RECOVERY OF ISOLATED PANCREATIC ISLETS DERIVED FROM GUT LEAK-INDUCED DIABETES TYPE II MOUSE MODEL

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การฟื้นฟูไอส์เลตที่แยกได้จากตับอ่อนของหนูเบาหวานชนิดที่ 2 ที่เหนี่ยวนำจากการเปลี่ยนแปลงของเยื่อบุทางเดินอาหาร

น.ส.อเดร็ตต้า โซดาร์มันโท

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By	Miss Adretta Soedarmanto
Field of Study	Veterinary Science and technology
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อเดร็ตต้า โซดาร์มันโท : การฟื้นฟูไอส์เลตที่แยกได้จากตับอ่อนของหนูเบาหวานชนิดที่ 2 ที่เหนี่ยวนำจากการเปลี่ยนแปลงของเยื่อบุทางเดินอาหาร. (RECOVERY OF ISOLATED PANCREATIC ISLETSDERIVED FROM GUT LEAK-INDUCED DIABETES TYPE II MOUSE MODEL) อ.ที่ปรึกษาหลัก : ผศ. น.สพ.ดร.เจนภพ สว่างเมฆ

ภาวะลำไส้รั่วในผู้ป่วยที่มีปัญหาโรคอ้วน เป็นหนึ่งในปัจจัยเสี่ยงที่ก่อให้เกิดโรคเบาหวานชนิดที่ 2 โดย อินแตอร์ลิวคิน 10 (interleukin-10, IL-10) ไซโตไคน์สำคัญที่ทำหน้าในการรักษาสมดุลของเยื่อบุผนังลำไส้ เพื่อป้องกันการเกิดภาวะลำไส้รั่ว อย่างไรก็ตาม ความเกี่ยวโยงระหว่างโรคเบาหวานชนิดที่ 2 และภาวะลำไส้รั่วจากความบกพร่องของอินแตอร์ลิวคิน 10 นั้น ยังไม่มีการศึกษายืนยันอย่างแน่ชัด นอกจากนั้น การปลูกถ่ายกลุ่มเซลล์ไอส์เลต ซึ่งเป็นที่ทราบกันดีว่าเป็นหนึ่งในการรักษาทางเลือกสำหรับผู้ป่วยโรคเบาหวาน แต่ยังคงมีข้อจำกัดในการใช้ เนื่องจากยังมีปัญหาในการเก็บรักษากลุ่มเซลล์ไอส์เลตที่สกัดมาจากตับอ่อนของผู้บริจาค ให้คงรูปร่างสัณฐาน ความมีชีวิตและหน้าที่ของกลุ่มเซลล์ไอส์เลตให้มีประสิทธภาพ ดังนั้น การศึกษาฉบับนี้ทางคณะผู้วิจัยมีวัตถุประสงค์ในการศึกษา 2 ประการ หนึ่งเพื่อพิสูจน์แนวคิดการเกิดโรคเบาหวานชนิดที่ 2 จากภาวะลำไส้รั่วในหนูทดลองที่บกพร่องในการผลิตอินแตอร์ลิวคิน 10 และสองเพื่อตรวจสอบวิธีการเก็บรักษาและการฟื้นฟูกลุ่มเซลล์ไอส์เลตที่สกัดมาจากตับอ่อนของหนูทดลอง โดยแบ่งหนูทดลองอายุ 13 อาทิตย์ ออกเป็น 3 กลุ่ม (จำนวน 6 ตัวต่อกลุ่ม) ประกอบด้วย กลุ่มควบคุมที่มียีนดังเดิมของหนูทดลอง (WT) กลุ่มควบคุมที่ถูกยับยั้งการแสดงออกของยีนอินแตอร์ลิวคิน 10 (IL-10KO) และกลุ่มศึกษาหนูทดลองที่ถูกยับยั้งการแสดงออกของยีนอินแตอร์ลิวคิน 10 และถูกกระตุ้นให้เกิดภาวะลำไส้รั่ว (GL-IL-10KO) โดยหนูทุกกลุ่มจะถูกป้อนด้วย dextran sulfate sodium (DSS) ทางปาก (gavage) เพื่อกระตุ้นให้เกิดภาวะลำไส้รั่ว หลังจาก 12 อาทิตย์ของการศึกษา หนูทดลองถูกการุณฆยาตและเก็บตับอ่อน เพื่อสกัดกลุ่มเซลล์ไอส์เลตและทำการตรวจสอบรูปร่างสัณฐาน ความมีชีวิตและหน้าที่ของกลุ่มเซลล์ไอส์เลต โดยกลุ่มเซลล์ไอส์เลตจากกลุ่มหนูทดลอง WT ถูกนำมาศึกษาประสิทธิภาพของอาหารเลี้ยงเซลล์ที่ถูกปรับปรุงขึ้นมาใหม่ 2 ชนิด ได้แก่ VSCBIC-1 และ VSCBIC-2 เปรียบเทียบกับอาหารเลี้ยงเซลล์ชนิด RPMI1640 ซึ่งใช้เป็นอาหารเลี้ยงเซลล์พื้นฐานในการปรับปรุงอาหารเลี้ยงเซลล์สูตรใหม่และถูกใช้เป็นกลุ่มควบคุม ผลการศึกษา พบว่ากลุ่มเซลล์ไอส์เลตที่สกัดจากกลุ่มหนูทดลอง GL-IL-10KO มีข้อบกพร่องในด้านความมีชีวิตและหน้าที่ สำหรับอาหารเลี้ยงเซลล์ที่ถูกปรับปรุงขึ้นมาใหม่ พบว่าอาหารเลี้ยงเซลล์ชนิด VSCBIC-1 สามารถเก็บรักษากลุ่มเซลล์ไอส์เลตที่สกัดจากกลุ่มหนูทดลอง WT ให้มีประสิทธิภาพเป็นเวลาอย่างน้อย 21 วัน ดังนั้นอาหารเลี้ยงเซลล์ชนิด VSCBIC-1 จึงถูกนำมาใช้ในการบ่มเพาะกลุ่มเซลล์ไอส์เลตที่สกัดได้จากกลุ่มหนูทดลอง GL-IL-10KO โดยผลการศึกษา พบว่าอาหารเลี้ยงเซลล์ชนิดดังกล่าว สามารถส่งเสริมอัตราการรอดชีวิตกลุ่มเซลล์ไอส์เลตจากกลุ่มหนูทดลอง GL-IL-10KO ให้ทัดเทียมกับอัตราการรอดชีวิตของกลุ่มเซลล์ไอส์เลตจากกลุ่มหนูทดลอง IL-10-KO และ WT ที่บ่มเพาะในอาหารเลี้ยงเซลล์ชนิด VSCBIC-1 เช่นเดียวกัน รวมถึง VSCBIC-1 สามารถส่งเสริมและเพิ่มประสิทธิภาพหน้าที่ของกลุ่มเซลล์ไอสเลตจากหนูทดลองทั้ง 3 กลุ่ม ตลอดการบ่มเพาะตั้งแต่วันที่ 0 จนถึงวันที่ 21 ดังนั้นจากการศึกษานี้ พบว่าภาวะลำไส้รั่วที่เกิดจากความบกพร่องของอินแตอร์ลิวคิน 10 สามารถส่งผลกระทบต่อประสิทธิภาพของกลุ่มเซลล์ไอส์เล็ต อย่างไรก็ตาม ทางคณะผู้วิจัยพบว่าอาหารเลี้ยงเซลล์ที่ถูกปรับปรุงขึ้นมาใหม่ VSCBIC-1 สามารถช่วยฟื้นฟูกลุ่มเซลล์ไอส์เลตที่เสียหายจากภาวะดังกล่าวได้ ในหลอดทดลอง

สาขาวิชา	วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี	ลายมือชื่อนิสิต .....
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Adretta Soedarmanto : RECOVERY OF ISOLATED PANCREATIC ISLETSDERIVED FROM GUT LEAK-INDUCED DIABETES TYPE II MOUSE MODEL. Advisor: Asst. Prof. Dr. CHENPHOP SAWANGMAKE, D.V.M., M.Sc., Ph.D.

Gut leak in the obese patient was known to be one of the predisposing factors causing diabetes type II. Indeed, the interleukin-10 (IL-10) plays a key role in gut mucosa homeostasis preventing the gut leak. Nevertheless, the linkage of diabetes type 2 caused by gut leak with IL-10 deficiency in the non-diabetes patient has not been investigated yet. Islets transplantation is known as the best alternative treatment for diabetes. The culture of isolated islets is still facing difficulties to preserve the morphology, viability, and functionality. Therefore, the present study was aiming to prove the concept of gut-leak on IL-10 deficient mice inducing diabetes type II and aiming to validate the way to preserve and recover the diabetes type II isolated islets using the newly established conditioned media. The 13-week-old age mice were separated into three groups (n=6/group) including background wildtype control (WT), transgenic control (IL-10KO), and gut leak-induced diabetes type II (GL-IL-10KO) which were induced by gavaging the dextran sulfate sodium (DSS). Then, mice were sacrificed, and the islets were isolated after 12 weeks nurtured and checked for morphology, viability, and functionality. The WT islets were used for validating two newly established conditioned media, VSCBIC-1 and VSCBIC-2, by using RPMI1640 as the basal and control medium. The GL-IL-10KO showed a defect in the viability and functionality of the isolated islets after isolation. Additionally, VSCBIC-1 was chosen as the best newly established conditioned media for preserving the WT isolated islets at least for 21 days. Then, VSCBIC-1 was used for culturing the GL-IL-10KO islets, and the results showed that the GL-IL-10KO could catch up the IL-10-KO and WT in term of viability. The functionality among GL-IL-10KO, IL-10-KO, and WT islets was upsurged gradually from day 0 to day 21. In brief, the GL-IL-10KO shows the defect on islets efficacy. Moreover, the newly established conditioned media VSCBIC-1 was able to recover the damaged islets in vitro.

Field of Study:	Veterinary Science and technology	Student's Signature .....
Academic Year:	2020	Advisor's Signature .....

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Adretta Soedarmanto

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CHAPTER I
INTRODUCTION

Importance and Rationale

Diabetes mellitus is a disease characterized by hyperglycemia caused by the decreased insulin secretion or insulin action which defects the glucose utilization and inevitable multi-organ impairment (Wu et al., 2014; Kang et al., 2019). Islets of Langerhans consist of several types of cells producing hormones that are involved in glucose homeostasis (Xavier, 2018). Insulin was synthesized, stored, and released by β-cells, as one of the constituents of islets of Langerhans (Marchetti et al., 2017).

Around 463 million adults were diagnosed with diabetes in 2019 and the number was predicted to be rising to 700 million by 2045 according to International Diabetes Federation-IDF (2019). About 90% of diabetes case is diabetes type II that linked to an unhealthy lifestyle, lack of vitamin D and K, heritable genetic, and gut metagenome (Wu et al., 2014; IDF, 2019).

Focusing on the gut metagenome risk factors, the gut bacteria in the intestinal ecosystem is providing gut homeostasis (Russler-Germain et al., 2017). The intestinal microorganism will generate symbiosis mutualism by promoting regulatory T cell (Treg) to produce IL-10 to suppressing the dendritic cells, macrophage, and T helper 1/T helper 17 (Th1/Th17), which are affecting inflammatory prevention (Keubler et al., 2015). Meanwhile, the increased growth of some opportunistic pathogens may cause a gut leak (Wu et al., 2014). The gut leak, is also known as increased intestinal permeability, is a condition where the tight junction of intestinal epithelial cells damaged. This condition is allowing the toxin, some ingesta, and microorganisms to escape into the bloodstream and stimulate systemic inflammation (Perkins, 2019). One of the leaky gut condition models was presented by interleukin 10 knockout (IL-10 KO) mouse treated with dextran sulfate sodium (DSS) induced colitis (Cardoso et al., 2018). Hence, the inflammation is resulting on the alteration in glucose metabolism, insulin resistance, energy imbalance, and body weight (Boulangé et al., 2016; McGuinness et al., 2018) which leads to diabetes type II.

The patients with diabetes can get the medication either perorally, parenterally, or transplantation (Marin-Penalver et al., 2016). The best treatment nowadays is the transplantation of islets of Langerhans since it has a 50% possibility to be insulin-independent for 3-5 years (Vantyghem et al., 2014; Rheinheimer et al., 2015). Nevertheless, a single patient needs number of

donors since the number of extracted islets of Langerhans from a single brain-dead donor is very limited (de Souza et al., 2017b). Moreover, the transplantation is also facing immune rejection, immunosuppressive, blood-mediated inflammatory reaction, and lack of vascularization factors (Zammit et al., 2019; Chen et al., 2020b).

One of the solutions is by enhancing the pancreatic islets morphology, viability, and functionality. Many studies were conducted to explore the way to achieve these goals by establish insulin-producing cells (IPCs) and co-culture the islets with MSCs. However, those findings are very important to understand the regeneration mechanism of pancreatic islets, the result is not yet satisfying in terms of morphology, viability, and functionality. Therefore, the better potency on recovery the damaged pancreatic islets as the consequences of IL-10 deficiency induced-diabetes type II, for maintaining their morphology, viability, and functionality is not fully understood.

Thus, this study aims to understand the consequences of gut leak-induced diabetes type II towards isolated pancreatic islets and enhance the morphology, viability, and functionality of isolated pancreatic islets on mouse models. Therefore, the result of this study may help to understand the effect of IL-10 deficient-induced diabetes type II towards the pancreatic islets on morphology, viability, and functionality. Additionally, the enhanced pancreatic islets protocol could be practically used including banking and transplantation.

Objectives

1) Study the effect of gut leak-induced diabetes type II mouse model on morphology, viability, and functionality of isolated pancreatic islets.
2) Study the enriched culture protocol for recovering the isolated pancreatic islets derived from gut leak-induced diabetes type II mouse model.

