and 10 mM β-glycerophosphate (Sigma-Aldrich).

According to our previous chondrogenic differentiation protocol (Sawangmake et al., 2014b; Nantavisai et al., 2020), 50,000 cells were divided into each well of a 24-well culture plate and differentiated into chondrogenic cells. The induction medium is basal medium consisting of 0.1 μM dexamethasone, 50 mg/ml 2-dihydrogen phosphate-L ascorbic acid (AA2P) (Sigma-Aldrich), 40 mg/ml S-Pyrrolidine-2-carboxylic acid (Sigma-Aldrich), 1% insulin-transferrin-selenium (ITS) (Thermo Fisher Scientific), 15% FBS, 10 ng/ml of transforming growth factor (TGF)-β3 (Sigma-Aldrich), and 2% antibiotics-antimycotics for 21 days.

Alizarin Red S staining

After osteogenic induction, the cells will be stained with a 0.5 % Alizarin Red S solution (Sigma-Aldrich) for 30 minutes at room temperature (RT) after washed with

PBS solution and fixed in 10% paraformaldehyde for 15 minutes (Sawangmake et al., 2016). Non-stained dye will be washed out with distilled water. The images will be taken by an invert microscope.

Oil Red O staining

After 28 days of adipogenic induction, the cells were washed with PBS and fixed in 10% paraformaldehyde for 30 minutes at RT. The cells were rinsed with 60% isopropanol for 2-5 minutes and then incubated with Oil Red O working solution for 10 minutes (Eom et al., 2018). Finally, the cells were washed with distilled water several times and the images were obtained under the light microscope.

Alcian blue staining

The chondrogenic differentiated cells were washed with PBS and then fixed with a cool 10% paraformaldehyde for 15 minutes at RT. Subsequently, they were incubated overnight with 1% alcian blue solution (pH 2.5) and then rinsed with 0.1N HCl for 3 times followed by PBS (Voga et al., 2019). The images were taken by an inverted microscope.

DE induction

When cAD-MSC reached 70-80% confluence, they were collected by trypsinization. Concentration of 10^6 cells were used for each DE induction condition. Two protocols were applied.

Protocol 1.1 (P.1.1): cells were induced in serum free media (SFM)-DMEM containing 1X ITS, 50 µM β-mercaptoethanol (Sigma-Aldrich), 1 mM sodium butyrate (Sigma-Aldrich), 1% bovine serum albumin (BSA) (Sigma-Aldrich), and 4 nM Activin A (Sigma-Aldrich) for 3 days (Sawangmake et al., 2014a).

Protocol 1.2 (P.1.2): cells were induced in SFM-DMEM supplemented 1X ITS, 50 µM β-mercaptoethanol, 1 mM sodium butyrate, 1% BSA, and 3 µM Chir99021 (Sigma-Aldrich) for 1 day. After that, the medium was changed to protocol 1.1 medium and maintained for next 2 days (Naujok et al., 2014).

The induced cells were collected on day 1 and day 3 to evaluate related mRNA markers. Morphology of colonies, size and total number of colonies were assessed on day 3 of post-induction.

PE induction

After choosing the best potential DE induction protocol, the DE cells from those protocols were induced into PE with three different protocols.

Protocol 2.1 (P.2.1): DE cells were differentiated in SFM-DMEM and added 1% BSA, 1X ITS, and 0.3 mM taurine (Sigma-Aldrich) for 5 days.

Protocol 2.2 (P.2.2): DE cells were differentiated in SFM-DMEM added 1% BSA, 1X ITS and 0.3 mM taurine, 20 ng/ml FGF2 (Sigma-Aldrich), and 2 μM RA (Sigma-Aldrich) for 2 days, and then medium was changed to SFM-DMEM added 1% BSA, 1X ITS and 0.3 mM taurine, 2 μM RA, 10 mM NIC (Sigma-Aldrich), 1 μM dorsomorphin (Sigma-Aldrich), 10 μM SB431542 (Sigma-Aldrich), 25 μM DAPT (Sigma-Aldrich), and 50 ng/ml EGF (Sigma-Aldrich) for 3 days.

Protocol 2.3 (P.2.3): DE cells were differentiated in SFM-DMEM added 1% BSA, 1X ITS and 0.3 mM taurine, 20 ng/ml FGF2, 2 μM RA, and 50 ng/ml EGF for 2 days, and then medium was changed to SFM-DMEM added 1% BSA, 1X ITS and 0.3 mM taurine, 2 μM RA, 10 mM NIC, 1 μM dorsomorphin, 10 μM SB431542, and 25 μM DAPT for 3 days.

Related mRNA markers, morphology of colonies, size and number of colonies were assessed on day 5 and day 8 since the first day of DE induction.

IPC induction

When PE induction protocol was verified, PE cells were continually differentiated toward IPCs after they were encapsulated with 2% alginate (Sigma-Aldrich). Two different media were utilized to induce PE cells to IPCs for 5 days.

Protocol 3.1 (P.3.1): SFM-DMEM supplemented 1.5% BSA, 1X ITS, 3 mM taurine, 100 nM glucagon-like peptide-1 (Glp-1) (Sigma-Aldrich), and 1 mM NIC, and 1X non-essential amino acids (NEAA) (Sigma-Aldrich).

Protocol 3.2 (P.3.2): SFM-DMEM supplemented 1.5% BSA, 1X ITS, 3 mM taurine, 100 nM Glp-1, 1 mM NIC, 1X NEAA, 10 μM Forskolin, 10 μM SB431542, and 10 μM LY294002 (Sigma-Aldrich).

Related mRNA markers, morphology of colonies, size and number of colonies along with IPCs' function were measured on day 13 since the first day of DE induction.

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

The total cellular mRNA was extracted by TRIzol™ reagent (Thermo Fisher Scientific) and Direct-Zol RNA Miniprep kit (ZymoResearch, USA) according to the manufacturer's protocol. Then, the complementary DNA (cDNA) was collected upon reverse transcription polymerase chain reaction (RT-PCR) of collected mRNA using ImProm-II™ Reverse Transcription System (Promega, USA). For quantitative polymerase chain reaction (qPCR), targeted genes were amplified and detected by FastStart Essential DNA Green Master (Roche Diagnostics, USA) and CFX96 Touch Real-Time PCR detection system (Bio-Rad, USA) with the specific primers which show in Table 1. The expression of mRNA was demonstrated as relative mRNA expression by normalizing with glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) as the reference gene.

Table 1. ***Primers sequences***

Gene	Accession number	Sequences	5'-3'	Length (bp)	Tm (°C)
Stemness markers					
Oct4	XM_538830.1	Forward	AGGAGAAGCTGGAGCAAAACC	100	60.55
		Reverse	GTGATCCTCTTCTGCTTCAGGA		59.50
Rex1	XM_003639567.1	Forward	AGGTTCTCACAGCAAGCTCA	199	59.24
		Reverse	CCAGCAAATTCTGCGCACTG		60.73
Proliferation marker					
Ki67	XM_014108788.1	Forward	GTGCAACTAAAGCACGGAGA	124	58.49
		Reverse	GAGATTCCTGTTTGCGTTTTCGT		58.49
Cell cycle regulator					
Cdkn1a	XM_532125.6	Forward	GACTTTCCCCACTGCCCTAC	108	60.04
		Reverse	GCCCTATCCACAGCGTCTAC		59.97
Osteogenic markers					
Runx2	XM_005642335.1	Forward	GGAAGAGGCAAGAGTTTCACC	209	58.84
		Reverse	GTGCTCACTTGCCAACAGAA		58.89
OCN	XM_547536.4	Forward	GCCAGCCTATGGTCTCCTCTG	249	61.90
		Reverse	CCACCAGCTCCTTCTGTTCTCT		54.55
Adipogenic makers					
Leptin	NM_001003070.1	Forward	TGTGGCTTTGGCCCTATCTG	147	60.03
		Reverse	CAGCGACCCTCTGTTTGGAG		60.67
LPL	XM_005635734.3	Forward	CTGGAGAGACTCAGAAAAAGGTAAT	148	58.29
		Reverse	TCCTTCTGTAGATTTGCTCAGGT		59.16
Chondrogenic markers					
Sox2	XM_005639752.2	Forward	AGAAGGATAAGTACACGCTGCC	130	60.16
		Reverse	TTCATGTGCGCGTAGCTGTC		61.35
Col2a1l	NM_001006951.1	Forward	ATGAAAGACTGCCTCAGCCC	103	60.03
		Reverse	TCTGTCCCTTTGGTCCTGGT		60.40
Mesendodermal markers					
Mixl1	XM_022417967.1	Forward	ACCCCTCAGTCCCCTGATTT	120	60.18
		Reverse	TCAGGGAGCTGGTGGTATGA		59.96
Eomes	XM_005634337.3	Forward	CAGTCGAGAAGGGCAGAAAG	161	58.28
		Reverse	GGAGGTTGACCGAAAAAGCA		58.69
GSC	XM_005623927.2	Forward	TGGAACAAGACCTCGTCCAAG	135	59.93

