OSTEOGENIC DIFFERENTIATION POTENTIAL BY MOUSE BONE MARROW-DERIVED MESENCHYMAL STEMCELLS (mBM-MSCs) DERIVED FROM GUT LEAK-INDUCED DIABETES TYPE II MOUSE MODEL

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ความสามารถในการเปลี่ยนแปลงสู่เซลล์กระดูกของเซลล์ต้นกำเนิดมีเซ็นไคม์ที่แยกจากไขกระดูกของหนูเบาหวานชนิดที่ 2 ที่เหนี่ยวนำจากการเปลี่ยนแปลงของเยื่อบุทางเดินอาหาร

น.ส.นาบิลา เซียรีฟาห์ จามิละห์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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By	Miss Nabila Syarifah Jamilah
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นาบิลา เซียรีฟาห์ จามิละห์ : ความสามารถในการเปลี่ยนแปลงสู่เซลล์กระดูกของเซลล์ต้นกำเนิดมีเซ็นไคม์ที่แยกจากไขกระดูกของหนูเบาหวานชนิดที่ 2 ที่เหนี่ยวนำจากการเปลี่ยนแปลงของเยื่อบุทางเดินอาหาร . (OSTEOGENIC DIFFERENTIATION POTENTIAL BY MOUSE BONE MARROW-DERIVED MESENCHYMAL STEMCELLS (mBM-MSCs) DERIVED FROM GUT LEAK-INDUCED DIABETES TYPE II MOUSE MODEL) อ.ที่ปรึกษาหลัก : ผศ. น.สพ.ดร.เจนภพ สว่างเมฆ

เป็นที่ยอมรับกันว่าโรคเบาหวานชนิดที่ 2 มีผลต่อหลายอวัยวะในร่างกายรวมไปถึงกระดูก ดังนั้นผู้ป่วยโรคเบาหวานชนิดที่ 2 จึงมีความเสี่ยงสูงที่จะเกิดการแตกหักของกระดูก สาเหตุความสำคัญที่เกี่ยวข้องทำให้เกิดโรคเบาหวานชนิดที่ 2 คือ มีระดับของสารป้องกันการอักเสบอินเตอร์ลิวคินเท็นในระดับต่ำร่วมกับสภาวะการเปลี่ยนแปลงของเยื่อบุทางเดินอาหาร ส่งผลให้เกิดการเหนี่ยวนำ ความก้าวหน้าและการกำเริบของโรคเบาหวานตามมา เซลล์ต้นกำเนิดมีเซ็นไคม์จากผู้ป่วยได้ถูกนำมาใช้อย่างกว้างขวางในการรักษาการหายของกระดูก เนื่องมาจากผลกระทบของโรคเบาหวานต่อเซลล์ต้นกำเนิดมีเซ็นไคม์และกระดูก การศึกษานี้จึงได้ถูกจัดทำขึ้นเพื่อศึกษาความสามารถในการเปลี่ยนแปลงสู่เซลล์กระดูกของเซลล์ต้นกำเนิดมีเซ็ นไคม์ที่แยกจากไขกระดูกของหนูเบาหวานชนิดที่ 2 ที่เหนี่ยวนำจากการเปลี่ยนแปลงของเยื่อบุทางเดินอาหาร ในการศึกษานี้กระดูกทิเบียได้ถูกพิจารณาให้เป็นส่วนที่ถูกวิเคราะห์การสูญเสียเนื้อกระดูกและกระดูกฟีเมอร์จจะถูกใช้เพื่อเก็บเซลล์ต้นกำเนิดมีเซ็นไคม์ที่แยกจากไขกระดูก โดยการศึกษาคุณสมบัติของเซลล์ต้นกำเนิดมีเซ็นไคม์ที่แยกจากไขกระดูกจะวิเคราะห์จากลักษณะของเซลล์ต้นกำเนิดมีเซ็นไคม์,เครื่องหมายบนผิวเซลล์, คุณสมบัติเซลล์ต้นกำเนิด, การแสดงออกของยีนเพิ่มจำนวน, การวัดการเพิ่มจำนวนของเซลล์และการสร้างหน่วยโคโลนี ผลการศึกษาที่ได้จากหนู 3 กลุ่มได้แก่ หนูกลุ่มควบคุม, หนูดัดแปรพันธุกรรมและหนูเบาหวานชนิดที่ 2 ที่เหนี่ยวนำจากการเปลี่ยนแปลงของเยื่อบุทางเดินอาหาร จะถูกนำมาศึกษาและเปรียบเทียบผลความสามารถในการเปลี่ยนแปลงเป็นเซลล์กระดูกจากการแสดงออกของยีนจำเพาะของเซลล์กระดูก, ความสามารถการทำงานของเอนไซม์อัลคาไลน์ฟอสฟาเทสและการย้อมสีการจับตัวของแร่ธาตุ จากผลการศึกษาพบว่าแม้ว่าหนูเบาหวานชนิดที่ 2 ที่เหนี่ยวนำจากการเปลี่ยนแปลงของเยื่อบุทางเดินอาหารจะเกิดการสูญเสียเนื้อกระดูกแต่เซลล์ต้นกำเนิดมีเซ็นไคม์ที่แยกจากไขกระดูกยังคงมีการแสดงออกของยีนและเครื่องหมายของคุณสมบัติเซลล์ต้นกำเนิด อย่างไรก็ตามหนูเบาหวานชนิดที่ 2 ที่เหนี่ยวนำจากการเปลี่ยนแปลงของเยื่อบุทางเดินอาหารแสดงคุณสมบัติความสามารถในการเปลี่ยนแปลงไปเป็นเซลล์กระดูกได้น้อย ดังนั้นผลการศึกษานี้สามารถนำไปใช้เป็นความรู้พื้นฐานสำหรับการวิเคราะห์ การนำไปใช้และประยุกต์ใช้เซลล์ต้นกำเนิดมีเซ็นไคม์ที่แยกจากไขกระดูกจากผู้ป่วยเบาหวานได้