Hypotheses

1) The gut leak-induced diabetes type II mouse model leads to pancreatic islets defective properties.
2) The established enriched culture protocol can recover the isolated pancreatic islets derived from gut leak-induced diabetes type II mouse model.

CHAPTER II

LITERATURE REVIEW

Part 1 Islets of Langerhans

1.1 Structure

Islets of Langerhans is discovered by Dr. Paul Langerhans in 1869 in the Pancreas (Xavier, 2018; Shahid and Singh, 2020). Around 1-2% of islets, part of the pancreas, can be found across parenchymal mostly at the tail of the pancreas (Lacy, 1974; Xavier, 2018) that act as an endocrine and also paracrine system (Jones and Persaud, 2017). Each islets composed of 1000-2000 of 5 types of endocrine cells that clustered into a 3D structure (Nam et al., 2010; Persaud et al., 2014). The islets also rich in vascularization and autonomic nerve supply to keep the islets works well (Jones and Persaud, 2017). However, the morphology, distribution, and cell structure of islets are varies among species (Paniccia and Schulick, 2017).

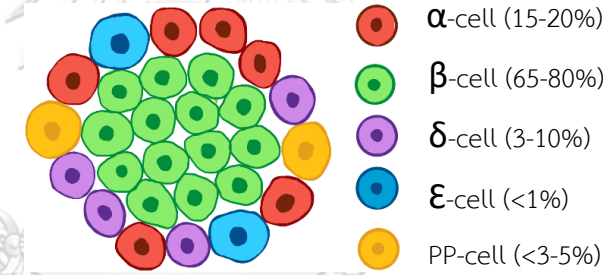


Figure 1 Mice islets' structure and cells distribution

The islets of Langerhans have a spheroid shape (Jo et al., 2007). The size of the mice islets is around 50μm to bigger than 250 μm with golden-brown color (Nam et al., 2010; Marzorati and Ramirez-Dominguez, 2015). In addition, islets size distribution which bigger than 250 μm is 15%, 100-250 μm is 35%, 50-10 μm is 30%, and less than 50 μm is 20% (Marzorati and Ramirez-Dominguez, 2015).

Specifically, the distribution of β-cells on mice islets is located at the core of the islets and surrounded by other cells which forming a mantle (Fig. 1) (Noguchi and Huising, 2019; Adams et al., 2020). β-cells are the major composer (65-80%) of the islets on mice compared to human, while α-cell is 15-20%, δ-cell is 3-10%, ε-cell is less than 1% and PP cells is 3-5% of the islets (Kilimnik et al., 2012; Roder et al., 2016).

The role-play of islets is to maintain the homeostasis of glucose, metabolism, and feeding behavior (Hartig and Cox, 2020). The function of islets of Langerhans is supported by the varies of the composed cells that secrete hormones. It acts as an endocrine and paracrine signal controlling the usage and repository of metabolic fuel that can be stimulated by the interaction of the cells with the autonomic nerve and GIT hormones (Jones and Persaud, 2017).

The pancreas was formed from two part of pancreatic buds, ventral and dorsal that fused together (Nandy and Mukhopadhyay, 2011). It has two lineages, the exocrine (the acinar and duct cell) and the endocrine (islets of Langerhans) (Henderson et al., 1981; Wilson et al., 2003). The endocrine lineage is where the pancreatic islets growth which regulated by the *Hnf-6* as one of the gene precursors Which promotes the *Ngn3* expression (Jacquemin et al., 2000). Matured pancreatic islets will express some genes according to the functions. The gene expressed in α-cell are *Gcg, Glp1-R*, *Nkx2.2*, *Maf-b*, and *Isl1* while the β-cell is expressing *Glp1-R*, *Nkx6.1*, *Isl1*, *Maf-a*, *Ins1*, *Ins2*, *Pdx1*, and *Glut2* (Portha et al., 2011; van der Meulen and Huising, 2015). The δ-cells is marked by the expression of *Pdx1* while ε and PP cells are expressing *Nkx2.2* (Mastracci and Sussel, 2012).

1.2 α-cell

α-cell is a part of islets that secrete glucagon hormones (Rix et al., 2000). The Glucagon hormones act in glucose homeostasis by increasing blood glucose levels (Sakata et al., 2019). Blood glucose level can be triggered by stimulate hepatic glucose production (Rix et al., 2000). This will provide enough amount of glucose in every organ by inducing glycogenolysis (liver glycogen breakdown) then increased glucogenesis (Hall and Guyton, 2011).

1.3 β-cell

Around 60% to 80% of the mice islets is composed of the β-cell (Noguchi and Huising, 2019). As the most constituted cell in the islets, β-cell plays key role in glucose homeostasis (Volta et al., 2019). Its secreting membrane-bound secretory granules containing insulin hormone (Do and Thorn, 2015). The elevation of blood glucose after meal stimulate the β-cell to produce insulin (Roder et al., 2016). The insulin mobilized the circulating glucose into the tissues and mediated by the glucose transporter protein (GLUT) to be metabolized (Satoh, 2014).

1.4 δ cells

δ-cells produce somatostatin hormone to inhibit the secretion of insulin and glucagon (e Drigo et al., 2019). The production of somatostatin is stimulated when the number of glucagon is too high since the function of the somatostatin hormone is to inhibit the insulin and glucagon secretion (Rorsman and Huising, 2018).

1.5 ε and PP cells

Occupy the least number as the pancreatic islets composer, ε and PP cells also have their role to the body. The ε-cell is secreting a Ghrelin hormone that stimulates the pituitary to release the growth hormones and activating the hypothalamic arcuate nucleus as the regulator of nutritional intake. Ghrelin is important in the developing periods of the pancreas, since it is stimulating the pituitary to release growth hormones. However, the number begins to decrease when the pancreas gets mature (Sakata et al., 2019). Meanwhile, the PP cell or also known as γ cell is secreting pancreatic polypeptides that work as the inhibitor of gastric and intestinal motor activity (Brereton et al., 2015).

Part 2 Diabetes Type II

2.1 Etiology

Diabetes type II is a condition related to insulin resistance and impaired insulin secretion (Sanghera and Blackett, 2012). As an indication of diabetes type II, insulin resistance also defects the autonomic and endothelial function (De Souza et al., 2017a). This condition is correlated with the downregulation of insulin action on the liver, skeletal muscle and adipocytes which lead to diabetes type II (Wang et al., 2015). Since the insulin action is declined, it will result in hyperglycemia, the accumulation of glucose in the bloodstream, as well as the continuity of insulin production in order to maintain glucose homeostasis (Goyal and Jialal, 2020).

Uninterrupted hyperglycemia condition will followed by the secretion of IL-1β (Donath et al., 2005) which can induce the production of another IL-1β which enchant the macrophage to produce other cytokines (Zhao et al., 2014). Over the time, the pancreatic islets gradually decreases its function caused by the IL-1β infiltration (Jeong et al., 2002) which causing the declined insulin secretion and poor of glucagon secretion inhibition (Spellman, 2010). The IL-1β will bind to the IL-

1β receptor on the islets and become a complex together with Toll-like Receptor (TLR) and MyD88, induce the Tumor necrosis factor Receptor-Associated Factor 6 (TRAF6) which stimulates the nuclear factor **K** B kinase (NF-**K**B) and conveyed to the nucleus inducing the nitric oxide (NO) production (Chen et al., 2020a). The NO may interact with the DNA fragments and prosthetic groups in the transcription factor which resulting on the downregulated of glucose oxidation, oxygen consumption and ATP synthesis (Wang et al., 2010). This will decline the function of the islets and lead to apoptosis (World Health Organization, 2019).

2.2 Predisposition and Pathogenesis

Diabetes type II is caused by either genetic factors involving impaired insulin secretion and insulin resistance or environmental factors or both (Kaku, 2010; Sirdah and Reading, 2020). The genetic factor is identified by numerous genetic variants (Zheng et al., 2019). Previously described by Cho et al. (2011), they found the loci involved in the diabetes type II development in Southeast Asia by genome-wide association studies (GWAS). It is also explained by Wu et al. (2014) that monozygotic twins have a high incidence by 96% compared to the dizygotic twins and the first-degree relatives (FDR) incidence rate is only 6% in the society. However, 40% of them are likely to suffering from diabetes type II (Sanghera and Blackett, 2012). Other than that, the diabetes type II case on the FDR is also affected by the epigenetic process which may contribute to the inherited risk until several generations, including the higher risk on siblings by adverse intrauterine and pregnancy-associated factors exposure during the first trimester and common environment which hard to be controlled in the studies (Ali, 2013; Zheng et al., 2020).

On the other hand, environmental predisposition is including obesity, aging and, lack of exercise (Association, 2008). These three factors lead to muscle reduction and increasing the fat deposit that dramatically rises in middle age and old age as the consequences of the increased food intake, without exercise (Kaku, 2010).

Those factors are the initial point of diabetes type II by causing inflammation on white adipose tissue. The continuous inflammation in obese white adipose tissue leads to resistance of insulin antilipolytic effect that cause the fat-binding capacity of the fat cells declined. The inflammation also elevated the non-esterified fatty acid (NEFA) levels and resulting in the impaired insulin gene expression and cell death which caused by the decrease of glucose-insulin secretion when exposed

to β-cell. Furthermore, it will affect the other organs by increased insulin resistance and impaired insulin secretion in muscle, liver, and β-cell in the pancreas (Petrovic et al., 2020). Stressed conditions of the endoplasmic reticulum (ER) may also take a part in the cytokine-induced β-cell death leading to β-cell loss and insulin resistance (Petrovic et al., 2020). Following that situation, it will be resulting in hyperglycemia continuously lead to degeneration of β-cell ruthlessly (Petrovic et al., 2020).

2.3 Therapy

The most popular among effective therapy nowadays are either peroral or parenteral drugs balanced with a healthy lifestyle (Marin-Penalver et al., 2016). This treatment may help the patient to manage hyperglycemia by decreasing the glucose levels (Steinberg and Carlson, 2019). Even though this treatment may help the patient to be able to have an euglycemic condition, but they are still insulin-dependent. The islets transplantation by Edmonton protocols was firstly published in 2000 using the death-brain donor healthy islets of Langerhans and transplanted via intrahepatic procedure (Shapiro et al., 2000). Islets transplantation is the best medication since it offers a promising chance by 80% to be insulin-dependent until 5 years or more (Vantyghem et al., 2014). This Edmonton protocol's then followed and reported by Brennan et al. (2016) after 11 years. The result shows that the patient still managed the islets graft function for at least 10 years (Brennan et al., 2016).

Part 3 Interleukin 10 on Mice Intestinal Mucosa

3.1 Interleukin-10 Function and Mechanism

Interleukin-10 (IL-10) is an anti-inflammatory cytokine produced by macrophages, dendritic cells, and T-cells which are mostly found in the intestine (Morhardt et al., 2019). The function of this cytokine is to prevent inflammation and autoimmune pathologies (Iyer and Cheng, 2012). Specifically in the intestine, it acts as a homeostasis system between the intestine with microbiota (Keubler et al., 2015). It is directly regulating goblet cells to induce the function of intestinal secretory cell by producing the mucous barrier specifically under stress conditions (Hasnain et al., 2013). It also plays an important role in wound healing (Andrews et al., 2018) and prevents the loss of epithelial function in the intestine (Mendes et al., 2019).

The mechanism of IL-10 role play in the intestine is determined by the macrophage. Once the macrophage failed to respond towards the IL-10, the proinflammatory cytokine could migrate causing spontaneous colitis (Mantovani and Marchesi, 2014). The IL-10 is also produced by macrophage and myeloid dendritic cells when the cell surface or cytoplasmic pattern recognition receptors (PRR) of APCs (e.g. macrophage and dendritic cells) observed the pathogens (Shouval et al., 2014). Interleukin-10 is produced by Treg induced by the bacteria (Keubler et al., 2015). The antigen of the bacteria will induce macrophage to secrete IL-1β which triggers the GM-CSF in the innate lymphoid cells 3 (ILC3). The GM-CSF re-induce back the macrophage reducing retinoic acid which promote the differentiation of Treg (Samarakoon et al., 2016). Crucially needed for wound healing, the IL-10 stimulates the synthesis of Wnt1-inducible signaling protein 1 to induce epithelial proliferation (Andrews et al., 2018). Together with lipopolysaccharide (LPS) or IL-10 itself, it is not only simply an inactivator of macrophage function but also regulating the transcription of immunity and inflammation control, extracellular matrix remodeling, and β-cell function (Mantovani and Marchesi, 2014). The LPS is taking part on TLR4 ligation which upregulating the IL-10 on the intestinal epithelial cells to stimulate cell proliferation and strengthen the mucosal barrier (Andrews et al., 2018).

3.2 Correlation between Gut leak, Interleukin-10 Deficiency and Diabetes Type II

The gastrointestinal have an important role in the body metabolism by digest and absorb the nutrition from the ingested food (Mu et al., 2017). Anatomically, the lumen of GI tract is covered with the mucosa layer composed of epithelial cells that act as absorption of nutrients and transporter, retain the environment to be humid, and prevent pathogens, and foreign particles to the body (Kong et al., 2018). The epithelial have tight junction which controlling ions, solutes, and water paracellular movement and detoxification system act as the physical barrier, protecting from microorganism, antigens, and xenobiotics (Lee et al., 2018). The tight junction are fibrils formed by at least 2 proteins, occludin and claudin family members, located between two epithelial cells. The contraction of cytoskeletal actin filament that connecting occludin and member of claudin family by intracellular scaffold proteins, zonula occluding protein-1, -2, and -3 (ZO-1, ZO-2, and ZO-3), regulates the paracellular permeability (de Kort et al., 2011).