		Reverse	TCTTTGTCCCCTGACCATCG		59.39
Definitive endoderm markers					
Gata4	NM_001048112.1	Forward	TCCCCACAAGGCTACACATC	112	59.38
		Reverse	ATGCAGTGATTATGTCCCCGT		59.51
Gata6	XM_022421725.1	Forward	TACAGCAAGATGAACGGCCT	140	59.39
		Reverse	GGTTCACCCTCCGCATTTCT		60.32
Cxcr4	NM_001048026.1	Forward	GTTGAGGCTGTGGCAAACTG	186	59.97
		Reverse	GTAGACCACCTTTTCCGCCA		59.96
Sox17	XM_544084.5	Forward	GGGGGAGAAAAATGTGGGGT	75	59.88
		Reverse	GGGTTCTGACAACGCAACTG		59.69
Pancreatic endoderm marker					
Pdx1	NM_001284471.2	Forward	AAGTCTACCAAGGCTCACGC	201	60.04
		Reverse	GTGCCTCTCGGTCAAGTTCA		59.97
Primitive gut and posterior foregut markers					
Hnf1b	XM_005624790.3	Forward	TGCTATTGAACTGAGCCACACA	170	60.22
		Reverse	AAGGGGAATGGAGGGGCAAT		61.23
Hnf6	XM_846134.5	Forward	CCCCAAACCCTGGAGCAAAC	108	61.47
		Reverse	TTGGGTGTGTTGCCTCTATCC		60.00
Hnf4a	XM_022408885.1	Forward	GCGACCTGCTGTTTGATGGT	105	61.24
		Reverse	GGAGGCGTGATAGCCAGATAG		59.80
Multipotent pancreatic progenitor markers					
Ptf1a	XM_845293.4	Forward	GAACAGCCAAAGTGTGGACC	90	59.33
		Reverse	ACTCAAAGGGCGGTTCGTTT		60.46
Sox9	NM_001002978	Forward	CAGCACAAGAAAGACCACCC	107	59.05
		Reverse	GAAATGTGCGTCTGTTCGGT		59.13
Nkx6.1	XM_544960.5	Forward	CAGGAGTTATGCAGAGCCCG	111	60.53
		Reverse	ACGTGGGTCTCGTGTGTTTT		60.11
caNotch pathway-related gene					
Hes1	XM_025478075.1	Forward	GAGAAGGCGGACATTCTGGA	137	59.46
		Reverse	ACCTCGTTCATACACTCGCTG		60.14
Pancreatic endocrine markers					
Nkx2.2	XM_542867.6	Forward	GACACCAACGACGAGGAAGG	169	60.67
		Reverse	AGCGCGTATATGGGTTGTCG		60.60
Pax4	XM_022427277.1	Forward	GCCCCTTAGACCTTGCTCTG	114	60.11
		Reverse	CTGCTTGGGCAGGATTAGGT		59.74
Ngn3	XM_546140.1	Forward	TCTGAGCAAGCAGCGACG	177	60.43

		Reverse	AGCGCCCAGATGTAGTTGTG		60.39
NeuroD1	XM_005640377.3	Forward	AGTTCGAGAAGAGCTACGCC	155	59.55
		Reverse	CTCGTGATGCGAATGGCTCT		60.53
Isl1	XM_848628.4	Forward	TGGCTTACAGGCAAACCCAG	171	60.54
		Reverse	GACATCGACGCCACTTCACT		60.39
Glut2	XM_545289.5	Forward	ACTCATCACAGGACGTGGAG	108	59.11
		Reverse	AGCTGAGTGTAGCGGTGAAG		59.76
Pre-mature endocrine marker					
MafB	XM_005635059	Forward	TGCCCAGTGGTGACCTAAAC	105	59.89
		Reverse	AAGGACGCGCTTGAAAGTTG		59.69
Mature pancreatic endocrine markers					
Glp1r	XM_014118246.1	Forward	CACGGTGGGCTATACACTCTC	116	59.93
		Reverse	AGGACGCAAACAGGTTCAGG		60.54
MafA	XM_003431814.3	Forward	GCTTCAGCAAGGAGGAGGTC	136	60.39
		Reverse	CTCTGGAGCTGGCACTTCTC		60.11
Glis3	XM_022421745.1	Forward	CAGGTCTTGTAGGCCTTGGG	109	60.04
		Reverse	GCTGGTCGTGGACATCAAAC		59.48
Insulin	NM_001130093.1	Forward	CGGCTTCTTCTACACGCCTA	202	59.55
		Reverse	GCGCCCCTAGTTGCAGTAAT		60.46
Glucagon	NM_001003044.1	Forward	TCCAATCGCGGTGTCAGAAG	197	60.39
		Reverse	ACCCTGAGAATGACGCTTGT		59.31
Hormone release-related markers					
Rfx6	XM_022419390.1	Forward	TGTTTAGGGCACAGCCTCAC	154	60.25
		Reverse	TAGCTGGAAGGTGGTCGAGA		59.96
Epac2	XM_014110689.1	Forward	GAACGATCCAGTGAAGATGTGG	174	59.07
		Reverse	GCCAGGACAGCATACCAGTT		60.04
Reference marker					
Gapdh	NM_001003142.1	Forward	CCAACTGCTTGGCTCCTCTA	100	59.38
		Reverse	GTCTTCTGGGTGGCAGTGAT		59.67

Immunocytochemistry

IPC colonies were fixed in cold methanol for 15 min at RT, then permeabilized with 0.1% Triton-X100 (Sigma) in PBS. After that, the background was blocked with 10% donkey serum on PBS for 1 hour. The primary antibodies, rabbit anti-insulin (Cell

signature technology, USA) at dilution 1:200 and mouse anti-rat glucagon (Abcam, USA) at dilution 1:200, were added and incubated overnight. Then, cyanine (Cy) 3-conjugated donkey anti-rabbit IgG (Bio Legend) and FITC-conjugated goat anti-mouse IgG (Bio-Rad) were used as secondary antibodies, respectively. After incubation with secondary antibodies for 2 hours, DAPI was used to stain the nucleus. The results and images were acquired using a fluorescent microscope equipped with Carl ZeissTM Apoptom.2 apparatus (Carl Zeiss, Germany).

Encapsulation

The protocol of colony encapsulation was followed by a previous report (Kuncorojakti et al., 2020), cAD-MSC-derived colonies were harvested and resuspended in 2% alginate solution. Sterile polystyrene syringe and 22G needle were used to generate alginate beads. Drops of beads were collected in 100mM $CaCl_2$ (Sigma-Aldrich) under stirring condition, and then washed by Krebs-Ringer- Hepes (KRH) containing $CaCl_2$ buffer. For double-layer encapsulation, a cold solution of 30% pluronic F127 was added to cover all surfaces of alginate beads at RT.

Maintenance of the alginate/Pluronic acid-encapsulated IPC's function

Subsequently of IPC induction, three different media were applied to consider the ability of the IPCs' function and viability *in vitro*. Medium 4.1 (M.4.1) is basic DMEM. Medium 4.2 (M.4.2) is an IPC induction medium (P.3.2) and 10 μM LY294002. Medium 4.3 (M.4.3) is VSCBIC-1 which was prepared following our previous study (Soedarmanto, 2020).

Live/dead staining

Encapsulated cAD-MSC-derived IPCs were evaluated for their viability using the NUCLEAR-ID Blue/Red cell viability reagent (GFP-CERTIFIED) (Enzo Life Science, USA), according to the manufacture protocol. The result was clarified under fluorescent microscopy (Carl Zeiss).

Functional test for IPCs

Generated IPCs or encapsulated IPCs were assessed by glucose-stimulated C-peptide secretion assay (GSCS). The IPCs were incubated in normal KRH bicarbonate (KRBH) adjusted pH 7.4 (120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.1 mM $MgCl_2$ and 25 mM $NaHCO_3$) for 1 hour as basal control, then in 5.55 mM of glucose anhydrous (Sigma-Aldrich) in KRBH for the next 1 hour and finally in 22 mM glucose anhydrous in KRBH for 1 hour. Enzyme-linked immunosorbent assay (ELISA) kit (Millipore) was used to detect the amount of generated C-peptide level based on manufacturing protocol. Total DNA (ng) and stimulation time (mins) were exercised to normalize the concentration of C-peptide.

Statistical analyses

The total number of colonies and their size were determined using ImageJ software. The results were illustrated by dot plot (n=4) using GraphPad Prism 9.0 (Graph Software Inc., San Diego, CA). SPSS statistics was utilized to analyze the results. Mann-Whitney U test was used to compare significant differences for two experiment groups while Kruskal-Wallis was employed to test for three or more experiment groups. Statistically significant difference is recognized if p-value <0.05.

CHAPTER IV

RESULTS

Isolation and characterization of cAD-MSC

cAD-MSCs showed adherent-dependent and fibroblast-like cells on the 2 dimensions (2D) culture (Figure 2A). The mRNA expression of stemness markers (*Oct4* and *Rex1*) and proliferative marker (*Ki67*) were detected by RT-qPCR (Figure 2B). In addition, MSC-related surface markers using flow cytometry revealed the strong expression of CD29 and CD90, moderate expression of CD44, low expression of CD73, but absent expression of CD45 (Figure 2C).

Moreover, *in vitro* multi-differentiation potential toward adipogenic, osteogenic, and chondrogenic lineages was observed. cAD-MSCs dramatically upregulated adipogenic-related genes (*Leptin* and *LPL*) while the production of lipid droplets was detected by Oil Red O staining (Figure 2D). Regarding osteogenic differentiation, the substantial upregulation of *Runx2* and *Ocn* was indicated upon exposed cells in osteogenic induction medium and osteocyte-produced calcium deposits were recognized by Alizarin Red S staining (Figure 2E). For chondrogenic differentiation, the upregulation of the *Col2a1* gene was significantly disclosed, and glycosaminoglycan accumulation was stained with Alcian blue (Figure 2F).

Thus, the isolated cAD-MSCs show homogeneous appearance and the differentiation potential toward other cell lineages.