สาขาวิชา	วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี	ลายมือชื่อนิสิต .....
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Nabila Syarifah Jamilah : OSTEOGENIC DIFFERENTIATION POTENTIAL BY MOUSE BONE MARROW-DERIVED MESENCHYMAL STEMCELLS (mBM-MSCs) DERIVED FROM GUT LEAK-INDUCED DIABETES TYPE II MOUSE MODEL . Advisor: Asst. Prof. Dr. Chenphop Sawangmake, D.V.M., M.Sc., Ph.D.

Diabetes type II were known to affect several organs in the body including bone, thus diabetes type II patients have higher risk of bone fracture. One of the important features in diabetes type II is low level of anti-inflammatory cytokine Interleukin-10 (IL-10) and gut leak condition. Both of these conditions have the role in diabetes induction, progression and exacerbation. Autogenous Mesenchymal stem cells (MSCs) have been widely utilized for enhancing bone healing treatment. Due to the diabetes condition affecting one of the most common and widely used MSCs source, the bone, this study was done to investigate the effect of IL-10 KO mice induced with gut leak as diabetes type II mouse model in term of its effect to bone loss, properties of mice bone marrow MSCs (mBM-MSCs), and osteogenic induction of mBM-MScs. Assessment of bone loss were done in mice tibia, and mBM-MSCs were isolated from the femur. Characterization of mBM-MSCs properties were done by comparing MSCs morphology, surface markers, stemness and proliferation gene expression, proliferation assay, and colony forming unit (CFU) of background control group, transgenic control group with the gut leak-induced diabetes type II mouse model. In vitro osteogenic differentiation properties were done by assessing expression of osteogenic gene, alkaline phosphatase activity, and mineralization staining. The outcome illustrated that despite of the bone loss condition, mBM-MSCs from gut leak-induced diabetes type II mouse model still expressing the stemness gene and show higher expression of stemness marker. However, it shows lower osteogenic differentiation properties. The obtained result can serve as baseline knowledge and suggestion for further exploration, utilization and application of BM-MSCs to and from diabetic individual

Field of Study:	Veterinary Science and technology	Student's Signature .....
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Nabila Syarifah Jamilah

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CHAPTER I

INTRODUCTION

Importance and Rationale

Chronic high blood sugar level or hyperglycemia is one of the prominent characters of diabetes mellitus (DM). Chronic hyperglycemia condition has been shown to trigger cellular inflammation which may lead to other diseases, such as bone loss, myocardial infarction, nephropathy, retinopathy, and neuropathy (Wongdee and Charoenphandhu, 2011; Barry et al., 2016; Rios-Arce et al., 2020)

Past studies have shown that diabetes type II patient have 69% higher risk of bone fracture than a healthy person (Wongdee and Charoenphandhu, 2011; Oei et al., 2013). In the bone, inflammation and hyperglycemic condition cause disturbance of normal bone homeostasis, thus, making diabetic patients more prone to bone fracture (Inzerillo and Epstein, 2004; Rios-Arce et al., 2020).