Zonulin is a protein produced by the intestinal induced by bacteria (Asmar et al., 2002). Once the secretion of zonulin dramatically increases in the intestinal lumen and binds to zonulin

receptor in the epithelial cells, it will up-regulate epithelial permeability by decreasing the interaction between ZO-1 and occludin and interrupt the tight junction complex integrity. This phenomenon leads to the increased intestinal permeability known as leaky gut (de Kort et al., 2011).

The appearance of microbiota on the gut is a key player for gut homeostasis (El Aidy et al., 2013). It is helping the gut mucosal on the prevention from pathogen bacteria, digesting the fibers to become the short chain fatty acid (Belkaid and Harrison, 2017), and metabolized the bile acid (Ramírez-Pérez et al., 2017). The microbiota (*Lactobacillus reuterri* and *Lactobacillus casei*) (Smits et al., 2005) that provide the protection for gut epithelial from the pathogenic bacteria (*C. rodentium*, *E. coli,* and *S. Thypimurium*) is by colonizing the intestinal epithelial surface area and strengthen the immunity via innate lymphoid tissue 3 (ILC3) (Pickard et al., 2017). On the other hands, the Gram-negative bacteria have the lipopolysaccharides (LPS) which can induce the inflammation of insulin receptor if permeate through the blood stream (Allin et al., 2015).

The butyrate producing bacteria is located at the colon, digesting the fiber and produced short chain fatty acids (SCFAs) (acetate, butyrate, and propionate) (Parada Venegas et al., 2019). The SCFAs is produced by the commensal microbiota in the intestine and interacted with IL-10 that modulates the epithelial function by inhibit the claudin -2 (Cldn-2), permeability promotor, to maintain the homeostasis (Mendes et al., 2019). According to Puddu et al. (2014), the butyrate producing bacteria is important for diabetes type II patient, preventing the obesity and diabetes type II. When the butyrate producing bacteria decreased, the energy source of the epithelial is also downregulated resulting on the decreased of tight junction function which lead to gut-leak (Liu and Lou, 2020). The bacteria also able to deconjugate the primary bile acid secreted from the liver into secondary bile acid (Allin et al., 2015). On the diabetes type II patient, it is reported that the pooled bile acid is altered (Prawitt et al., 2011).

In the last few years, it is known the importance of IL-10 on intestinal immune regulatory. The IL-10 deficiency is explained clearly by the model of IL-10 KO. On mice model, the knockout of IL-10 affecting the proinflammatory cytokine production and Th1-like response upregulated lead to chronic enterocolitis spontaneously (Mendes et al., 2019). Meanwhile, on humans, the research was conducted in intestinal epithelial cell lines by knockdown of the IL-10 receptor 1 and resulting on interrupted membrane permeability. In short, the IL-10 deficiency on the intestine causing the turnover of the intercellular tight junction function (Andrews et al., 2018).

Continuously, the alteration of tight junction may lead to diabetes type II. On a normal condition of intestinal mucosal barrier, only water, nutrients, and bioactive compounds could pass the tight junction. Once the barrier is damaged and causing a leaky gut, the microbial and foreign particles may permeate through the epithelium barrier (Gomes et al., 2017). When the Gram-negative bacteria LPS is circulating in the blood, leads to metabolic endotoxemia (Radilla-Vázquez et al., 2016). In regard of this, the LPS is suggested to be the initiator of inflammation related to insulin resistance (Boulangé et al., 2016). The LPS plasma level on the leaky gut condition is upregulated and modulate the Toll-like receptor 4 (TLR-4), which can be found in the insulin targeted tissue including liver, fat tissue, skeletal muscle, β-cell, blood vessel, and brain (Kim and Sears, 2010) to activate the innate and adaptive immune response (Sato et al., 2017).

This is caused by the composer of the LPS, lipid A (Gomes et al., 2017) that have an ability to pass the intestinal mucosal barrier to the bloodstream via leaky gut tight junction or infiltrating chylomicrons (Salguero et al., 2019). Additionally, the binding between LPS lipid A with the LPS-binding protein (LBP) (Neves et al., 2013) activates the receptor protein CD14 in the macrophage plasma membrane and becoming a complex (Salguero et al., 2019). Furthermore, the complex bind to TLR4 and myeloid differentiation factor 2 (MD2) (Salguero et al., 2019) and activate two signaling cascades, the induction of proinflammatory cytokines and induction of type 1 interferon genes. These pathways will initiate both positive feedback via IL-6 and TNFα and negative feedback via activation of IκB (Kim and Sears, 2010). Moreover, the proinflammatory regulators will change the expression of IRS-1, glucose transporter 4 (GLUT-4), and PPAR-α lead to insulin sensitivity (Boulangé et al., 2016).

Part 4 Gut Leak-induction by Dextran Sulfate Sodium

Synthetic sulfated polysaccharides which consist of dextran and sulfated anhydro-glucose unit is known as dextran sulfate sodium (DSS) (Jamwal and Kumar, 2017). Dextran sulfate sodium, as a negative charge caused by the sulfate groups, is water-soluble, stable at room temperature in a powder form, toxic to the epithelium of the colon which leads to erosion that interferes with the gut barrier (Chassaing et al., 2014). This condition will result in an increase of intestinal epithelial permeability, also known as gut leak (Andrews et al., 2018).

Although the mechanism is still unclear, the DSS has the possibility to induce gut leak by altering epithelial mucosa permeability, disturbing the ZO-1 protein of tight junction (Solomon et al., 2010). Since the DSS is well known to induce the erosion of the colon epithelium, it is widely used for understanding the mechanism of acute or chronic colitis and other related diseases in mice models (Mizoguchi et al., 2020).

Part 5 Principal of Islets Isolation and Islets Culture

After the big success on islets transplantation by Shapiro and his colleagues also known as Edmonton's protocol (Shapiro et al., 2000). The science was developing rapidly until nowadays, supporting Edmonton's protocol. The Edmonton's protocol has been successfully treating the diabetes mellitus by transplanting the islets of Langerhans obtained from brain dead donor patient (Shapiro et al., 2006). The result was interestingly maintaining the euglycemic conditions for more than 5 years (Pepper et al., 2016). However, the donors were not always available and surely, the donors' number was insufficient, and the isolated islets need to be transplanted as soon as possible to prevent further apoptosis (Rother and Harlan, 2004). Several techniques were established for isolating the islets of Langerhans and for culturing medium to obtain the right size, morphology, viability, and functionality of the islets of Langerhans (Rother and Harlan, 2004).

5.1 Islets Isolation

The islets can be obtained from different techniques with different results. The principal of islets isolation is exposing the islets from the pancreas tissue and separated from surrounding cells without disrupting the islets structure (Rheinheimer et al., 2014). This is depending on the digestion solution, digestion techniques, times, and the materials that used (Corbin et al., 2021). It has been known that to digest the pancreas can use the digestion enzyme, such as the Proteolytic Enzymes V, and various type of collagenase (Qi et al., 2009; Saliba et al., 2017; Villarreal et al., 2019). The function of this digestion enzyme is to interrupt the peptide bond of collagen in the pancreatic tissue, resulting the separation of the cells in the tissue (Kin et al., 2010). However, high concentration of collagenase will affect the vitality which harm the cells (Feng et al., 2018).

Meanwhile, the Cell Recovery Solution, a Non-enzymatic solution from Corning® which was widely used for dissolving the Matrigel (Abe et al., 2018). Matrigel is an extracellular matrix (ECM)

that used *in vitro* originates from Engelbreth-Holm-Swarm (EHS) mouse tumor (Mullen, 2004). However, there is no reported data yet about the effect on the islets isolation.

The techniques for pancreas digestion can be done by mainly two ways, perfusion and mechanical with the digestion solution (Yin et al., 2016). The advantages of the isolating islets by using perfusion technique are allowing the digestion enzyme perfuse to every corner of the pancreas anatomically and minimize the wound of the islets (Carter et al., 2009). However, this technique must be done in the right process to get higher yield of the islets (Yin et al., 2016). Mechanical islets isolation is easy to be performed. Yet, the technique may cause more damage to the islets because the pancreas was cut into pieces and disrupt the pancreas tissue by shaking continuously (Carter et al., 2009).

5.2 Islets Culture

Islets culture facing many problems in terms of maintaining the viability and also the functionality in *in vitro* culture (Saliba et al., 2017; Komatsu et al., 2018). Many small molecules, antioxidants, growth factors, and other supplements can be used to maintain the viability and the function. On another hands, the environment of culture condition can support the viability and the function (Daoud et al., 2010; Komatsu et al., 2018).

Small molecule, a substance with small molecular weight, have the ability to assisting the biochemical process in the body to treat or diagnose the diseases (Ngo and Garneau-Tsodikova, 2018). It can be called as the small molecules when the compound can pass through the membrane cells to do the activation of the biochemical processes, which known should have size less than 1,000 kDa (Thierauch, 2011).

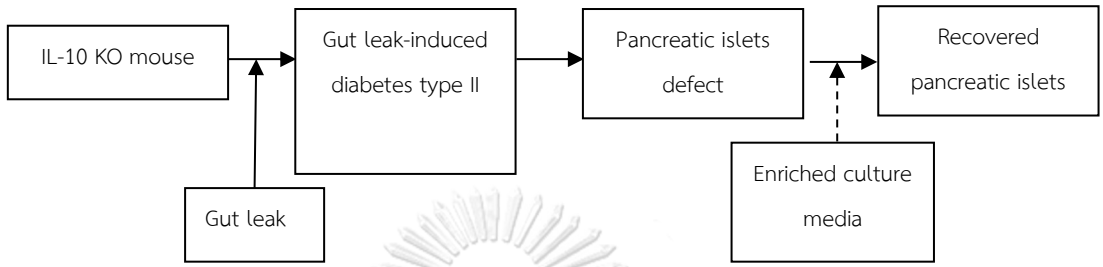
The antioxidant was widely used on islets culture. On the *in vitro* condition, the islets loss many of endogenous anti-oxidants which promotes the reactive oxygen species, leading to apoptosis (Bottino et al., 2004). Meanwhile, it has been reported of overexpression of anti-oxidant in culture condition will prevent the oxidative stress of the islets (Myasnikova et al., 2019). Additionally, it also protects the islets on the transplantation process by minimizing the apoptosis reaction (Miki et al., 2018). The role play of anti-oxidant on islets culture were observed to increase the viability, insulin secretion, and the islets yield on previous study (Mohseni Salehi Monfared et al., 2009).

Growth factor is an active polypeptide which induced the growth of cells by binding to the receptor, affecting the metabolism in the cell (Goustin et al., 1986; Stone et al., 2021). The act of the growth factor was by either activating several pathways or deactivating, upregulates the islets viability, functionality, and the engraftment (Tsuchiya et al., 2015). Other supplements, such as antibiotics, antimycotics, were widely used as prophylactic agent for contaminations during culture (Campos et al., 2012). Additionally, serum was often be used on the islets culture with the purpose of maintaining the cell physiological stability which promotes the cell viability and also cell growth (Yang and Xiong, 2012).

CHAPTER III

METHODOLOGY

Conceptual Framework



Experimental Plan

The study was using mouse as the animal models which they were separated into three groups (n=6), namely background control group (mouse strain C57BL/6NJcl; wildtype/WT), transgenic control group (IL-10 KO mouse, strain B6.129-IL10^{tm1Cgn}/J; IL-10KO), and gut leak-induced diabetes type II model group (IL-10 KO mouse, strain B6.129-IL10^{tm1Cgn}/J with DSS induction; GL-IL-10KO) throughout the study period. The animals were checked for the blood glucose, homeostasis model assessment (HOMA) of insulin resistance (IR) and HOMA β-cell function (HOMA β) and additional for the gut leak-induced diabetes type II model group was given DSS as shown below (Fig. 2).

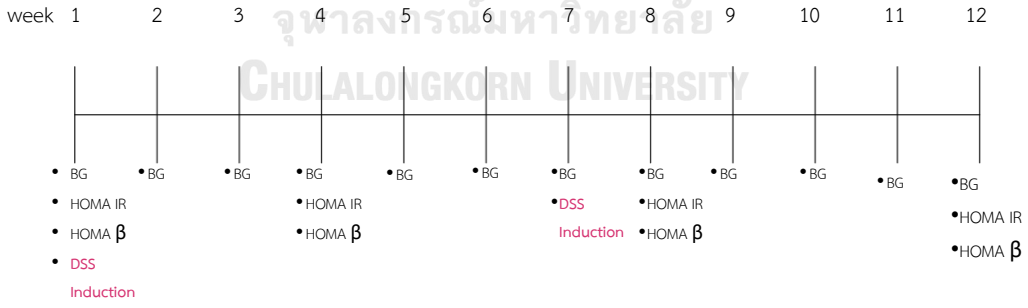


Figure 2 Scheme of DSS induction schedule.

The DSS was given perorally on the first week and seventh week.

Study the effect of gut leak-induced diabetes type II mouse model on morphology, viability, and functionality of isolated pancreatic islets

1a) Establishment of pancreatic islets isolation protocol from gut leak-induced diabetes type II mouse model

- The protocols will be modified from previous reports using three mice groups (n=6), background control group (mouse strain C57BL/6NJcl), transgenic control group (IL-10 KO mouse, strain B6.129-IL10tm1Cgn/J), and gut leak-induced diabetes type II model group

1b) Exploration of the isolated pancreatic islets properties derived from gut leak-induced diabetes type II mouse model.