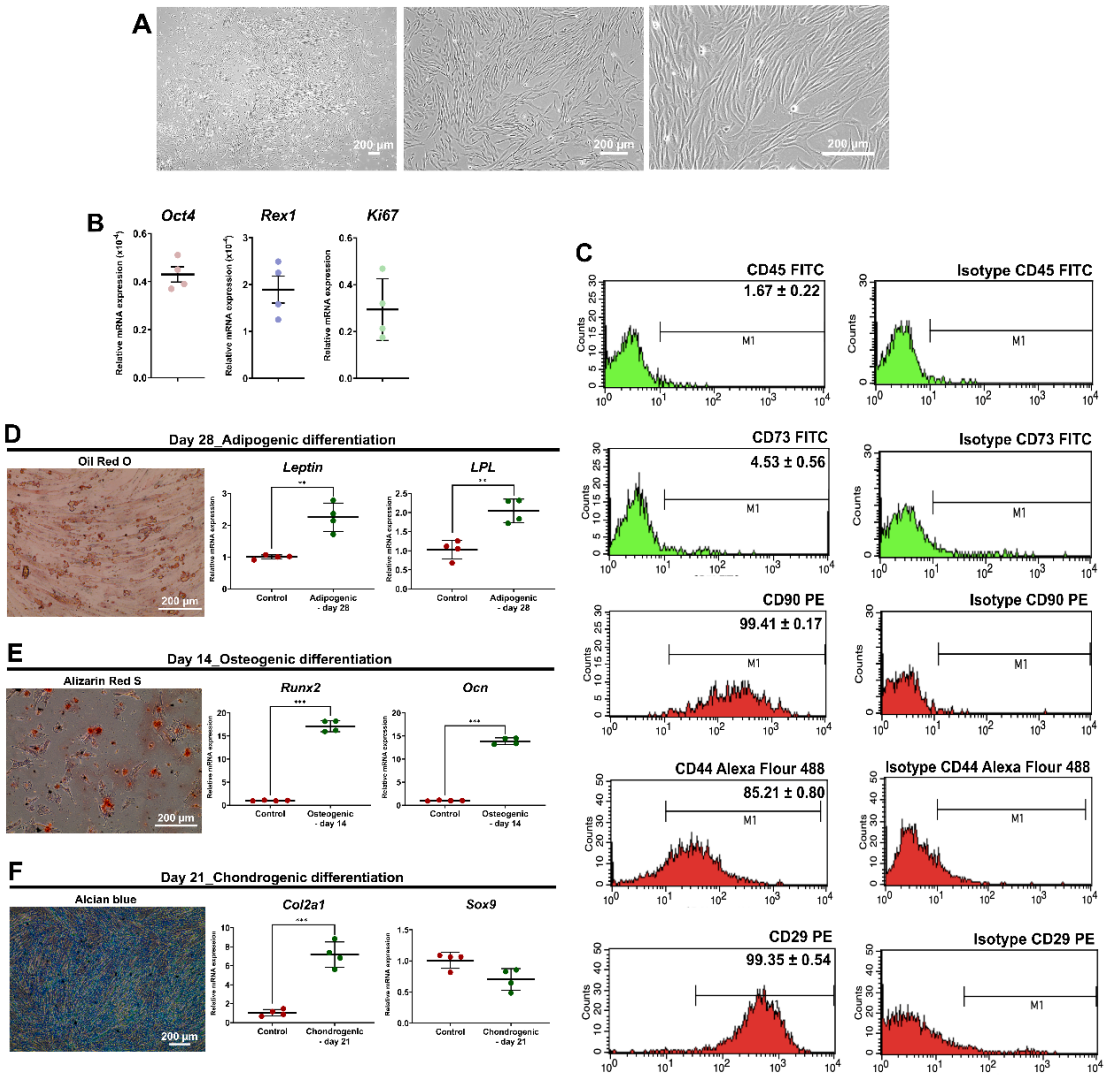


Figure 2 **cAD-MSC characterization**

Morphological appearances of cAD-MSCs were observed under a light microscope with magnification of 4X, 20X, and 40X (**A**). The mRNA expression of stemness markers (*Oct4*, and *Rex1*) and a proliferation marker (*Ki67*) were analyzed by RT-qPCR (**B**), normalized with the reference gene (*Gapdh*). Expression of surface markers exhibiting MSC property were revealed using flow cytometry (**C**). Adipogenic differentiation potential at day 28 post-induction was stained with Oil Red O, and adipogenic related-mRNA expression was determined (**D**). Osteogenesis was confirmed using Alizarin Red S staining at day 14 post-induction, and the expression of osteogenic mRNA markers were assessed (**E**). Chondrogenic differentiation potential at day 21 post-induction was demonstrated by Alcian blue staining, and chondrogenic mRNA markers were determined (**F**). The expression of mRNA genes related to multilineage differentiation was normalized with the reference gene and the undifferentiation control. Bars indicates the significant differences (*, *p* value <0.05).

Differentiation of cAD-MSCs into definitive endoderm (DE)

First, formation of DE, a germ cell layer, is an essential initiation step for giving rise to pancreatic cells (Kubo et al., 2004; Shim et al., 2007). DE were generated from cAD-MSCs using DE induction media supplemented with Activin A alone for 72 hours (P.1.1) or Chir99021 for first 24 hours and following with Activin A for 48 hours (P.1.2) (Figure 3A). Small three-dimension (3D) clusters were formed from dissociated cAD-MSCs cultured in suspension for 24 hours, then size and density of colonies were increased (Figure 3B). On day 3, the total colony counts (medium) were 1610.75 and 1637.25 colonies per batch ($1x10^6$ seeding cells) in P.1.1 and P.1.2 groups, respectively (Figure 3C). Interestingly, the distribution of size-based colonies showed that the small-to-medium colony size (100-299 μm) occupied the most population in both protocols, and P.1.2 yielded significantly more medium-size colonies (300-499 μm) than P.1.1 (Figure 3D).

Levels of mRNA expression related to mesendoderm (ME) and DE were analyzed and compared to undifferentiated cells. The ME-related markers (*Eomes* and *GSC*) in P.1.2 showed the upregulated expression in a time-dependent manner (Figure 3E). In contrast, the expression of *Mixl1* was upregulated in P.1.1, while P.1.2 was downregulated. For the DE-related markers, both protocols upregulated all genes comparing with undifferentiated control (Figure 3F). However, P.1.2 was able to increase the expression of *Gata4*, *Gata6*, and *Sox17* on the last day of DE induction.

Generally, the result revealed that the cooperation of Chir99021 and Activin A is effectively on the generation of DE from cAD-MSCs with small-size and the crucial DE-related markers.

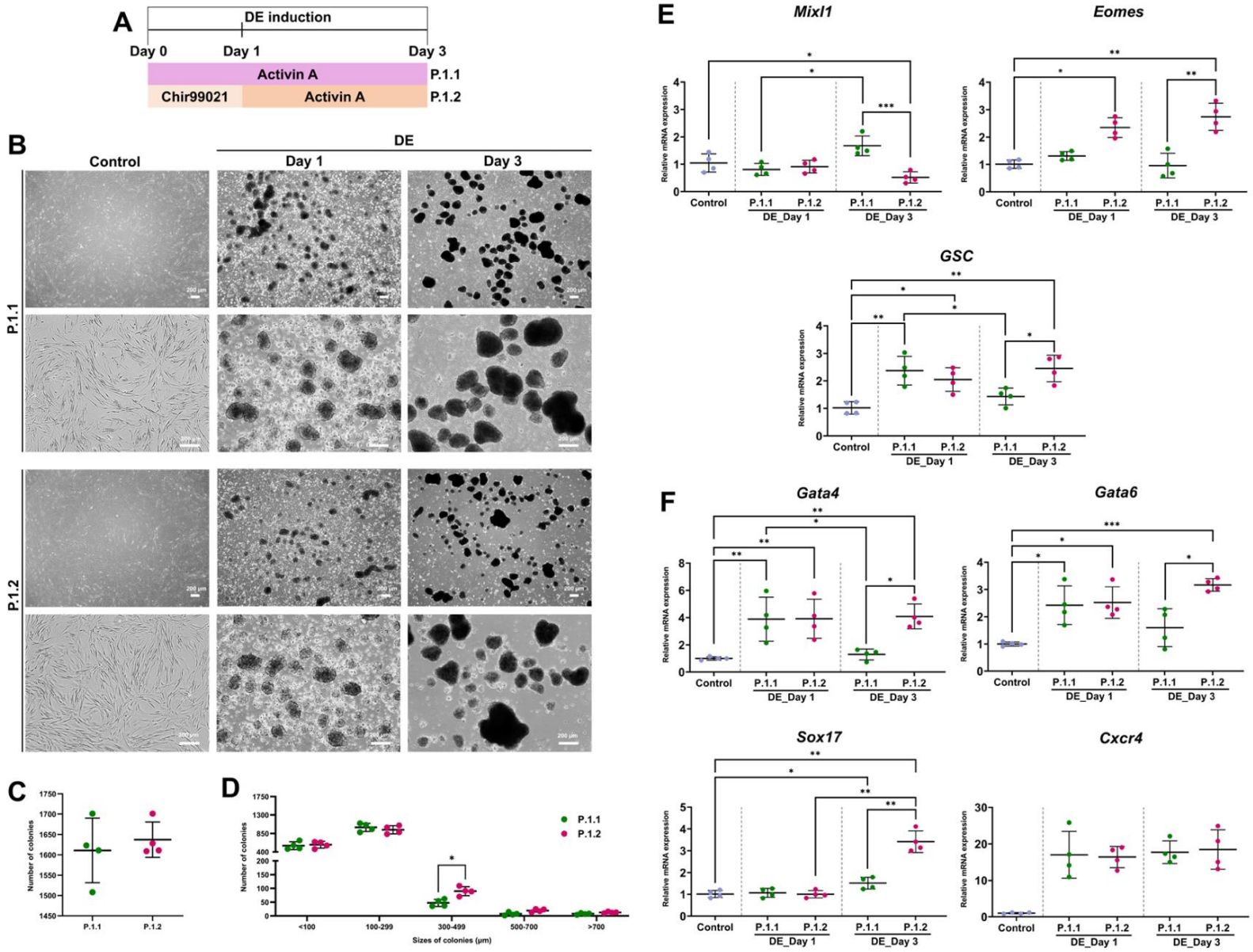

A
DE induction
Day 0
Day 1
Day 3
Activin A
P.1.1
Chir99021
Activin A
P.1.2
B
Control
DE
Day 1
Day 3
P.1.1
P.1.2
C
Number of colonies
P.1.1
P.1.2
D
Number of colonies
P.1.1
P.1.2
<100
100-299
300-499
500-700
>700
Sizes of colonies (μm)
E
Mixl1
Eomes
GSC
Relative mRNA expression
Control
P.1.1
P.1.2
DE_Day 1
DE_Day 3
F
Gata4
Gata6
Sox17
Cxcr4

Figure 3 **Generation of cAD-MSC-derived DE cells**

The diagrams of two protocols used for the induction of cAD-MSC derived DE cells are shown in (**A**). Differentiation of morphological appearances of cAD-MSCs toward DE cells was observed at day 1, and 3 (**B**). The total colony number (**C**) and the distribution of colony sizes (**D**) were evaluated. The expression of mRNA markers relating to mesendoderm status (**E**), and definitive endoderm status (**F**) were analyzed by RT-qPCR at day 1, and 3 post induction. Relative mRNA expression was normalized with the reference gene, and the undifferentiation control. Bars indicates the significant differences (*, *p* value <0.05; **, *p* value <0.01; ***, *p* value <0.001).

Differentiation of cAD-MSC derived DE into pancreatic endocrine (PE) precursors

Pancreatic endocrine (PE) stage is the vital second stage on pancreatic development process according to its important roles in cell fate modulation on DE cell toward pancreatic cell types (Arda et al., 2013; Aigha and Abdelalim, 2020). In this part, three established protocols were explored; P.2.1, P.2.2, and P.2.3 (Figure 4A). All protocols exposed the 3D floating colonies which became bigger and denser along the culture period (Figure 4B). All protocols showed the similar trend of total colony count between each protocol group, however, there were gently decreasing by the induction day (Figure 4C). For the sized-based colony number, the number of colonies was mostly found in 100-299 μm, nevertheless, the large-size colonies were slightly increasing during the induction period (Figure 4D).