One of the cytokines that play crucial roles both in diabetes and bone regulation is interleukin (IL)-10. It has been demonstrated that IL-10 inhibits secretion of pro-inflammatory cytokines such as IL-1, IL-6, tumor necrosis factor-alpha (TNF-α) by the immune cell (van Exel et al., 2002; Barry et al., 2016). In the pathogenesis of diabetes type II, IL-10 is known to effectively inhibit the production of TNF-α, by which TNF-α contributes to the development of insulin resistance in diabetes type II. In the bone, IL-10 has been reported to plays major role in bone remodeling process (Zhang et al., 2014), as it moderates several regulation activities such as, controlling the osteoclast formation and osteoblast differentiation (Dresner-Pollak et al., 2004; Evans and Fox, 2007). Therefore, lack of IL-10 production may initiate the development of diabetes type II and disturbs normal bone homeostasis that causes bone loss in animal model.

In bone disease condition, bone takes a long time to heal. The remodeling phase of indirect fracture itself takes about 3-4 weeks to be (Marsell and Einhorn, 2011). Moreover, months of remodeling process were needed before regeneration of

bone structure is completed (Ghiasi et al., 2017). During the bone healing process, endogenous mesenchymal stem cells (MSCs) from periosteum, endosteum, and bone marrow will be recruited to the impacted site (Knight and Hankenson, 2013; Jiao et al., 2015). The fact that MSCs is an important contributor in bone healing (Ghiasi et al., 2017), MSCs therapy have become one of cell therapy which is widely studied and used both in human and animal to treat bone and other regenerative diseases (Oryan et al., 2017; Wang et al., 2020).

Bone marrow- and adipose-derived mesenchymal stem cells (BM-MSCs, AD-MSCs) are the most common types of stem cells used in regenerative studies with clinical outcomes (Wang et al., 2020). Stem cell therapy using BM-MSCs has been reported to improve bone healing and provide a quicker recovery as it has the ability to differentiate into osteogenic cells (Rosset et al., 2014). Currently, allogenous stem cells derived from other individuals of the same species are widely used, however, this method poses some risks of rejection by the immune system (Oryan et al., 2017). Therefore, MSCs treatment using autogenous stem cells is more preferred due to its non-immunostimulatory properties (Lohan et al., 2017). Nevertheless, there might be several difficulties in applying MSCs therapy from diabetes type II individual due to low IL-10 level. As IL-10 plays major roles both in DM and bone homeostasis, lack of IL-10 may change the osteogenic differentiation ability of MSCs derived from diabetes type II individual. Therefore, it is important to investigate the osteogenic differentiation potential of MSCs from diabetic type II individual to know its potential as autogenous stem cell therapy.

There are several ways to induce diabetic type II and insulin resistance in animal models such as, genetic modification and experimental attempts. (Srinivasan and Ramarao, 2007; Al-Awar et al., 2016). It has been known that lack of IL-10 contributes to the development of diabetes type II. Therefore, transgenic IL-10 KO mice have been used as one of the genetic modifications in diabetic animal model (Bobe et al., 2020). To induce diabetes type II in animal model using an experimental attempt, chemical substance such as dextran sulfate sodium (DSS) administration has been used to induce gut leak (Sorini et al., 2019). Gut leak is characterized by the change of gut barrier integrity which leads to infection and inflammation and is considered as one of

the pathogenesis processes that induce insulin resistance in DM (Winer et al., 2016; Rios-Arce et al., 2020). Based on these facts, this proposed study will use IL-10 KO mice with gut leak induction as animal model for type II diabetes.

Understanding the bone loss condition in type II diabetes animal model is important because bone loss may change the bone homeostasis cues. The change of bone homeostasis cues possibly will also affect the properties of MSCs in bone marrow that utilized for MSCs therapy. Osteogenic differentiation determines the efficacy of MSCs therapy in bone healing, however, the MSCs properties and osteogenic induction of mice BM-MSCs (mBM-MSCs) from gut leak-induced diabetes type II mouse model is still unknown. Hence, the study about MSCs properties and osteogenic induction from gut leak-induced diabetes type II mouse model is critical to further explore the possibility of applying mBM-MSCs therapy for bone healing in diabetes type II individual.