- Morphology: microscopical analysis, histological examination
- Viability: Viability percentage using Trypan Blue staining and cell viability in the colony using Live/Death staining
- Functionality: Glucose Stimulating C-peptide Secretion and pancreatic islet related marker gene expression by RT-qPCR

Study the enriched culture protocol for recovering the isolated pancreatic islets derived from gut leak-induced diabetes type II mouse model

2a) Establishment of the enriched culture protocol for recovering isolated pancreatic islets

- Culturing the isolated pancreatic islets from background control group (mouse strain C57BL/6NJcl), transgenic control group (IL-10 KO mouse, strain B6.129-IL10tm1Cgn/J), and gut leak-induced diabetes type II model group using the enriched culture medium

2b) Exploration of the enriched culture protocol's effect on the isolated pancreatic islets derived from gut leak-induced diabetes type II mouse model

- Morphology: microscopical analysis, histological examination
- Viability: Viability percentage using Trypan Blue staining and cell viability in the colony using Live/Death staining
- Functionality: Glucose Stimulating C-peptide Secretion and pancreatic islet related marker gene expression by RT-qPCR

Materials and methods were set up according to the proposed objectives as follows:

Specific aim **1) "Study the effect of gut leak-induced diabetes type II mouse model on morphology, viability, and functionality of isolated pancreatic islets."**

Strategies 1a) Establishment of pancreatic islets isolation protocol from gut leak-induced diabetes type II mouse model

The pancreatic islets isolation protocol was modified from previous reports (Li et al., 2009; Graham et al., 2016; Saliba and Farès, 2019; Villarreal et al., 2019). The digestion of pancreas was using Cell Recovery Solution (Sigma-Aldrich, USA) a substitution of collagenase, by perfused to the pancreas via biliary duct. The inflated pancreases were snatched and put into an ice-cold condition of transport medium containing $CaCl_2$ (Sigma-Aldrich), 1x HBSS (Thermo Fisher Scientific, USA), and 2x antibiotic-antimycotic (Thermo Fisher Scientific), then incubated for 10 mins at 37°C with 5% CO_2. Following the digestion, the centrifugation was performed at 4°C 300 rpm for 3 min, and the supernatant was replaced with the ice-cold condition of transport medium to stop the digestion process. The islets were collected after dissociated mechanically from other pancreas tissue using Cell Strainer (Sigma-Aldrich) and was used for further experiments.

Strategies 1b) Exploration of the isolated pancreatic islets properties derived from gut leak-induced diabetes type II mouse model.

The isolated pancreatic islets were analyzed the morphology, viability, and functionality between three groups (n=6), namely wildtype (WT), IL-10KO, and GL-IL-10KO groups. The morphology of isolated pancreatic islets was assessed by the histology, total number, size, color, and shape. Additionally, the islets of Langerhans were checked for their viability using 0.4% Trypan Blue staining (Sigma-Aldrich) and Live/Dead staining using Enzo® (ENZO LIFE SCIENCES, INC, USA) or Calcein AM/ propidium iodide (PI) (Thermo Fisher Scientific). On the other hand, the functionality of isolated pancreatic islets was tested with glucose stimulated C-peptide secretion assay and the expression of pancreatic endoderm, pancreatic islets, and islets-related gene markers by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Specific aim **2) "Study the enriched culture protocol for recovering the isolated pancreatic islets derived from gut leak-induced diabetes type II mouse model."**

Strategies 2a) Establishment of the enriched culture protocol for recovering isolated pancreatic islets.

Our enriched culture protocol was established and tested using pancreatic islets isolated from wildtype (WT) group. The isolated pancreatic islets were cultured using the enriched culture medium directly after isolation and kept for 21 days with previous preliminary study. On the previous study, three different kinds of media were used to culture the isolated pancreatic islets together with the assessment on their morphology, viability, and functionality. The best enriched culture medium was used for further study.

Strategies 2b) Exploration of the enriched culture protocol's effect on the isolated pancreatic islets derived from gut leak-induced diabetes type II mouse model.

The selected enriched culture medium was used to culture the isolated pancreatic islets derived from three different groups: wildtype (WT), IL-10KO, and GL-IL-10KO groups. The effect of the selected enriched culture medium was assessed based on the islet's morphology, viability, and functionality at 6-time points (0, 3rd, 5th, 7th, 14th, 21st day respectively) and was analyzed statistically among three groups.

Materials and Methods

The experiment was conducted after the protocol approved by the Chulalongkorn University-Institutional Animal Care and Use Committee (CU-IACUC) (Protocol No. 1973029). The animals were housed at the Chulalongkorn University Laboratory Animal Center (CULAC).

Detailed Material and Methods

1. Animals and Groups

The experiment was using 18 mice aged 13-week-old divided into three groups (n=6/group): WT, IL-10KO, and GL-IL-10KO, and were housed for 84 days. The GL-IL-10KO group mice were treated with 2% Dextran Sulphate Sodium (DSS) perorally on week 1 and 7 to induce gut leak. The animal was sacrificed at week 12 of the experiment.

2. Pancreas Histology with Hematoxylin & Eosin (H&E) Staining

The pancreas from each group was collected in 4% paraformaldehyde and embedded in paraffin. Prior staining, the paraffin-embedded of the pancreas was sectioned and deparaffinized using xylene. The sections were rehydrated with several dilution of ethanol and stained with H&E staining (Nurdiana et al., 2017). The samples were checked under light microscope.

3. Pancreatic Islets Isolation

The isolation of islets of Langerhans was using previous protocols (Li et al., 2009; Graham et al., 2016; Saliba and Farès, 2019; Villarreal et al., 2019) with some modification. Shortly, after the mice are sacrificed, the abdomen was opened and moving aside all the intestine, exposing the common bile duct of the pancreas. The pancreas was digested by injecting 1ml of Cell Recovery Solution (Thermo Fisher Scientific) via common bile duct using 27.5G needle and 3 ml syringe (NIPRO, Japan). The pancreas was then dissected from the abdomen and put on the transport media (1ml working solution with the ice-cold condition). The working solution was composed 1:1 of 1% HBSS (Thermo Fisher Scientific) and $CaCl_2$ 2 mM (Sigma-Aldrich) with the additional of 2% antibiotic-antimycotic solution (Thermo Fisher Scientific).

To isolate pancreatic islets, the pancreas was directly incubated for 10 mins followed by centrifugation at 300 g 3 mins and 4°C. After that, the supernatant was discarded to remove the unwanted materials and to stop the digestion by adding 1ml of the ice-cold condition of working

solution before re-centrifuge on 300 g 3 mins at 4°C. The supernatant was removed, and 1 ml of working solution ice-cold condition was added and gently tapped. The pancreas was then poured through the 70 µm Cell Strainer (Thermo Fisher Scientific). The Cell Strainer was directly washed with 1 ml working solution three to five times and transferred over a new 50 ml tube container. The pancreas was mashed on the strainer until smooth. The flow through was centrifuged for 5 mins at 200 g, 4°C, and the supernatant was discarded. The pellet was mixed with the culture media and cultured on the 60 mm cell culture dish (Thermo Fisher Scientific). The culture media was changed every 48 hours.

4. Enriched Culture Condition for Pancreatic Islets Recovery

The enriched culture condition medium was freshly made prior to usage, namely VSCBIC-1 and VSCBIC-2, and compared with the basal medium (Table 1). The recipes of enriched culture condition medium were using the Roswell Park Memorial Institute 1640 (RPMI1640) (Thermo Fisher Scientific) as the basal medium supplemented with 10% Fetus Bovine Serum (FBS) (Thermo Fisher Scientific), 1% antibiotic-antimycotic (Thermo Fisher Scientific), and 1% of GlutaMax (Thermo Fisher Scientific) added with other supplements. Due to the confidential contract, the supplements cannot be disclosed.

Table 1. Composition of new enriched culture protocols

VSCBIC-1	**VSCBIC-2**
Serum	Serum
Antibiotic-antimycotic	Antibiotic-antimycotic
Essential amino acids	Essential amino acids
Non-essential amino acids	Non-essential amino acids
Antioxidant	Antioxidant
Small molecule-Induce islets' function	Small molecule-Induce islets' function
-	Small molecule-Induce cells proliferation
-	Growth factors

5. Cell Counting and Morphology Analysis

The isolated pancreatic islets were counted using a 96-well-plate under inverted microscope. The counted number was then timed to the total volume to get the total number of islets. The distribution size number was counted by capturing 5 field views and measured using ImageJ software (National of Institutional Health, USA).

6. Trypan Blue Staining

The trypan blue staining was used for viability percentage calculation. A 0.4% Trypan Blue (Sigma-Aldrich) was mixed with the islets and counted under an inverted microscope. The counted result was then calculated using the formula:

$$\left(\frac{\boldsymbol{total\ viable\ cells}}{\boldsymbol{all\ cells}} \times 100\%\right).$$

7. Live/Death Staining

The Live/Death staining was performed for evaluating the condition of the isolated pancreatic islets. The islets were stained by incubating 30 mins with 50 µl of the staining solution containing Enzo® (ENZO LIFE SCIENCES, INC) or Calcein AM-PI (Thermo Fisher Scientific) with sterile PBS pH 7.4 at ratio 1:1000. After incubation, the islets were washed with phosphate buffer saline (PBS) (Sigma-Aldrich) and checked under a fluorescence microscope.

8. Glucose Stimulated C-peptide Secretion

The glucose stimulated C-peptide secretion assay was performed to evaluate the C-peptide towards the total DNA/hour by using three conditions of Krebs-Ringer bicarbonate HEPES (KRBH) buffer; normal KRBH, KRBH with glucose 5.5 mM, and with glucose 22 mM. Shortly, the isolated pancreatic islets were incubated with the KRBH solution respectively for 1 hour each at 37°C with 5% CO_2, then they were centrifugated for 5 mins at 500 rpm, 15°C. The supernatant of each incubation results was then collected into a tube for checking C-peptide level by ELISA (Sigma-Aldrich) according to its manufacturer protocol. At the last step of incubation, the pellet was then collected for DNA extraction using Proteinase K/Papain (Worthington Biochemical, New Jersey, USA) and calculated the concentration by Qubit® Kit (Thermo Fisher Scientific) according to the manufacturer protocol.

9. Real Time-quantitative Polymerase Chain Reaction (RT-qPCR)

The RT-qPCR was conducted to observe the pancreatic endocrine, pancreatic islets, and islets-related gene expression at six-time points during culture (day 0, 3rd, 5th, 7th, 14th, 21st). The RNA was obtained from the islets of Langerhans by collecting in the TRIzol-RNA isolation reagent (Thermo Fisher Scientific) and mixed well with chloroform by vortex mixer followed by centrifugation for 5 mins at 12.000 g, 4°C. The supernatant was collected, processed with DirectZol-RNA isolation kit (ZymoResearch, USA) and measured quantitatively using QUBIT® (Thermo Fisher Scientific). The collected RNA was converted into complimentary DNA (cDNA) using ImProm-II® reverse transcription kit (Promega, USA) according to the manufacturer's methods. Following the conversion, the cDNA was used for RT-qPCR by using PowerUp™ SYBR™ Green Master Mix Kit (Thermo Fisher Scientific) and Real Time PCR detection system (Bio-Rad) according to manufacturer protocol using the listed gene primers (Table 2). Relative mRNA expression described the level of gene expression by normalizing with housekeeping gene expression and control.

Table 2. Genes Primer

Genes	Accession No	Seq	Primer Sequence (5' - 3')	Tm (°c)	Length size (bp)
			House keeping		
Rpl13a	NM_009438.5	F	TGAATACCAACCCCTCCCGA	60.25	99
		R	CTCTCTTGGTCTTGTGGGGC	60.32	
			Pancreatic endoderm markers		
Ngn3	NM_009719.6	F	GCTGCTTGACACTGACCCTA	59.68	194
		R	AGGTGGGGTGGAATTGGAAC	59.89	
Nkx-2.2	NM_001077632.1	F	TCGCTACAAGATGAAACGTGC	59.28	96
		R	CTTGCGGACACTATGGGCA	60.08	
Pdx1	NM_008814.4	F	CCTTTCCCGAATGGAACCGA	60.04	132
		R	TTCCGCTGTGTAAGCACCTC	60.32	
Hnf-6	NM_008262.3	F	GCAACGTGAGCGGTAGTTTC	59.84	162
		R	GTCCTTGCTGGGAGTTGTGA	59.89	
			Pancreatic islets markers		
Nkx-6.1	NM_144955.2	F	GCACGCTTGGCCTATTCTCT	60.46	200
		R	TTCGGGTCCAGAGGTTTGTT	59.16	
Maf-a	NM_194350.2	F	CGCACCCGACTTCTTTCTGT	60.60	95
		R	CAGAGTCTGAACCGAGACCG	59.83	
Glut-2	NM_031197.2	F	ACCGGGATGATTGGCATGTT	60.03	140
		R	GGACCTGGCCCAATCTCAAA	59.96	
Isl1	NM_021459.4	F	CCCAGAGTCATCCGAGTGTG	59.83	127
		R	GAGTTCCTGTCATCCCCTGG	59.46	
Insulin1	NM_008386.4	F	AATGGGCCAAACAGCAAAGT	58.87	160
		R	TAGGAAGTGCACCAACAGGG	59.60	
Insulin2	NM_001185083.2	F	GCAAGCAGGAAGGTTATTGTTTCA	60.26	156
		R	CACACACCAGGTAGAGAGCC	59.75	
			Islets-related markers		
Glp-1R	NM_021332.2	F	GGGCCAGTAGTGTGCTACAA	59.68	111
		R	CTTCACACTCCGACAGGTCC	60.04	
Gcg	NM_008100.4	F	TCTACACCTGTTCGCAGCTC	59.76	172
		R	GTCCTCATGCGCTTCTGTCT	60.11	
Maf-b	NM_010658.3	F	GGCAACTAACGCTGCAACTC	60.11	109
		R	CGGAAGGGACTTGAACACCA	59.89	

10. Data analysis

The dot plot (n=4) graph was used for represented the study results by GraphPad Prism software (GraphPad Company, USA), and the statistical analysis was analyzed by SPSS software (IBM Corporation, USA). The significance of differences between groups was calculated using analysis of variance (ANOVA) and followed by the Post hoc test, Dunnett. The significance level was accepted if $p < 0.05$.