Except for the *Glut2* gene, the expression of other mRNA markers relating to the development of PE precursors exhibited significant differences among the protocols. Compared to the undifferentiated cells and DE cells on day 3, the expression of pancreatic endoderm marker (*Pdx1*) was upregulated in groups treated with P.2.2 and P.2.3 on day 5 and day 8, while there was not detected in P.2.1 (Figure 5A). The expression of *Hnf1b*, *Hnf4a*, and *Hnf6*, known as primitive gut and posterior foregut markers, was upregulated on day 5 and day 8 post-induction in P.2.2 and P.2.3 (Figure 5B). The multipotent pancreatic progenitor (MPP) marker (*Ptf1a*) was upregulated on day 5 in three protocols, however, the trend of *Ptf1a* downregulation was found in

P.2.2 and P.2.3 on day 8 (Figure 5C). On other hand, P.2.2 and P.2.3 exhibited the upregulation trend of the other MPP markers (*Sox9* and *Nkx6.1*) on the last day of PE induction (Figure 5C). All of the protocols showed the increased expression of the PE-related mRNA markers such as *Nkx2.2*, *Pax4*, *Ngn3*, *NeuroD1*, and *Isl1* on day 8, compared to undifferentiated cells (Figure 5D). On day 8, it is noticed that the highest expression of *Nkx2.2* and *NeuroD1* was found in P.2.1, whereas the greatest expression of *Ngn3*, a master key for endocrine specification, was found in P.2.3. In addition, the immature pancreatic endocrine marker, *MafB*, was tremendously upregulated in P.2.2 and P.2.3 on day 8 compared to the undifferentiated cells (Figure 5E). Moreover, P.2.2 and P.2.3 displayed the significant downregulation of *Hes1*, Notch target gene, on the last day of PE induction (Figure 5F). Besides, compared with cAD-MSCs, P.2.1 increased the expression of *Cdkn1a*, a cell cycle regulator, on day 8, while the decreased expression was observed in P.2.2 and P.2.3 (Figure 5G).

Regarding all the results, the new combination of signaling modulators supports the PE development. Importantly, the obtained PE cells from P.2.3 showed the greatest PE-related mRNA expression.

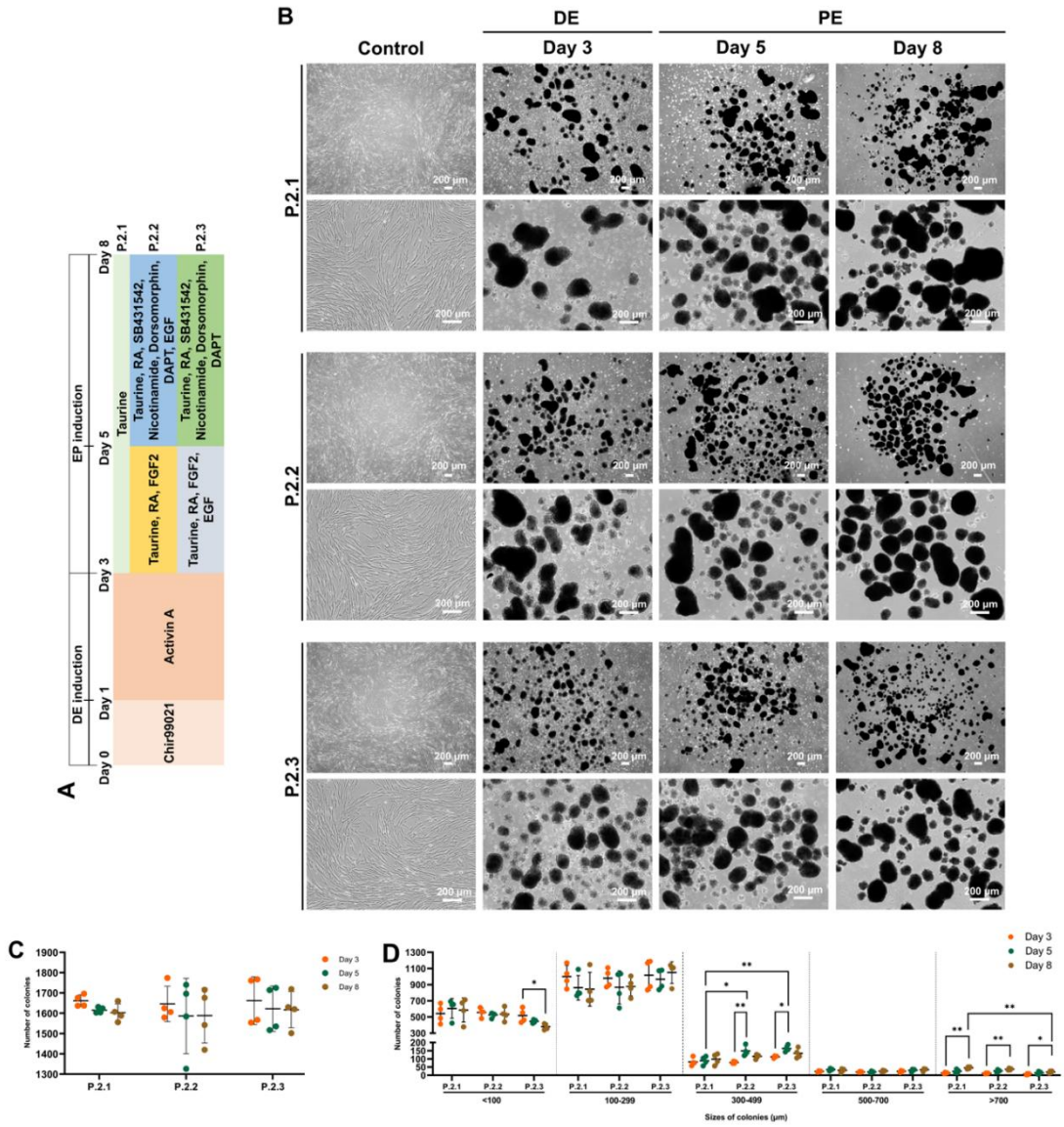


Figure 4 **Generation of cAD-MSC-derived PE cells**

The diagrams of three protocols used for the induction of cAD-MSC-derived PE cells are shown in (**A**). Differentiation of morphological appearances of cAD-MSCs toward PE cells was observed at day 3, 5 and 8 (**B**). The total colony number (**C**) and the distribution of colony sizes (**D**) were evaluated. Bars indicates the significant differences (*, *p* value <0.05; **, *p* value <0.01; ***, *p* value <0.001).

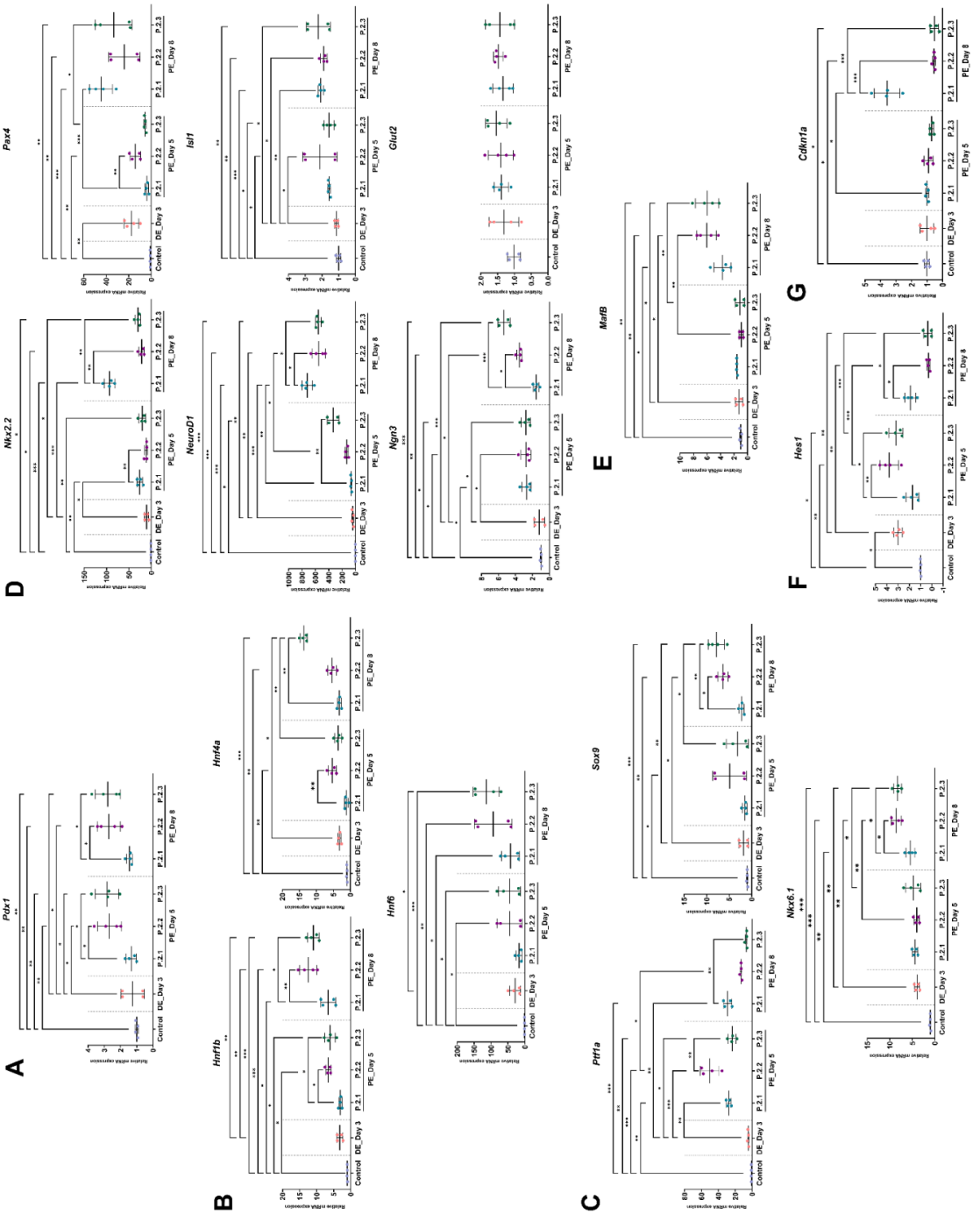

A
Pdx1
B
Hnf1b
Hnf4a
Hnf6
C
Ptf1a
Sox9
Nkx6.1
D
Nkx2.2
Pax4
NeuroD1
Isl1
Ngn3
Glut2
E
MafB
F
Hes1
G
Cdkn1a
Control
DE_Day 3
P-2.1
P-2.2
P-2.3
PE_Day 5
PE_Day 8

Figure 5 **PE-related marker expression**

The expression of mRNA markers relating to pancreatic endoderm (**A**), primitive gut and posterior foregut (**B**), pancreatic multipotent progenitor (**C**), endocrine precursors (**D**), immature pancreatic endocrine (**E**), caNotch pathway target gene (**F**), and a cell cycle regulator (**G**) were analyzed by RT-qPCR at day 3, 5 and 8 post induction. Relative mRNA expression was normalized with the reference gene, and the undifferentiation control. Bars indicates the significant differences (*, *p* value <0.05; **, *p* value <0.01; ***, *p* value <0.001).