CHAPTER IV
RESULTS AND DISCUSSION

Results

The appearance of fresh isolated Islets from DSS-induced gut leak on IL-10KO mice

The H&E histological stains showed no defect on the pancreas from the gut leak-induced diabetes type II model group (GL-IL-10KO) compared to the transgenic control group (IL-10KO) and the background control group (WT) (Fig. 3A). Morphologically, the freshly isolated islets from all groups demonstrated the uniformly spheroid shape (Fig. 3B). However, the number of isolated islets among groups was significantly different. The GL-IL-10KO showed the highest number of isolated islets followed by WT control and the IL-10KO control, respectively (Fig 3C). Moreover, GL-IL-10KO revealed a higher trend of the number of islets with the diameter size less than 20 μm, while it showed the significant lowest number in islets diameter size 20-50 μm compared to WT (Fig 3D).

The viability of the isolated islets was observed in quantitative and qualitative aspects. The quantitative viability of the freshly isolated islets from the GL-IL-10KO group was significantly lower compared to IL-10KO and WT groups (Fig 3E). This finding was supported by the live/dead stained where the spherical shape of the islets was stained with bright green fluorescence of Calcein for live cytoplasm and red fluorescence of PI for dead cells (Fig 3F).

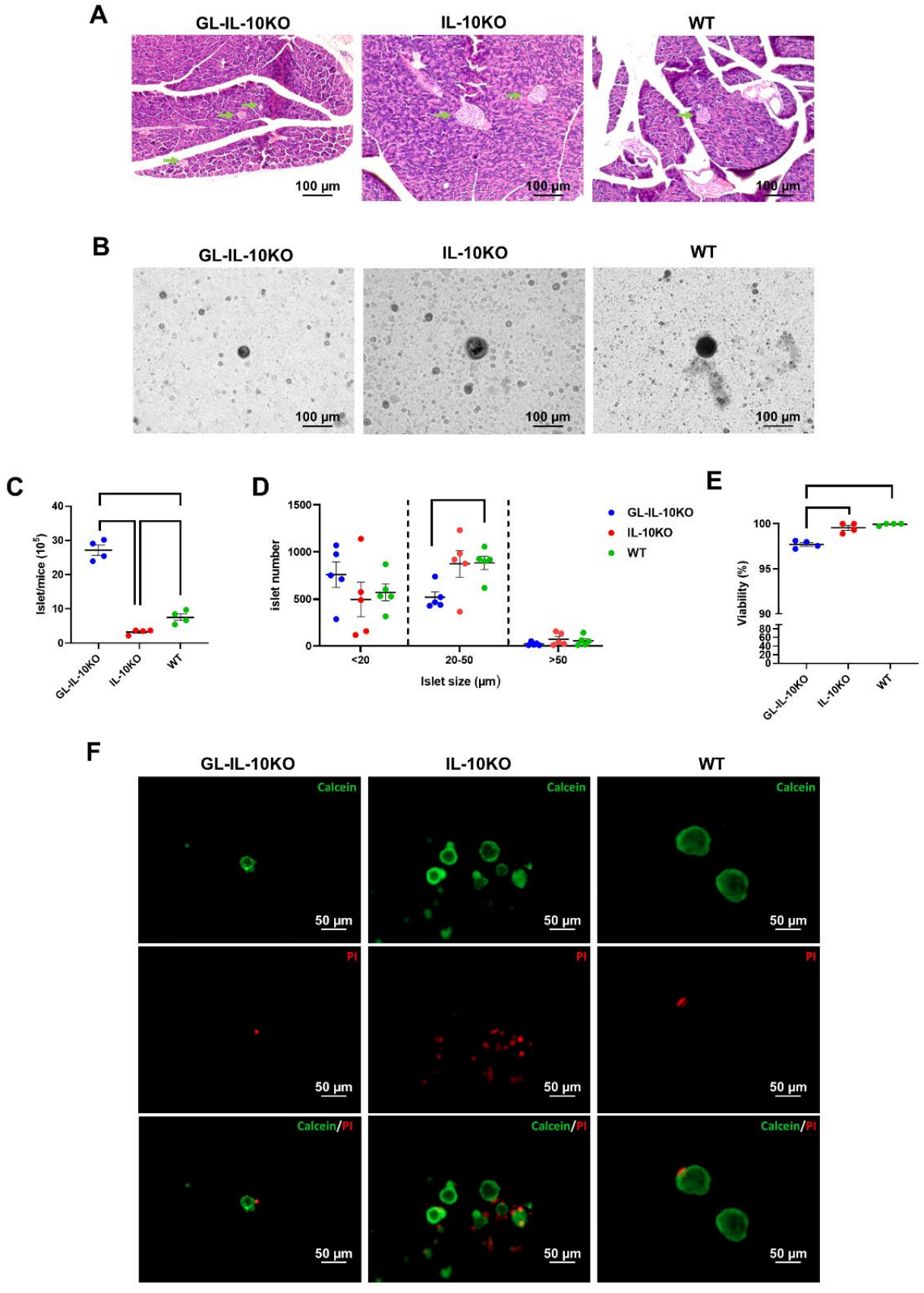

A
GL-IL-10KO
IL-10KO
WT
100 μm
B
GL-IL-10KO
IL-10KO
WT
100 μm
C
Islet/mice (10^5)
GL-IL-10KO
IL-10KO
WT
D
islet number
Islet size (μm)
<20
20-50
>50
GL-IL-10KO
IL-10KO
WT
E
Viability (%)
GL-IL-10KO
IL-10KO
WT
F
GL-IL-10KO
IL-10KO
WT
Calcein
PI
Calcein/PI
50 μm

Figure 3. Morphology and viability of fresh isolated islets among three groups.

Hematoxylin & eosin (H&E) staining of pancreas tissue from all groups of mice, the islets of Langerhans were shown with the green arrow (A). The fresh isolated islets morphological appearance showed under light microscope (B). The number of fresh isolated islets from each group (C) and islets diameter size differences among groups (D) were determined. The percentage of viable isolated islets were analyzed using trypan blue staining (E), and the qualitative results of viable islets were evaluated by Calcein/PI staining under fluorescent microscope (F). Bars indicated significant difference ($p < 0.05$).

The functional property of fresh isolated Islets from DSS-induced gut leak on IL-10KO mice

The functionality and the pancreatic islets-related mRNA expression of freshly isolated islets were explored (Fig. 4). Three groups of the genes of interest consisting of pancreatic endodermal markers (Fig. 4A), pancreatic islets markers (Fig. 4B), and islets-related markers (Fig. 4C) were analyzed by normalized with the housekeeping gene, *Rpl13a*, and WT control group. The pancreatic endodermal markers showed upregulated in both the Gl-IL-10KO and the IL-10KO control group, except *Pdx1* of IL-10KO control significantly showed downregulation (Fig. 4A). The upregulation of pancreatic islets markers showed on both GL-IL-10KO and IL-10KO control. However, *MafA* was downregulating comparing with WT control (Fig. 4B). The pancreatic islet-related markers were expressed in both GL-IL-10KO and IL-10KO except the *Glp1r* in the IL-10KO control was reducing (Fig. 4C).

The functional test was also performed to understand the ability of the islets to produce insulin by analyzing the C-peptide concentration/mg DNA in an hour (Fig 4D). The result revealed that islets derived from three groups were secreting the C-peptide, but they were decreasing followed the increase of glucose concentration. Nevertheless, the significant differences of C-peptide secretion level were noticed at glucose concentration 5.5 and 22 mM between GL-IL-10KO and IL-10KO control, and GL-IL-10KO and WT control, respectively.

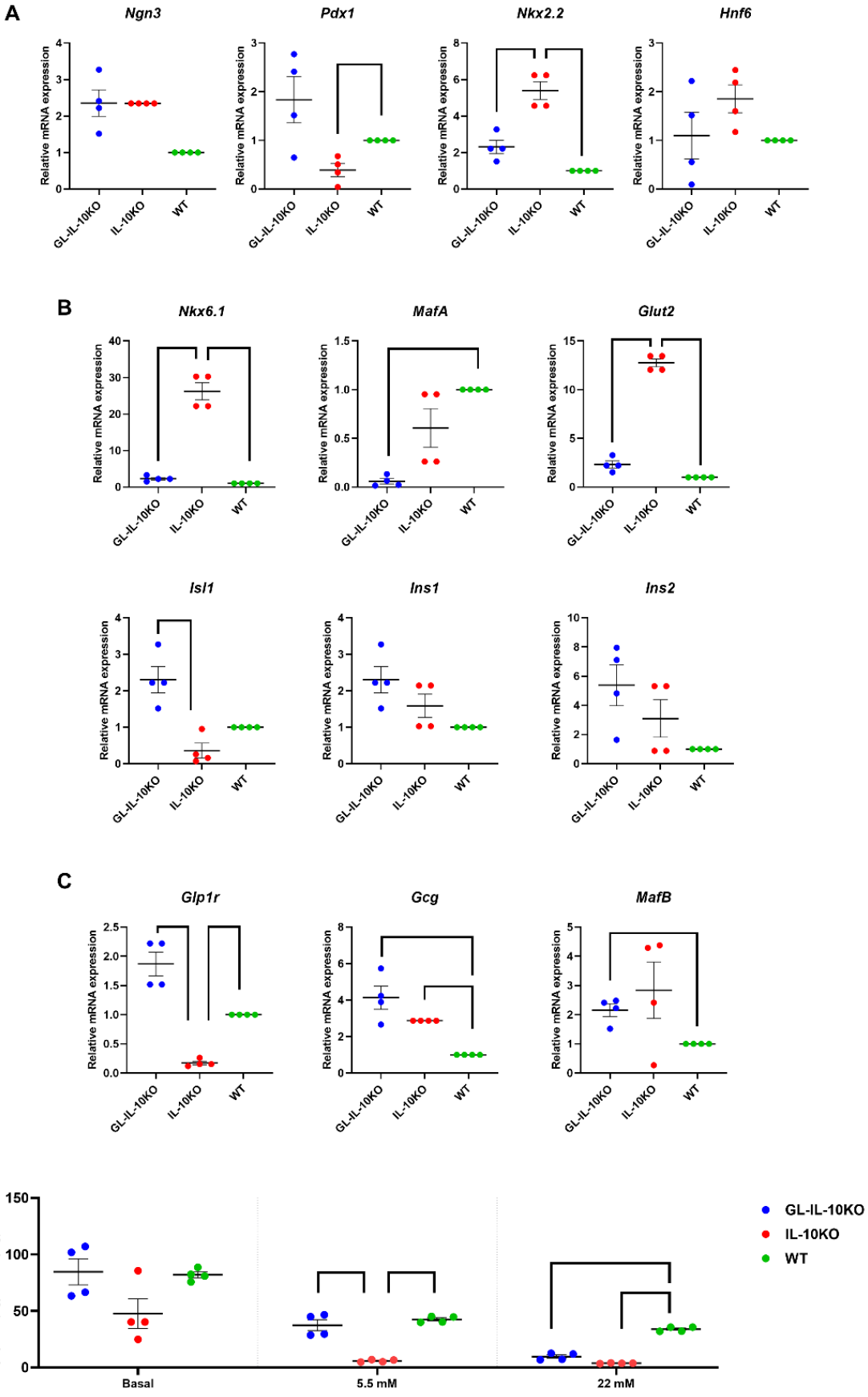

A
Ngn3
Pdx1
Nkx2.2
Hnf6
Relative mRNA expression
GL-IL-10KO
IL-10KO
WT
B
Nkx6.1
MafA
Glut2
Isl1
Ins1
Ins2
C
Glp1r
Gcg
MafB
D
C-peptide (ng) / DNA (mg) / 60 min
Basal
5.5 mM
22 mM
GL-IL-10KO
IL-10KO
WT

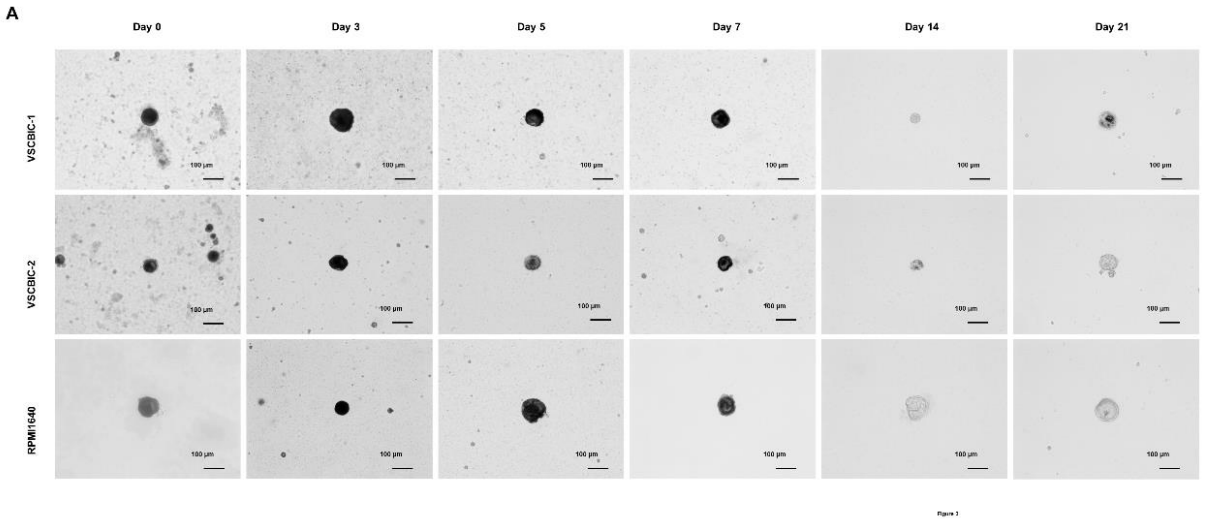
Figure 4. Functionality of fresh isolated islets among mice groups.