Differentiation of cAD-MSC derived PE into insulin-producing cells (IPCs)

Last induction, two cocktail media were established to effectively induce functional IPCs from PE precursors; formulas of P.3.1 and P.3.2 are defined in Figure 6A. PE colonies on day 8 were encapsulated in alginate gels, then the encapsulated PE were induced toward IPCs. Both protocols were still showing a 3D colony morphology during IPC induction period (Figure 6B).

Except for *Nkx6.1* gene, the mRNA expression of PE progenitor-related markers (*Pdx1*, *NeuroD1*, *Isl1*, *Ngn3*, and *Glut2*) was upregulated in P.3.2, compared to undifferentiated cells and PE cells on day 8 (Figure 6C). Conversely, the downregulation trend of *Nkx6.1*, *NeuroD1*, *Isl1* was found in P.3.1 compared to PE cells (Figure 6C). The expression of mature endocrine markers, including *Glis3*, *MafA*, *Insulin*, and *Glucagon*, was upregulated in both of protocols, compared with those from undifferentiation cells and PE cells (Figure 6D). However, the highest expression of mature endocrine markers was explored in P.3.2. Furthermore, P.3.2 showed the upregulation of hormone release-related markers (*Epac2* and *Rfx6*), compared to undifferentiated cells and PE cells (Figure 6E). Although P.3.1 showed the trend of increasing *Rfx6* expression, the downregulation of *Epac2* was found in this protocol (Figure 6E).

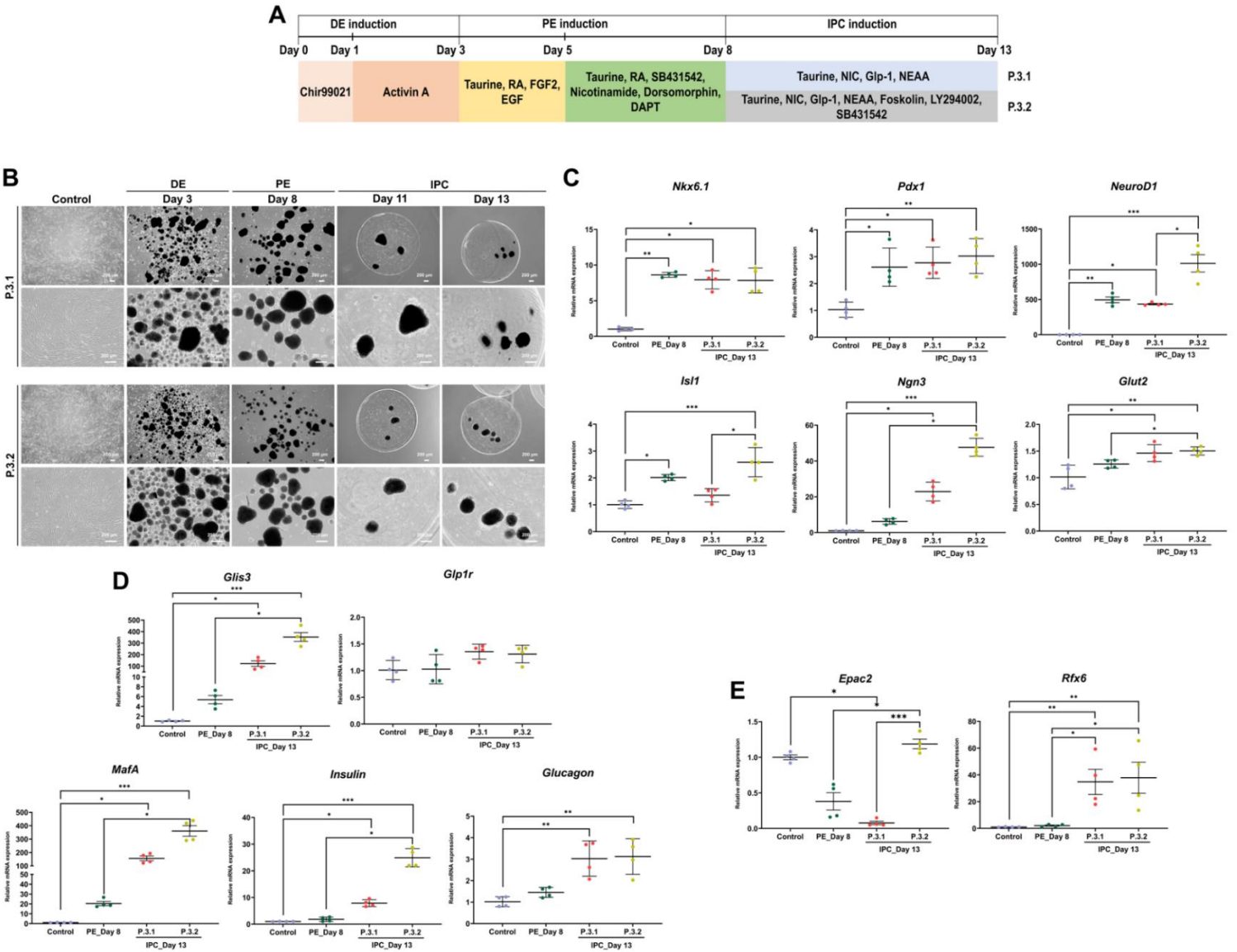

A
DE induction
PE induction
IPC induction
Day 0
Day 1
Day 3
Day 5
Day 8
Day 13
Chir99021
Activin A
Taurine, RA, FGF2, EGF
Taurine, RA, SB431542, Nicotinamide, Dorsomorphin, DAPT
Taurine, NIC, Glp-1, NEAA
Taurine, NIC, Glp-1, NEAA, Foskolin, LY294002, SB431542
P.3.1
P.3.2
B
Control
DE
PE
IPC
Day 3
Day 8
Day 11
Day 13
P.3.1
P.3.2
C
Nkx6.1
Pdx1
NeuroD1
Isl1
Ngn3
Glut2
Relative mRNA expression
Control
PE_Day 8
P.3.1
P.3.2
IPC_Day 13
D
Glis3
Glp1r
MafA
Insulin
Glucagon
E
Epac2
Rfx6

Figure 6 **Generation of cAD-MSC-derived IPCs**

The diagrams of two protocols used for the induction of cAD-MSC derived IPCs are shown in (**A**). Differentiation morphological appearances of cAD-MSCs toward IPCs were observed at day 8 and 13 post induction (**B**). The expression of mRNA markers relating to PE precursors (**C**), mature endocrine progenitors (**D**), hormone release-related markers (**E**). Relative mRNA expression was normalized with the reference gene, and the undifferentiation control. Bars indicates the significant differences (*, *p* value <0.05; **, *p* value <0.01; ***, *p* value <0.001).

At day 13 of IPC induction, cAD-MSC-derived IPCs in both P.3.1 and P.3.2 were further evaluated the functional potential regarding the production of C-peptide upon glucose stimulation at two different concentrations, 5.5 mM and 22 mM. The findings showed that IPCs in both the protocols yield C-peptide under a basal condition, the higher production of C-peptide was found in P.3.2 (Figure 7A). Moreover, IPCs secreted C-peptide in concentration-dependent manner upon high (22 mM) glucose stimulation, compared to those from basal control and P.3.1 (Figure 7A). In addition, immunocytochemistry staining was employed to confirm the expression of the crucial pancreatic islet-related hormones, insulin and glucagon. The result suggested that the expression of these proteins was observed on cAD-MSC-derived IPCs in both protocols on day 13 post induction (Figure 7B).

Taken together, the results suggested that P.3.2 which used forskolin, PI3K inhibitor, and TGF-β inhibitor could have more positive effects on IPCs' maturation from cAD-MSC-derived PE cells.

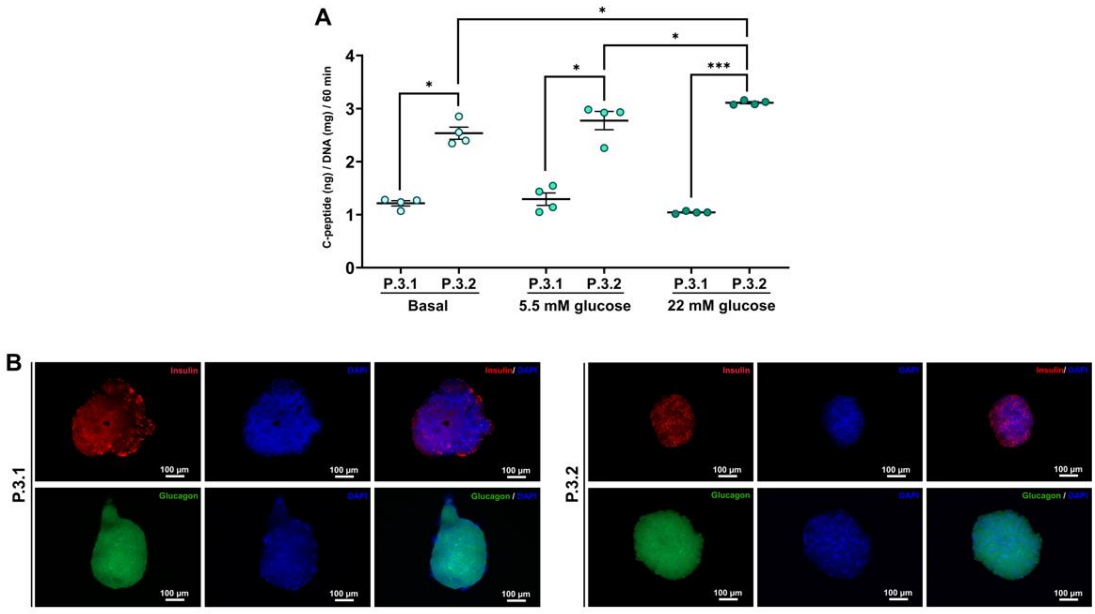


Figure 7 **Functionality of cAD-MSC-derived IPCs**

C-peptide secretion was determined by glucose-stimulated C-peptide secretion (GSCS) assay (**A**). The expression of Insulin and Glucagon by cAD-IPCs was detected by immunocytochemistry on day 13 by immunocytochemistry and observed under fuorescent microscope ZEISS Apotome.2 (Carl Zeiss, Germany) incorporated with Axio Observer Z1 and ZEN pro sofware (ZEISS International, Germany) (**B**). Bars indicates the significant differences (*, *p* value <0.05; **, *p* value <0.01; ***, *p* value <0.001).

Preservation of cAD-MSC derived IPCs encapsulated in pluronic F127/alginate layers

It is essential to generate ready-to-use (RTU) IPCs for in vivo application. Maintenance of ALGPA-encapsulated cAD-MSC-derived IPCs *in vitro* is required. Hence, three different media were chosen to the ALGPA-encapsulated cAD-MSC-derived IPCs *in vitro* after 13 days of IPC induction process (Figure 8A). M.4.1 is normal DMEM, M.4.2 is an IPC induction medium (P.3.2), and M.4.3 is VSCBIC-1, our specialized medium (Soedarmanto, 2020). PE progenitors on day 8 would be double encapsulated in ALGPA instead of only alginate. Morphological changes were observed for 2 weeks after day 13 of IPC induction process (Figure 8B). Damaged IPCs were not found in ALGPA, but the colonies became smaller in all three media. All maintenance media showed the

viability of ALGPA-encapsulated cAD-MSC-derived IPCs until day 27 by live/death staining (Figure 8C). Conversely, the mass of non-viable cells was lower detected in M.4.3, while the signal of dead cells expanded in M.4.1 and M.4.2 along the maintenance period. In addition, cAD-MSC-derived IPCs on day 27 in M.4.2 and M.4.3 exhibited the upregulation trend of PE mRNA markers (*Nkx6.1*, *Pdx1*, *Isl1*, and *Glut2*) compared IPCs from 13 and IPCs from M.4.1 (Figure 9A). Although the decreasing of *Ngn3* and *NeuroD1* was found in all media compared with IPCs on day 13, M.4.3 showed the insignificant decline (Figure 9A). Interestingly, the increasing of *Nkx2.2* was showed in M.4.1 (Figure 9A). In other hand, the upregulation of mature endocrine markers (*Glis3*, *MafA*, *Insulin*, *Glucagon*, and *Glp1r*) and hormone release-related markers (*Epac2*, and *Rfx6*) was explored in M.4.2 and M.4.3 compared with undifferentiated cells, IPCs from day 13, and IPCs from M.4.1 (Figure 9B,C). Although the proliferation marker, *Ki67*, of IPCs was lower expressed in M.4.2 and M.4.3 than M.4.1, the upregulation of a cell cycle regulator, *Cdkn1a,* was found in M.4.2 and M.4.3 groups (Figure 9D,E).

The functional property was evaluated by the level of C-peptide secretion (Figure 9F). The result showed the trend of concentration-dependent response upon 22 mM glucose stimulation in M.4.2 and M.4.3 groups, while M.4.1 exhibited the releasing of C-peptide level response to 5.5 mM glucose stimulation. Additionally, the protein expression of Insulin and Glucagon was done using immunocytochemical staining. The result confirmed that both hormones were detected in all maintenance media (Figure 9G).

According to the results, the utilization of VSCBIC-1 reveals the preservation effect of ALGPA-encapsulated cAD-MSC-derived IPCs together with the maturation of produced IPCs.

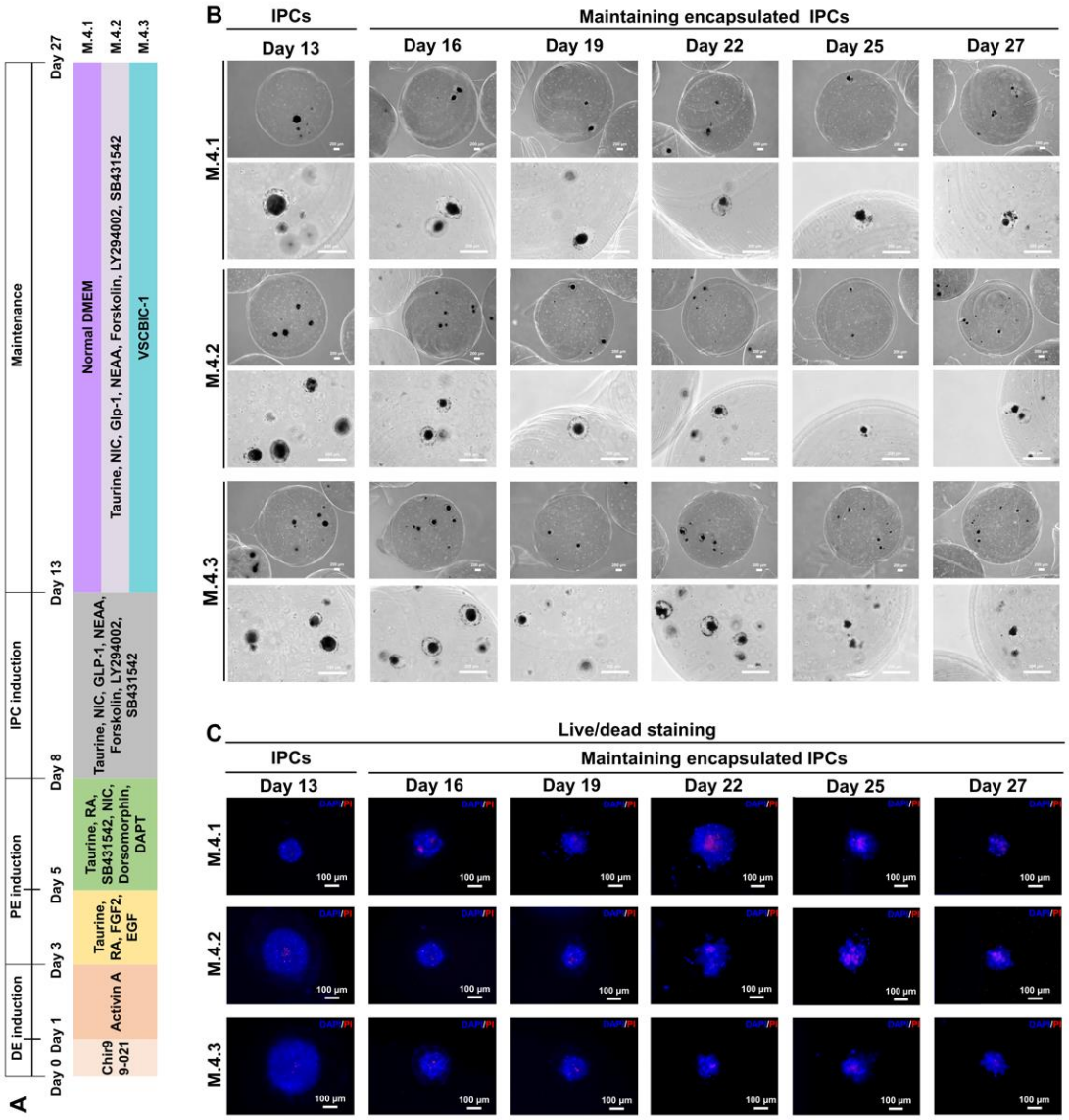


Figure 8 **Morphological and viability evaluation of ALGPA-encapsulated cAD-MSCs-derived IPCs**

The cAD-MSC-derived IPC induction diagram and media maintaining IPCs encapsulated in ALGPA are shown in (**A**). ALGPA-encapsulated cAD-MSCs-derived IPCs' morphological appearances were observed at day 13, 16, 19, 22, 25, and 27 post induction (**B**). The viability evaluation of encapsulated cAD-MSC-derived IPCs was determined by live/dead staining (**C**).