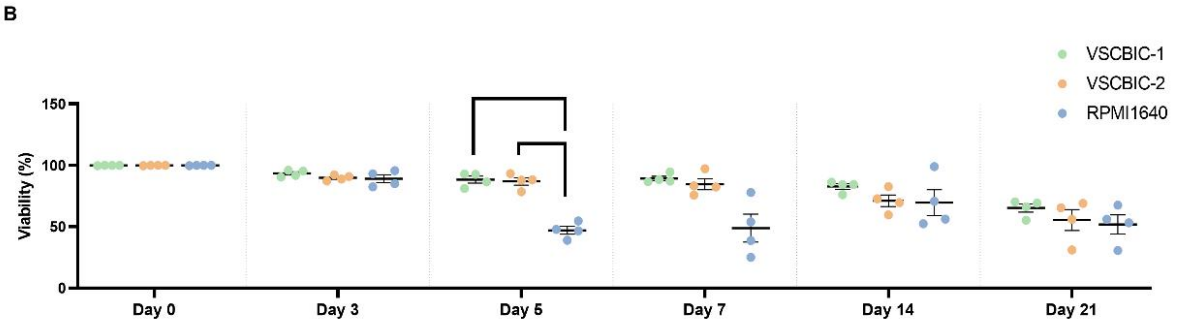
Relative mRNA expression of pancreatic endoderm markers (A), pancreatic islets markers (B), and islets-related markers (C) were analyzed by RT-qPCR. The islets function on secreting C-peptide was detected by glucose stimulating c-peptide secretion with basal glucose concentration, 5.5mM concentration, and 22mM concentration of glucose (D). Bars indicated significant difference ($p < 0.05$).

VSCBIC-1, a newly established conditioned medium, recovers the isolated islets appearance

The enriched culture protocol was obtained by comparing the isolated islets from WT control mice which were cultured in two media types, namely VSCBIC-1 and VSCBIC-2. These media were compared with the normal culture medium, RPMI1640 supplemented with 10% FBS, 1% antibiotic-antimycotic, and 1% GlutaMax. The morphology was checked under the light microscope with 6 times points (Fig. 5A). The islets cultured with RPMI1640 medium showed rough edges of the islets on day 7. Yet, the different results were observed in the other 2 newly established conditioned media, the cultured islets with VSCBIC-1 and VSCBIC-2 were started to show morphological changes on day 14. Even though, the islets appearance from VSCBIC-2 and normal RPMI1640 revealed the clear spheroid shape islets on day 21, the islets from VSCBIC-1 showed the dark spots of accumulated cells inside the spheroidal shape layer.

Furthermore, the percentage of islets viability by trypan blue appeared the dramatical significance reducing of islets viability from the normal RPMI1640 medium on day 5. Still, the cultured islets from both newly established conditioned media presented the slightly decreasing of viable islets in a time-dependent manner (Fig. 5B). This result was confirmed by live/dead staining using Enzo® staining kits, where the islets cultured with VSCBIC-1 showed the red fluorescence of PI as dead cell indicator was lesser than islets from VSCBIC-2 and RPMI1640 (Fig. 5C). All islets were counterstain with DAPI as the nuclei staining.


A
Day 0
Day 3
Day 5
Day 7
Day 14
Day 21
VSCBIC-1
VSCBIC-2
RPMI1640
100 µm


B
VSCBIC-1
VSCBIC-2
RPMI1640
Viability (%)
150
100
50
0
Day 0
Day 3
Day 5
Day 7
Day 14
Day 21

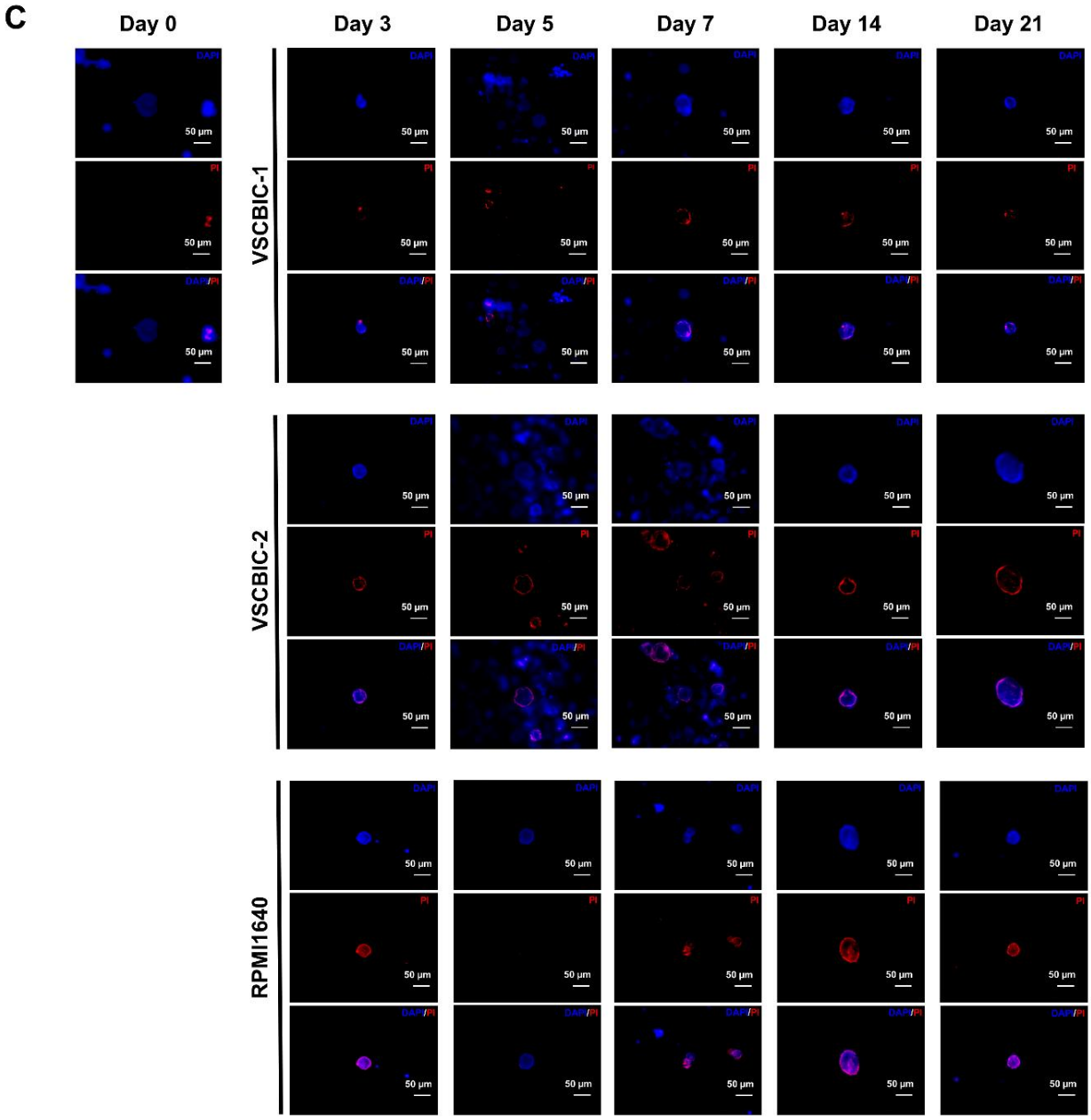

C
Day 0
Day 3
Day 5
Day 7
Day 14
Day 21
VSCBIC-1
VSCBIC-2
RPMI1640
DAPI
PI
DAPI/PI
50 μm

Figure 5. Morphology and viability of cultured islets with different media.

Microscopic result of isolated islets under light microscope on day 0, 3, 5, 7, 14, and 21 (A), number of viable islets by trypan blue staining comparing on day 0, 3, 5, 7, 14, and 21 (B), and qualitative results of viable islets by Enzo® staining under fluorescent microscope on day 0, 3, 5, 7, 14, and 21 (C). Bars indicated significant difference ($p < 0.05$).

VSCBIC-1, a newly established conditioned medium, recovers the isolated islets functional property

The effect of the media on islets functional recovery was evaluated by assessing the islets-related mRNA expression throughout the culture periods. To study the possibility of the islets to develop or regenerate, the pancreatic endoderm markers were analyzed (Fig. 6A). The *Ngn3* was expressed by the islets cultured in VSCBIC-2 and RPMI1640 on day 7 and 14, which significantly different compared to the islets cultured in VSCBIC-1. This finding was parallel with the expression of *Hnf6*, the islets cultured in VSCBIC-1 was expressed at the early culture, while it was expressed at the middle of the cultured period of islets in VSCBIC-2 culturing. However, on the late day of day 21, the islets from both newly established conditioned media showed significant downregulation of *Hnf6* comparing with normal RPMI1640 medium. In addition, the *Pdx1* were significantly different on day 5 from islets cultured with VSCBIC-1, then they were remaining at low expression until day 21. Unlike the other three genes, the *Nkx2.2* expression was diverse in all culture media during the culture period. On day 5, the pancreatic islets markers (*Nkx6.1*, *Isl1*, and *Ins1*) of cultured islets with VSCBIC-1 showed an upregulated significant difference (Fig 6B). Moreover, the cultured islets from VSCBIC-1 had shown a significant increase of *MafA* from day 5 to 14. Meanwhile, *Ins2* from VSCBIC-1-cultured islets was also significantly upregulated on day 7 and 14. *Glut2* was lowly expressed in all culture media.

To confirm the medium with the best effect of media for retaining the islets condition whether they were able to mimic in the natural environment, the islets-related markers were tested (Fig. 6C). The result showed neither *Glp1r*, *Gcg*, nor *MafB* were continuously expressed until day 21. However, only the *Glp1r* and *Gcg* were expressed during the early culture period until day 5 and continued to be downregulated.

To support the findings of the expressed mRNA, the functional test of glucose stimulating C-peptide secretion was performed (Fig. 6D). All conditions showed a good trend of C-peptide secretion on responding glucose stimulating in glucose concentration dependent manner from day 3 until day 7. Eventually on day 21, the islets cultured with VSCBIC-1 showed significantly higher C-peptide releasing compared to the islets cultured with VSCBIC-2 and normal RPMI1640 media in all glucose stimulating C-peptide secretion condition.

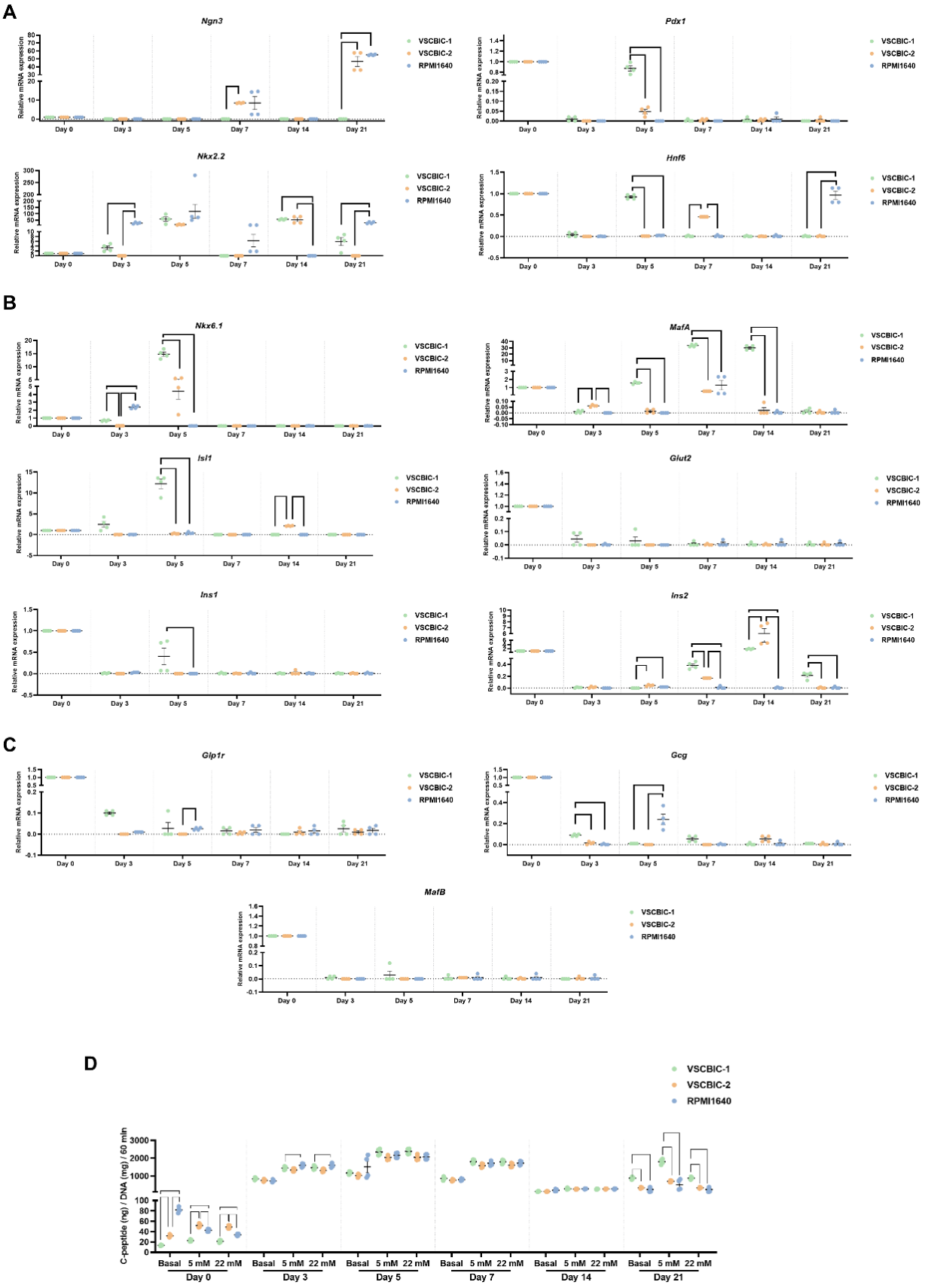
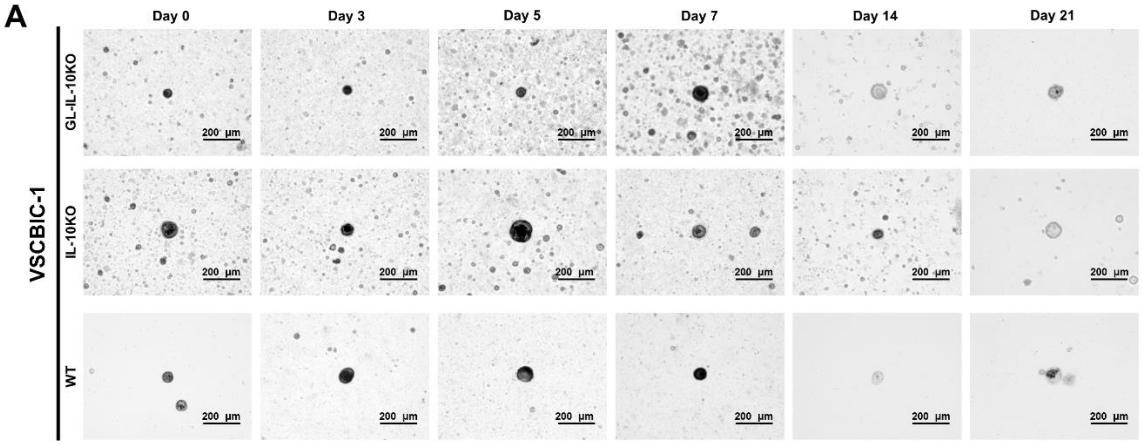
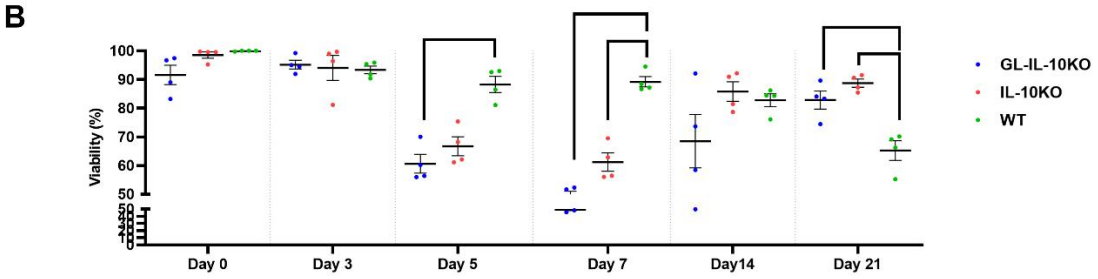

A
Ngn3
Pdx1
Nkx2.2
Hnf6
B
Nkx6.1
MafA
Isl1
Glut2
Ins1
Ins2
C
Glp1r
Gcg
MafB
VSCBIC-1
VSCBIC-2
RPMI1640
Relative mRNA expression
Day 0
Day 3
Day 5
Day 7
Day 14
Day 21
D
C-peptide (ng) / DNA (ng) / 60 min
Basal
5 mM
22 mM
Day 0
Day 3
Day 5
Day 7
Day 14
Day 21

Figure 6. Functionality of isolated islets cultured on three different medium comparison. Relative mRNA expression of pancreatic endoderm markers (A), pancreatic islets markers (B), islets-related markers (C) were analyzed. The islets function on secreting C-peptide were detected by glucose stimulating C-peptide secretion with basal glucose concentration, 5.5mM concentration, and 22mM concentration of glucose (D). Bars indicated significant difference ($p < 0.05$).

Application of VSCBIC-1 medium for recovering the appearance of isolated pancreatic islets derived from DSS-induced gut leak on IL-10KO mice

The VSCBIC-1 medium was selected as the isolated islets recovery medium according to the prior findings and it was used for culturing the isolated islets from GL-IL-10KO as the gut leak induced type II diabetes model, IL-10KO group as the transgenic control, and WT as the background control. The islets were observed for 21 days with 6-time points. The morphological outcome demonstrated the islets from GL-IL-10KO and WT group started to change their appearance on day 14, while the IL-10KO group was changing their morphology later on day 21 (Fig. 7A). Nonetheless, both GL-IL-10KO and WT groups the aggregated cells inside the spheroid layers on day 21. This finding was then followed by the viability assessment which was revealed the highest viability of WT group-isolated islets from day 5 until day 14 of culture (Fig. 7B). However, on the cultured day 21 of, the GL-IL-10KO and IL-10KO overtook the WT group together with the results from live/dead staining (Fig. 7C).


A
Day 0
Day 3
Day 5
Day 7
Day 14
Day 21
VSCBIC-1
GL-IL-10KO
IL-10KO
WT
200 µm


B
Viability (%)
100
90
80
70
60
50
Day 0
Day 3
Day 5
Day 7
Day14
Day 21
GL-IL-10KO
IL-10KO
WT

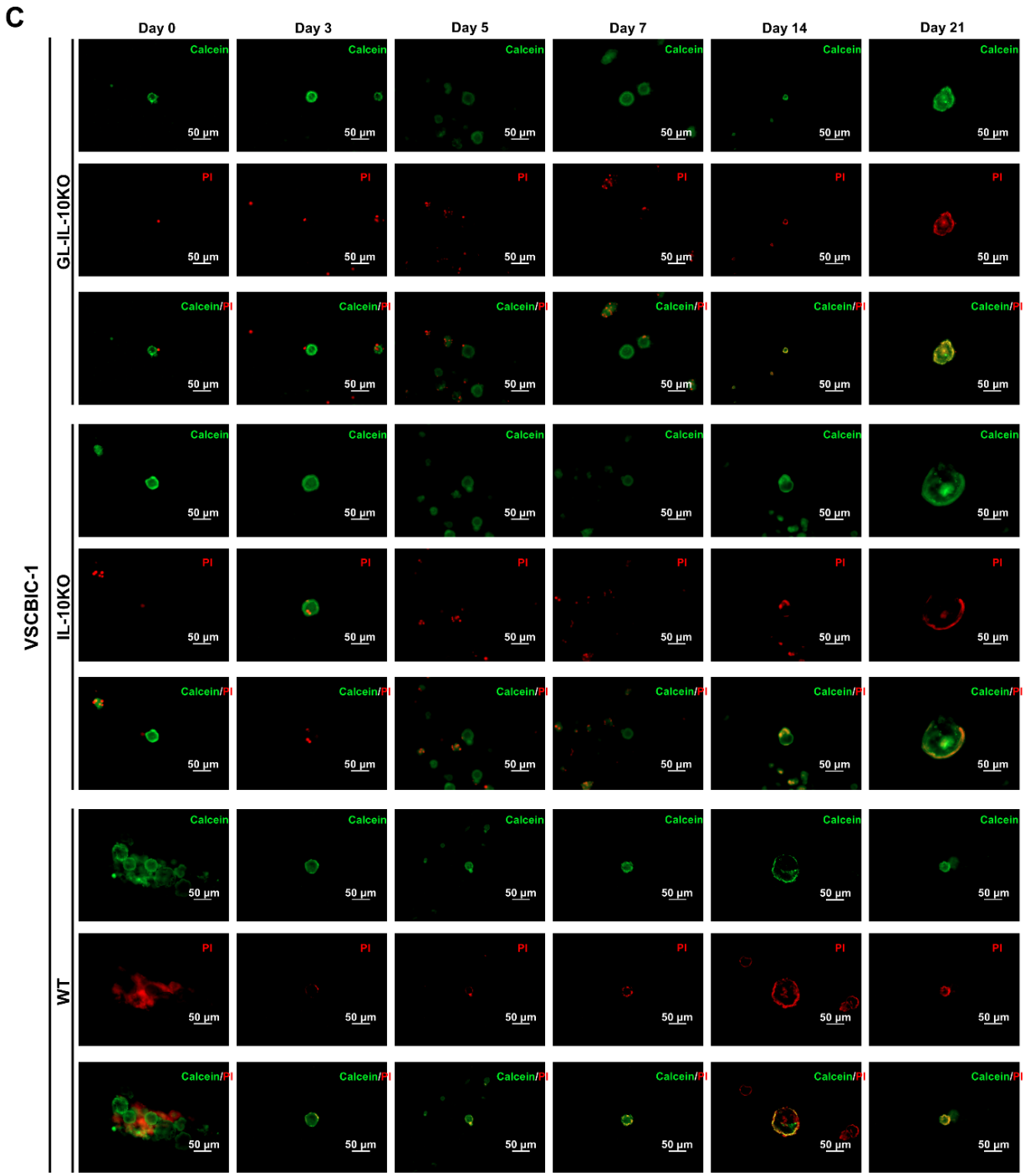


Figure 7. Morphology and viability of islets from GL-IL10KO, IL-10KO, and WT mice cultured with VSCBIC-1 medium.

Microscopic images of isolated islets under light microscope on day 0, 3, 5, 7, 14, and 21 (A), the number of viable islets by trypan blue staining on day 0, 3, 5, 7, 14, and 21 (B), and the qualitative results of viable islets by Calcein/PI staining under fluorescent microscope on day 0, 3, 5, 7, 14, and 21 (C). Bars indicated significant difference ($p < 0.05$).

VSCBIC-1 medium retains the functional property of isolated pancreatic islets derived from DSS-induced gut leak on IL-10KO mice

Concurrent to the morphology and viability findings, the expression of pancreatic progenitor was interestingly fluctuating (Fig. 8A). The *Ngn3* was expressed by the GL-IL-10KO and IL-10KO groups for 7 days, and it was abruptly dropping on day 21. Also, the *Pdx1* was upregulating expression until day 7 in the GL-IL-10KO group, and it was dramatically expressed on day 14 in the IL-10KO group. In addition, both *Nkx2.2* and *Hnf6* were expressed throughout the culture period and highly significantly expressed in GL-IL-10KO on day 21.

The pancreatic islets markers analysis (Fig. 8B) revealed that the *Nkx6.1*, *Isl1*, and *Ins2* were expressed on early culture, and then were dramatically downregulated until day 21. While the *MafA* was highly expressed on day 7 in all derived islets group, *Glut2* was significantly expressed by the IL-10KO group on day 14. Since *Ins1* showed stable expression since day 7 in IL-10KO group, the GL-IL-10KO appeared a high expression on day 14.

Additionally, the islets-related markers were also observed (Fig. 8C). The result showed that the *Glp1r* was expressed until day 7 in the IL-10KO group. *Gcg* was also expressed significantly higher on day 7, however, GL-IL-10KO showed the expression from day 3 until day 14. Even though, the *MafB* expression was increasing by the day-dependent manner in GL-IL-10KO and IL-10KO groups, it was the highly significant expression on day 14 comparing with IL1-10KO and WT groups.

The function of the isolated islets from GL-IL10KO, IL-10KO, and WT groups were analyzed using glucose stimulating C-peptide secretion assay with 6-time points for 21 days. The outcome revealed that all groups started to respond the glucose stimulation on day 3 compared to day 0 (Fig. 8D).