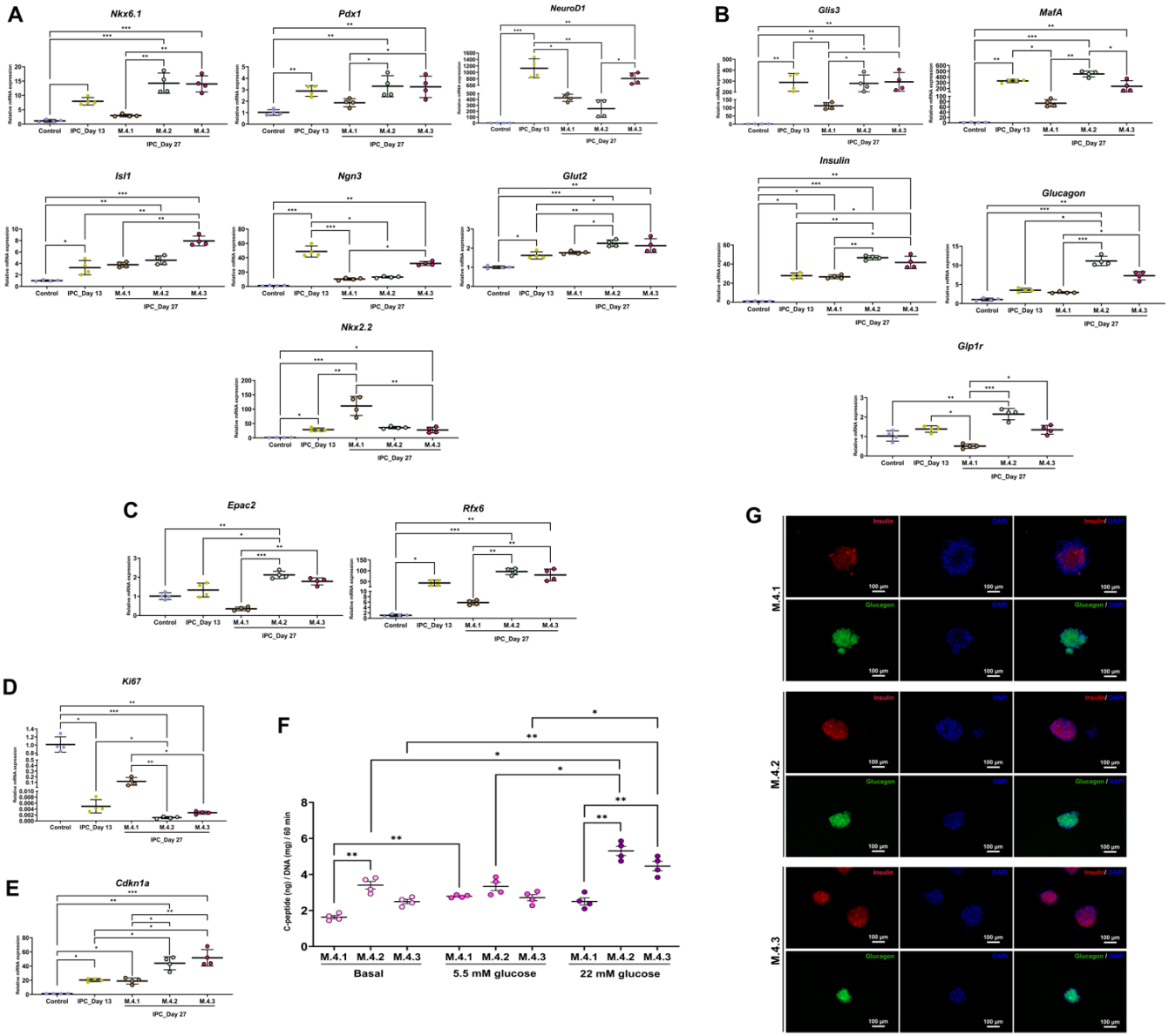

A
Nkx6.1
Pdx1
NeuroD1
Isl1
Ngn3
Glut2
Nkx2.2
B
Glis3
MafA
Insulin
Glucagon
Glp1r
C
Epac2
Rfx6
D
Ki67
E
Cdkn1a
F
C-peptide (ng) / DNA (mg) / 60 min
Basal
5.5 mM glucose
22 mM glucose
G
Insulin
DAPI
Glucagon
M.4.1
M.4.2
M.4.3
Control
IPC_Day 13
IPC_Day 27
Relative mRNA expression
100 μm

Figure 9 **Sustainable functionality of VSCBIC-1 on ALGPA-encapsulated cAD-MSCs-derived IPC maintenance**

The expression of mRNA markers relating to PE precursors (**A**), mature endocrine progenitors (**B**), hormone-release-related markers (**C**), a proliferation marker (**D**), and a cell cycle regulator (**E**) were analyzed by RT-qPCR at day 27 post induction. C-peptide secretion was determined by glucose-stimulated C-peptide secretion (GSCS) assay (**F**). The expression of Insulin and Glucagon by cAD-IPCs was detected on day 27 by immunocytochemistry and observed under fluorescent microscope ZEISS Apotome.2 (Carl Zeiss, Germany) incorporated with Axio Observer Z1 and ZEN pro software (ZEISS International, Germany) (**G**). Relative mRNA expression was normalized with the reference gene, and the undifferentiation control. Bars indicates the significant differences (*, *p* value <0.05; **, *p* value <0.01; ***, *p* value <0.001).

CHAPTER V

DISCUSSION

The concept of stem cell therapy holds gigantic promise for treating diabetes (Aggarwal and Pittenger, 2005), MSCs provides an auspicious platform to produce clinically applicable IPCs (Soria et al., 2000; Lindner et al., 2010; Hass et al., 2011; Betts and Tobias, 2015; Duan and Lopez, 2018; Gabr et al., 2018; Grohová et al., 2019). In this study, cAD-MSC induction protocol toward mature IPCs and the enriched medium for preserving AGLPA-encapsulated cAD-MSC-derived IPCs was established to produce RTU IPCs. The cAD-MSCs were isolated, cultured, and expanded following our previous report (Rodprasert et al., 2021). These isolated cAD-MSCs were then defined following the previous published studies and International Society for Cellular Therapy (ISCT) (Dominici et al., 2006; Kolf et al., 2007; Kim et al., 2016; Ivanovska et al., 2017; Nantavisai et al., 2019), they presented fibroblast-like cell shape, adherent to culture plastic. In addition, pluripotent markers *Oct4* and *Rex1* were expressed, which reflects the multipotent and proliferative properties (Kolf et al., 2007; Nantavisai et al., 2019). Furthermore, MSCs were required to exhibit CD73, CD90, CD44, and CD29 surface markers, and lack expression of CD45 marker (Dominici et al., 2006; Kim et al., 2016; Ivanovska et al., 2017). Similar to our result, other previous studies also illustrated low expression of CD73 on various types of cMSCs, even on MSCs of some other animals (Ivanovska et al., 2017; Nantavisai et al., 2020). It is noted that CD73 expression could be different among various sources and species. Moreover, the isolated cells showed capacity toward adipogenic, chondrogenic, osteogenic differentiation as same as previous studies (Kirkham and Cartmell, 2007; Grzesiak et al., 2011; Molly McAllister, 2016; Sawangmake et al., 2016; Sasaki et al., 2018; Nantavisai et al., 2019; Teshima et al., 2019). All these evidences reflect the potential and homogeneity of the isolated cAD-MSCs.

Currently, various differentiation protocols of IPCs were published (Hori et al., 2002; D'Amour et al., 2006; Wang et al., 2009; Champeris Tsaniras and Jones, 2010; Zhu et al., 2011; Tan et al., 2019). The strategies of these protocols were established on embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC). However, ESC and iPSC encounter ethical issues and safety concerns (Pavathuparambil Abdul Manaph et al., 2019). Compared to ESC and iPSC, MSCs which known as adult stem cells, offer a good candidate for IPC generation (Pavathuparambil Abdul Manaph et al., 2019). The MSC-based protocols mainly comprise of three differentiation stages (Sun et al., 2007; Sawangmake et al., 2014a; Pavathuparambil Abdul Manaph et al., 2019; Sawangmake et al., 2020; Rodprasert et al., 2021). The different stages for MSCs are DE, PE progenitors, and IPC maturation. Although IPCs could be produced from cAD-MSCs with the simple three-stepwise protocol (Sawangmake et al., 2014a; Sawangmake et al., 2020), their function is still hampered (Rodprasert et al., 2021). Here, we illustrated that the modified three-stepwise protocols could improve the *in vitro* functional IPC differentiation from cAD-MSCs.

As the first step of IPC-derived MSCs, DE formation is a prerequisite for generating efficient pancreatic lineage from MSC (Al Madhoun et al., 2016). In previous studies, Activin A and Chir99021 were known as the potential small molecules on DE development (D'Amour et al., 2005; Sawangmake et al., 2014a; Sawangmake et al., 2020; Rodprasert et al., 2021). Although the independent utilization of Chir99021 and Activin A could induce DE from stem cells (Kunisada et al., 2012; Illing et al., 2013; Naujok et al., 2014; Teo et al., 2014), the cooperation of Chir99021 and Activin A could optimize for inducing DE cells (Naujok et al., 2014; Teo et al., 2014). Here, Chir99021, a strong indirect activator of the canonical Wnt-pathway via inhibition of GSK-3β signaling pathway, allows MSCs differentiate into ME, a mesendoderm lineage (Naujok et al., 2014; Teo et al., 2014). Subsequently, DE is induced from ME by treating with Activin A which is known as an endogenous noggin (Kubo et al., 2004; D'Amour et al.,

2005; Tada et al., 2005; Shim et al., 2007). The cascade of ME specification before DE is considered as physiologically relevant (Wlizla and Zorn, 2015). In this study, the expression of ME markers, *Eomes* and *GSC*, was found the significant upregulation after treating Chir99021 for 24 hours. The later generation of DE was defined by Activin A through the upregulated expression of *Sox17*, *Gata4*, *Gata6*. Together, these results demonstrated that Chir99021 synergizes Activin A to effectively generate DE cells from cAD-MSCs.

Next, DE cells from previous step were induced into PE cells. Importantly, PE progenitors represent a critical step of *in vitro* IPC differentiation (Jennings et al., 2015; Márquez-Aguirre et al., 2015). In natural pancreatic development, it is required the development of DE into primitive gut, posterior foregut, and PE precursors, respectively (Caronna et al., 2013; Jennings et al., 2015; Márquez-Aguirre et al., 2015). Our study found that the combination of small molecules in PE induction medium, which consisted of taurine, retinoic acid (RA), FGF2, EGF, SB431542, dorsomorphin, nicotinamide (NIC), and DAPT, show the positive outcome, especially P.2.3. Here, the group treated with taurine alone revealed the hasty differentiation by the greater expression of endocrine precursor markers (*Nkx2.2*, *Pax4*), however, they showed the lowest expression of *Pdx1* and *Nkx6.1*, pancreatic endoderm and multipotent progenitor markers, which could cause the undesired commitments of the cell's fates (Zaret and Grompe, 2008; Jiang and Morahan, 2011). It is noticed that EGF, fibroblast growth factor, could promote the proliferation of *Sox9/Pdx1*-positive pancreatic progenitors (Konagaya and Iwata, 2019). We found that the using of EGF in early pancreatic differentiation (P.2.3) resulted the most efficient induction of Ngn3-positive PE precursors, while the using of EGF in late pancreatic differentiation stage (P.2.2) confined the differentiation of Ngn3-positive PE cells. Previous study reported that the presence of EGF was able to repress the differentiation of PE cells (Cras-Méneur et al., 2001). In several research on vertebrates, the important role of RA in pancreatic