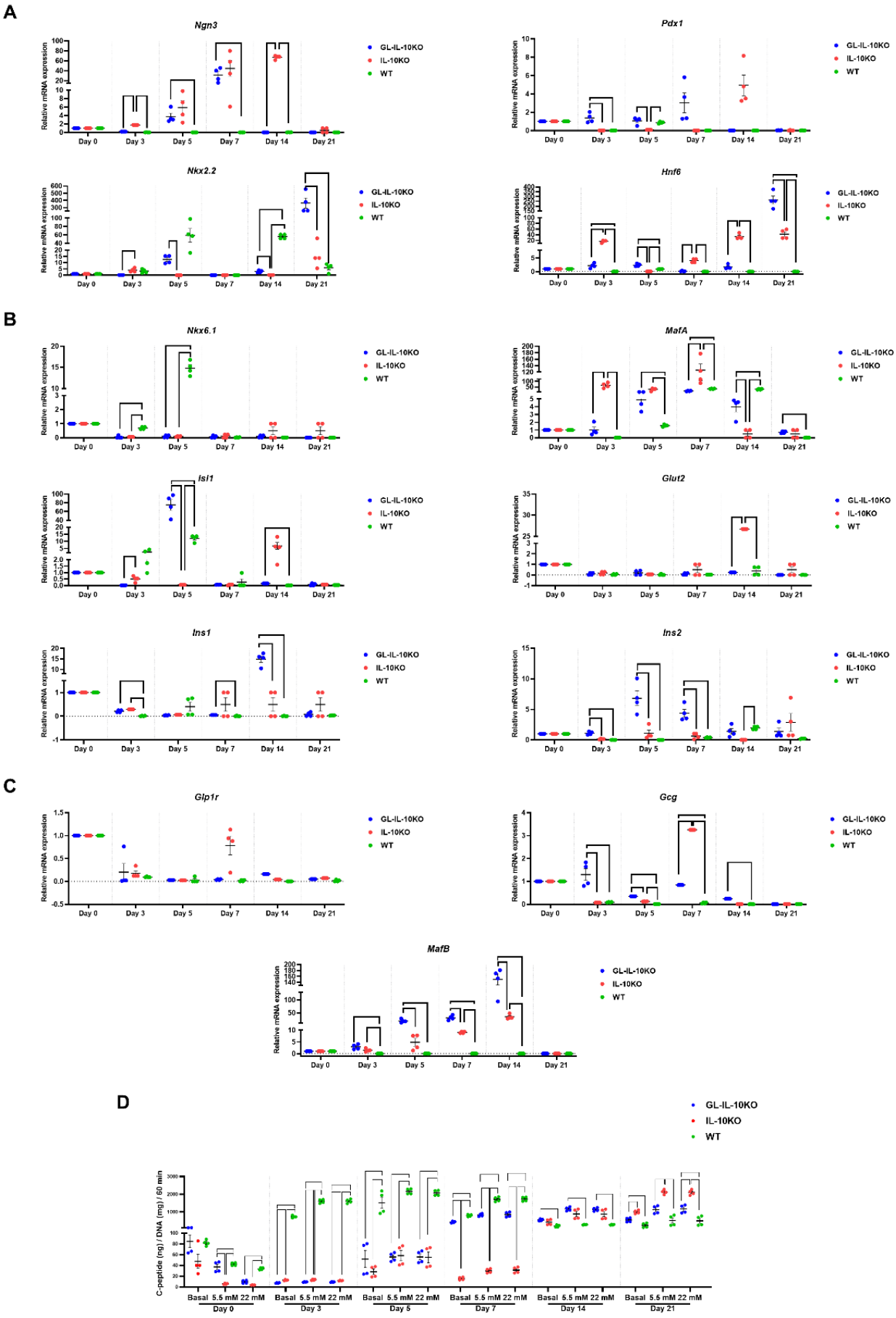

A
Ngn3
Pdx1
Nkx2.2
Hnf6
B
Nkx6.1
MafA
Isl1
Glut2
Ins1
Ins2
C
Glp1r
Gcg
MafB
D
Relative mRNA expression
C-peptide (ng) / DNA (mg) / 60 min
GL-IL-10KO
IL-10KO
WT
Day 0
Day 3
Day 5
Day 7
Day 14
Day 21
Basal
5.5 mM
22 mM

Figure 8. Functionality of GL-IL-10KO, IL-10KO, and WT isolated islets cultured on VSCBIC-1 medium comparison.

Relative mRNA expression of pancreatic endoderm markers (A), pancreatic islets markers (B), and islets-related markers (C) were determined by RT-qPCR. The C-peptide secretion upon glucose stimulating with basal glucose concentration, 5.5mM concentration, and 22mM concentration of glucose were evaluated by ELISA (D). Bars indicated significant difference ($p < 0.05$).

Discussion

The type II diabetes is known as the result of insulin resistance. Obese-induced leaky gut is assumed as one of the predisposition factors which is causing by the shifting of bacterial in the intestine (Chakaroun et al., 2020). The previous finding suggested that the decrease of butyrate can promote bacteria production resulting in the downregulated of *Glp1* and the increase of Gram-negative bacteria may cause endotoxemia, which may lead to diabetes type II (Aw and Fukuda, 2018). Additionally, IL-10 is recognized as an anti-inflammatory cytokine which abundantly available on the intestinal tract (Morhardt et al., 2019). The deficiency of IL-10 may slower the gut leak healing process, and it may be able to develop spontaneous colitis (Morhardt et al., 2019). To validate this concept, this present study used the IL-10KO mice which were treated with DSS, a capable chemical for inducing the gut leak.

To answer the first hypothesis of the present study, the pancreases from three groups of mice comprising GL-IL-10KO, IL-10KO, and WT, were collected and processed for histological purposes and islets isolation. According to Westermark and Westermark (2008), the diabetes type II islets show amyloid depositions inside the islets with little endocrine cells. In this study, even though, the GL-LI-10KO islets did not detect any amyloid depositions when stained with hematoxylin & eosin, the islets have mostly small size.

The freshly isolated islets from GL-IL-10KO, IL-10KO, and WT illustrated the similar of islets morphological appearance accepting to the previous study conducted by Jo et al. (2007). Meanwhile, the number of the freshly isolated islets from the GL-IL-10KO group was higher than the other groups which the mostly size of islets was very small, lower than 20 μm in diameter. However, the size of islets from the WT group was around 20-50 μm in diameter which was also in the range of previous mice islets diameter size (20-350 μm of diameter) (Huang et al. (2018). The islets viability of the freshly isolated islets also showed significant different between GL-IL-10KO towards IL-10KO and WT. Perhaps, the viability of the islets likely caused by the effect of the DSS induced gut leak.

On pancreatic islets development, it must go through several stages including the definitive endoderm, pancreatic endoderm, endocrine progenitor, and pancreatic islets (Takeuchi et al., 2014). To understand the underlying genes affected by DSS induction on GL-IL-10KO, the pancreatic endoderm, pancreatic islets, and islets-related markers were determined. In the present study, the

freshly isolated islets of GL-IL-10KO was expressing all of the pancreatic endoderm markers (*Ngn3, Pdx1, Nkx2.2,* and *Hnf6*), however, *Hnf6* showed a low expression not different from WT which is good for maintaining spheroid islets shape and β-cell function (Gannon et al., 2000). The pancreatic islets markers, *Nkx6.1, MafA,* and *Glut2*, are important for maturing the β-cell, preventing the impaired β-cell glucose intolerance and insensitivity, and regulating the glucose metabolic (Aigha and Abdelalim, 2020; Huang et al., 2020) which were low expressed by freshly isolated islets of GL-IL-10KO group. However, the GL-IL10KO was expressing the *Isl1, Ins1,* and *Ins2,* which are necessary for regulating islets development, β-cell differentiation, and insulin secretion (Hickey et al., 2013; Ediger et al., 2014). The fresh Isolated islets from all groups also expressing the islets-related marker *Gcg,* and *MafB*. However, the *Glp1r* was not expressed by the IL-10KO group which is important for β-cell production and insulin promotor (Zhu et al., 2019). This result implies that the islets may develop some mild defect of insulin resistance.

Additionally, the function of the islets for secreting C-peptide to respond to the glucose stimulation is implying the endogenous insulin. Generally, insulin is synthesized together with C-peptide derived from proinsulin (Wahren et al., 2012). Therefore, the secretion of C-peptide upon stimulation with three different glucose concentrations (0, 5.5, and 22 mM of glucose) from isolated islets of GL-IL-10KO, IL-10KO, and WT groups, present a good sensitivity and ability on insulin production and secretion. However, at the highest glucose concentration, the C-peptide secretion level of GL-IL-10KO and IL-10KO groups was lower than WT, this result illustrates the reduction of islets ability.

Rendering the second hypothesis, the newly established conditioned media protocols to recover the isolated islets, i.e., VSCBIC-1 and VSCBIC-2, were studied and selected as a suitable protocol for further recovering the islets from GL-IL-10KO. To evaluate the best protocols, the isolated islets obtained from WT mice were cultured with VSCBIC-1 and VSCBIC-2 compared with the RPMI1640 medium as the basal or control medium. Previously, mice islets have spheroid shape (Huang et al., 2018) which was noted in the result of this study. Additionally, it was remarkably on day 21 the islets from VSCBIC-1 cultured medium showed the intact cells inside the spheroid mantle, suggesting the growth of the islets resembling the previous study induced by Islets Neogenesis Associated Protein ($INGAP^{104-118}$) (Jamal et al., 2005). Nevertheless, it cannot be confirmed since the IHC/ICC was not performed for exploring the pancreatic duct-like cells.

Re-enforce the morphology and viability result, the functionality was investigated by the expression of pancreatic islets-related mRNA and the glucose stimulating C-peptide secretion. The *Ngn3* is the key player on early pancreatic development to be endocrine composing cells (Rukstalis and Habener, 2009), and it was expressed by islets cultured with VSCBIC-2 and RPMI1640 medium but not found in the islets cultured with VSCBIC-1. This result was also supported by the *Hnf6* expression which showed in the islets cultured with VSCBIC-1 on early culture, whilst was expressed on the late cultured period by the islets cultured with VSCBIC-2 and RPMI1640. The *Hnf6* is also known as the early markers on pancreatic growth, but then the late expression of *Hnf6* is also important for duct epithelial development (Zhang et al., 2009). In contrast, the *Pdx1* was only significantly different expressed on the islets cultured with VSCBIC-1 at day 3, and then sudden downregulated on the next days during cultured periods. As it is recognized widely, the *Pdx1* is one of the important genes helping in driving the pancreatic progenitor cells toward endocrine cells, and enhancing the growth, the number, and the function of β-cells (Gao et al., 2014). Furthermore, the *Nkx2.2* was expressed vary during culture, this gene is well-known for the longer period of expression even in the mature islets (Watada et al., 2003). The pancreatic islets markers were adopted for checking the function of the islets, i.e. *Nkx6.1*, *MafA*, *Isl1*, *Glut2*, *Ins1*, and *Ins2*. The highest expression of *Nkx6.1*, *Isl1*, and *Ins1* were observed on day 5 of islets cultured with VSCBIC-1, indicated that their function was better than the islets cultured with VSCBIC-2 and RPMI1640. These three genes are important in the adult pancreas, mainly involve in glucose metabolism regulation (Brolén et al., 2005; Aigha and Abdelalim, 2020). Interestingly, the *Ins2* was also expressed by the islets cultured with VSCBIC-1 and noted significance different on day 21, over the expression by the islets cultured with VSCBIC-2 and RPMI1640. Both *Ins1* and *Ins2* were important on insulin secretion but on $Ins1^{+/-}$ with $Ins2^{+/+}$ resulting in diabetes in male mouse models (Babaya et al., 2006). The islets cultured with VSCBIC-1 was humbly expressing *MafA* at day 3, but it was noticed to be altered drastically and significantly different compared with the islets from VSCBIC-2 and RPMI1640 until day 14. Based on a previous publication, the expression of *MafA* indicates the maturation of β-cells, and the islets are not collected from diabetes type II patients (Matsuoka et al., 2015). Meanwhile, *Glut2* expression was not significant difference on the islets cultured among the three media. Conferring to (Leturque et al., 2009), the encoded *Glut2* is used in the glucose metabolic process as the glucose receptor of β-cells. Concomitantly, the islets-

related markers including *Glp1r* and *Gcg*, which little expressed, and *MafB* that expressed on day 0 indicating none of the culture media could maintain to mimics the native condition. The glucose stimulating C-peptide secretion results suggest that the VSCBIC-1 has the highest ability to maintain the C-peptide secretion. This finding was in line with *Ins2* expression which marks the secretion of insulin. The C-peptide was cleavage together with insulin from the proinsulin, therefore it indicates the insulin secretion.

As the VSCBIC-1 medium showed the highest rate of viability and functionality on WT mice, the VSCBIC-1 medium was decided as the isolated islets recovery medium for the GL-IL-10KO. On the earlier result, the GL-IL-10KO was known to be affected on viability and functionality of islets. Therefore, the VSCBIC-1 was used to recover the isolated islets from GL-IL-10KO.

During 21 days of culture, the islets still retain in the spherical shape. The intact cells found on day 21 on GL-IL-10KO and the WT that might be the developed cells originated from duct-like structures (Jamal et al., 2005). However, it should be confirmed with the IHC/ICC staining. The VSCBIC-1 medium cannot preserve the islets viability perfectly from GL-IL-10KO until day 7 culture. However, a noticeable increase of viability on day 21 was observed, which means that the VSCBIC-1 medium can regain the viability of the islets isolated from GL-IL-10KO.

This result was supported by the pancreatic islets-related mRNA expression, where the pancreatic endodermal markers, the *Ngn3, Nkx2.2, and Hnf6* which are usually expressed on the pancreas development (Dassaye et al., 2016), were expressed during the culture time. Additionally, these results were analogous with the little expression of *Pdx1* in all groups, indicating the development of the islets (Gao et al., 2014). On the other hand, the pancreatic islets-related markers, *Nkx6.1* only expressed in the WT for 5 days after isolation. This finding was strengthened with the *Glut2* expression result, which shows no groups can express in our study. The *Nkx6*.1 and the *Glut2* were working in synergy, regulating glucose metabolism (Aigha and Abdelalim, 2020). Therefore, in this study, the glucose metabolism could not be able to well-maintain until day 21. In contrast, *MafA, Ins1,* and *Ins2* were expressed throughout culture periods, which are involving in β-cells function and insulin secretion (Hang et al., 2014; Nishimura et al., 2015; Ramzy et al., 2017). The *Isl1* was also greatly expressed on day 5 which is working on controlling the development of islets by regulating the transcription of β-cells differentiation. The glucose stimulating C-peptide

secretion also confirms the ability of the islets on C-peptide secretion during culture time. The C-peptide secretion is important to mark the insulin that can be produced by the islets.

Shortly, the GL-IL-10KO islets have some defect on the health of the pancreas and islets compared to the other 2 groups. Meanwhile, the best medium selected for recovering the GL-IL-10KO was the VSCBIC-1 medium.

CHAPTER V
CONCLUSION

In conclusion, the concept of IL-10 deficiency on gut-leak causing diabetes type II was not clear yet. However, the DSS induced-gut leak on IL-10 knockout shows viability and functionality defect. Furthermore, the newly established conditioned medium, VSCBIC-1, was able to maintain the isolated islets morphology and functionality, although the trend was gradually downregulated. Therefore, it may become an alternative medium to keep the isolated islets for a long time.

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