developments were detailly described (Öström et al., 2008; Mattin et al., 2014), while FGF2, a factor of notochord, is required to initiate for pancreatic development by inhibiting Shh expression (Hebrok et al., 1998; Ameri et al., 2010; McCracken and Wells, 2012). Our result also defines the role of RA and FGF2 in the early pancreatic development by showed the increased expression of the primitive gut and posterior foregut markers (*Hnf1b*, *Hnf4a*, *Hnf6*) and pancreatic endoderm marker (*Pdx1*). Combination of RA, dorsomorphin, and SB431542 achieved the effective commitment on Pdx1-positive cell induction (Kunisada et al., 2012). Also, another previous study showed that the cocktail including EGF, BMP inhibitor, and NIC boosted the induction of *Nkx6.1*, another crucial regulator of pancreatic islet development (Nostro et al., 2015). DAPT, known as an inhibitor of Notch signaling pathway, directly represses the expression of *Hes1* (Notch targeted gene) (Gomez et al., 2015). Transcription of *Hes1* gene inhibits the promotion of *Ngn3* gene (Lee et al., 2001), while *Ngn3* expression is essentially required for endocrine cell development (Gomez et al., 2015). Our previous studies proved that the inhibition of Notch signaling during PE induction benefits IPC generation from MSCs (Sawangmake et al., 2014a; Rodprasert et al., 2021). Although RA could also suppress the *Hes1* expression indirectly (Öström et al., 2008), further analyses are still required. BMP antagonism dorsomorphin inhibited Smad1/5/8 phosphorylation to expand the levels of *Insulin* expression which were affected the production of essential hormone "Insulin" in β-cell development process (Mfopou et al., 2010; Nostro et al., 2011). Moreover, the inhibitor of TGF-β type I receptor, SB431542, also promotes the increased expression of *Insulin* (Nostro et al., 2011). Interestingly, our discovery revealed that the upregulation of *Sox9*, *Nkx6.1*, *Pax4*, *NeuroD1*, *Isl1*, and *Ngn3* was found by combined treatment with RA, SB431542, dorsomorphin, NIC, and DAPT. Furthermore, our study also found that a modified medium P.2.3 restricted the expression of *Cdkn1a*, a cell cycle regulator, during PE induction process. *Cdkn1a* was known that it plays a critical role in the cellular

mediation by the overexpression of *Cdkn1a* results in cell cycle arrest (Bendjennat et al., 2003). Therefore, the suppression of this marker means the healthy and proliferative ability of IPCs. However, the further evaluation is still required. In summary, cAD-MSC-derived PE progenitors could be enriched when cAD-MSC-derived DE cells were treated with the combination of taurine, RA, FGF2, and EGF for 2 days, and then the mixture of taurine, RA, SB431542, dorsomorphin, NIC, and DAPT for 3 days.

Regarding the natural pancreatic development, PE cells can give rise to all types of pancreatic islets (alpha, beta, delta, epsilon, and upsilon) (Caronna et al., 2013; Jennings et al., 2015; Márquez-Aguirre et al., 2015). Therefore, the generation of islet β-like cells which can secrete insulin responding to glucose stimulation, is the aim of *in vitro* IPC induction (Gabr et al., 2018), (Caronna et al., 2013; Jennings et al., 2015; Márquez-Aguirre et al., 2015). Unfortunately, cAD-MSC-derived PE colonies tend to lose their shape and decrease their number after the PE induction process (not shown data), this problem was also found in a previous study (Jara et al., 2020). Meanwhile, the entrapment of 3D-PE organoids not only maintains their shapes, but also improves their differentiation property and hormone synthesis (Richardson et al., 2014; Jara et al., 2020; Legøy et al., 2020; Liu et al., 2020a). Hence, cAD-MSC-derived PE colonies were encapsulated in alginate gel before they were induced to IPCs. In this study, the functional IPC generation was found when PE precursors were treated with the mixture of taurine, NIC, Glp-1, NEAA, forskolin, LY294002, and SB431542. Forskolin and Glp-1 play an important role in insulin releasing process via the membrane adenylate cyclase (AC) cascade to covert ATP to cAMP, then modulate insulin secretion via PKA and PKC pathway by forskolin directly stimulates AC, while Glp-1 will stimulate after the binding to its receptor (Hermansen, 1985; Wan et al., 2004; Leech et al., 2011). Moreover, the inhibition of SB431542 via he TGF-β/ALK5 pathway could induce the expression of *MafA* and *NeuroD1* because of the upregulation of Foxo1 protein (Leech et al., 2011).

The inhibition of phosphoinositide 3-kinases (PI3K)/AKT pathway, LY290042, also potentiates the functional maturation of IPCs (Hori et al., 2002; Aoyagi et al., 2012). Here, *MafA*, *Insulin*, and *Glucagon* were the essential markers for islet maturation (Adams et al., 2018), while *Glut2, Epac2* and *Rfx6* were reported to increase the IPC-related hormone secretion capacity (Hermansen, 1985; Wan et al., 2004; Leech et al., 2011; Piccand et al., 2014; Thorens, 2015). Interestingly, we found that the expression of *MafA*, *Insulin*, *Glucagon*, *Glut2, Epac2* and *Rfx6* were upregulated in cAD-MSC-derived IPCs treated with P.3.2 (the combination of taurine, NIC, Glp-1, NEAA, forskolin, LY294002, and SB431542). Subsequently, the trend of concentration-dependent response upon 22 mM glucose stimulation was also discovered. Moreover, the higher expression of *Ngn3* was found on day 13 compared to PE at day 8, thus this cocktail, named "P.3.2" could stimulate the production of PE precursors alongside the mature IPCs. Hence, the IPCs on day 13 contained not only mature IPCs but also Ngn3-expressing PE cells, the similar results were also found in earlier studies (Hwang et al., 2019; Liu et al., 2020b; Xu et al., 2020). These evidences indicate that cAD-MSC-derived PE can more likely to undergo differentiation into IPCs by treatment with the combination of taurine, NIC, Glp-1, NEAA, forskolin, LY294002, and SB431542.

The entrapment of IPCs in alginate not only maintains 3D-floating colony shape, but also enhances differentiation and hormone synthesis (Richardson et al., 2014; Jara et al., 2020; Legøy et al., 2020; Liu et al., 2020a). Besides, the aims of encapsulation are to immobilize the implants as well as build a wall from the body's immune system for *in vivo* application (Yang et al., 2015). Several previous studies were reported that the encapsulation of pancreatic islet, IPCs from ESC and iPSC using alginate could maintain the viability and functionality both *in vivo* and *in vitro* (Wang et al., 2009; Richardson et al., 2014; Westenfelder et al., 2017; Kojayan et al., 2019; Liu et al., 2019; Legøy et al., 2020). However, a greatest obstacle of monolayer capsule is the protrusion of cells. Our previous study showed that ALGPA-encapsulated hDPSC-

derived IPCs can be preserved their viability and functionality (Kuncorojakti et al., 2020). Thus, ALGPA encapsulation may solve protrusion of IPCs and create favorable conditions for clinical treatments. In this study, we hypothesized that the *in vitro* preservation of ALGPA-encapsulated cAD-MSC-derived IPCs can provide the availability of IPCs for *in vivo* application requirements.

Here, three media were employed to investigate the preservation of ALGPA-encapsulated cAD-MSC-derived IPCs; normal DMEM, M.4.2, and M.4.3 (VSCBIC-1). The specialized medium, VSCBIC-1, maintain the morphology, viability, and functionality of mouse islets (Soedarmanto, 2020). In addition, VSCBIC-1 could resuscitate the impaired islets derived from gut leak-induced IL-10 knockout mice (Soedarmanto, 2020). Excitingly, the result showed that the best-preserved viability and function of ALGPA-encapsulated cAD-MSC-derived IPCs in VSCBIC-1 after 13 days of IPC induction protocol. Moreover, the maintained upregulation of *Ngn3* was also found in VSCBIC-1. Likewise, it has been reported that taurine, an essential amino acid, affects the pancreas development, enhances and maintains the endocrine function, and increases the size and number of the islets (El Idrissi et al., 2009). Also, NIC, a form of vitamin B, acts as a poly (ADP-ribose) polymerase inhibitor that is used to promote MSCs homing functional PE/IPCs and preserve the islet viability and function by protection of NAD^+/NADH ratio (Yang et al., 2015; Woodford et al., 2020). Thus, both taurine and NIC are used during differentiation into PE/IPCs and in vitro preservation. Which both small molecules were contained in both M.4.2 and M.4.3. On other hand, our observations suggested that the long-term induction with forskolin, LY294002, and SB431542, resulted in over-maturation of cAD-MSCs-derived IPCs as the extremely higher expression of markers in regard to IPC's maturation by the lower expression of *Ngn3* and the tremendously highest C-peptide secretion detected on day 27 post-induction. Briefly, VSCBIC-1 medium preserves the colonies in both PE and premature/mature IPC statuses until day 14 since the last day of the IPC induction, while the IPCs in P.4.2

medium was seemingly found in mature IPC status. Additionally, the colony population in VSCBIC-1, which contains both PE and premature/mature IPCs, is suitable for transplantation with the following reasons. First, *in vitro* generated PE/premature IPCs would get more maturation after transplantation (Castaing et al., 2005; Kroon et al., 2008; Rezania et al., 2012; Shahjalal et al., 2014; Ma et al., 2018; Ikemoto et al., 2019). Second, PE cells could able to take a longer than mature IPCs for achieving the reversed hyperglycemia in DM patients due to the prolonged maturation period *in vivo* (Rezania et al., 2012; Rezania et al., 2014; Agulnick et al., 2015; Russ et al., 2015). Third, downregulation of *Ki67* from IPCs in VSCBIC-1 indicated that the proliferation is inhibited, this might be safe for transplantation because of restricted invasion capacity (Choudhury et al., 2007; Velasco-Velázquez et al., 2009; Sarmadi et al., 2020). Fourth, the ability on releasing insulin/C-peptide upon high glucose stimulation was still responded when they were maintained in VSCBIC-1. Moreover, a lower expression of HLA on hESC-derived PE/premature IPCs permits maintenance of immune privilege (Azzarelli et al., 2017), this also indicated that PE/premature IPCs are more suitable for transplantation. Taken together, the potential of VSCBIC-1 medium is not only in preservation of IPCs, but also in production of RTU cAD-MSC-derived IPCs.

CHAPTER VI

CONCLUSION

Briefly, we established the three-stepwise protocol for generating IPCs from cAD-MSCs for 13 days. Combined management of small molecules could induce proficient differentiation into DE, PE precursors and IPCs. Our findings suggested that cAD-MSC-derived IPCs could be well-generated with our modified protocol. Moreover, these present results approved that the viability and functionality of ALGPA-encapsulated cAD-MSC-derived IPCs can be preserved for 14 days in specialized medium VSCBIC-1. For the further study, *in vivo* study is required to evaluate the safety and potential of clinical application of ALGPA-encapsulated cAD-MSC-derived IPCs.

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