Objectives of the study

Objective 1) “To investigate bone loss in gut leak-induced diabetes type II mouse model”. Strategies 1) To explore the effect of gut leak-induced diabetes type II mouse model on bone loss.

Objective 2) “To investigate properties of mBM-MSCs derived from gut leak-induced diabetes type II mouse model”. Strategies: 2a) To establish the isolation protocol of mBM-MSCs derived from gut leak-induced diabetes type II mouse model. 2b) To explore the characteristic of mBM-MSCs derived from gut leak-induced diabetes type II mouse model.

Objective 3) “To investigate the osteogenic induction of mBM-MSCs derived from gut leak-induced diabetes type II mouse model”. Strategies: 3a) To explore osteogenic differential protocol establishment of mBM-MSCs derived from gut leak-induced diabetes type II mouse model. 3b) To characterize and check for osteogenic differentiation properties assay of the mBM-MSC derived from gut leak-induced diabetes type II mouse model.

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Hypothesis

Gut leak-induced diabetes type II mouse model causes defects of bone and mouse bone marrow-derived mesenchymal stem cells (mBM-MSCs) according to their characteristics and osteogenic differentiation potential.

CHAPTER II

LITERATURE REVIEW

Diabetes Mellitus

Diabetes mellitus (DM) is a metabolic disease with chronic increase of blood glucose level (Parveen et al., 2017). This disease is caused by disturbance or absence of insulin (American Diabetes Association, 2013). Disturbance in insulin secretion leads to abnormal metabolism of carbohydrate, lipid, and protein in body (Kharroubi and Darwish, 2015). In more complicated condition this disease can cause several other diseases in several organ such as kidney, eyes, heart, bone and nervous system (Rios-Arce et al., 2020). In worse case of uncontrollable DM, a coma and death are possible. According to American Diabetes Association there are several types of DM, which is Type I, Type II, and gestational DM (Kharroubi and Darwish, 2015; Goyal and Jialal, 2020).

Diabetes type II

Diabetes type II accounts for approximately 90-95% of all DM cases (Maraschin et al., 2010). This disease has multifactorial pathogenesis such as genetic traits of disturbances in insulin secretion and environmental factor which leads to insulin resistance (Ozougwu et al., 2013; Kharroubi and Darwish, 2015). Environmental factors for diabetes type II are; obesity, food intake, age, and sedentary life habit. High food intake and sedentary life may also lead to obesity. In addition, obesity synergies with high body fat percentage and accumulation of visceral fat, which may induce insulin resistance through lipo-toxicity (Kohei, 2010; Goyal and Jialal, 2020). Several studies in twins have reported that genetics is highly involved factor of diabetes type II (Kharroubi and Darwish, 2015). Several genes that play roles in insulin secretion or mechanism of insulin action such as, glucokinase gene and insulin receptor gene (Yoshida et al., 2007; Ardon et al., 2014), by which a mutation on those genes may cause abnormality of

insulin action and secretion (Accili et al., 1989; Bell et al., 1993). Disturbances in insulin secretion mean that the body response to glucose is decreased, which in early phase of diabetes type II resulting in impaired glucose tolerance (IGT) (Kohei, 2010). The IGT will cause high blood glucose levels that can induce glucotoxicity. Together, lipotoxicity and glucotoxicity could cause deterioration of pancreatic β cell function (Poitout and Robertson, 2008; Kohei, 2010). In support of this, previous study by Kluth et al. (2011) which uses diabetic mice model, has demonstrated that glucose- and lipotoxicity could cause β cell apoptosis which leads to insulin resistance.

Insulin resistance is one of the key characteristics of diabetes type II that happens when insulin is not sufficient to normalize blood glucose level. In diabetes type II, Insulin resistance makes the target organs (liver and muscle) needs of insulin increase (Kharroubi and Darwish, 2015). Furthermore, in more complicated conditions, dysfunction of β cells was unable to meet the demand of insulin secretion due to genetic factors. Time by time, due to the destruction of β cells, insulin needs were unable to be fulfilled (Cerf, 2013). Insulin resistance due to accumulated fat tissue initiates inflammatory reaction. Thus, causing dysregulation of adipokine and increased release of free fatty acid (FFA) (Jung and Choi, 2014). This condition will lead to decreased level of glucagon-like peptide 1, abnormalities in gut microbiome, increased level of glucagon in blood, and renal glucose reabsorption which will subsequently worsen the insulin resistance (Kohei, 2010; Goyal and Jialal, 2020)

Diabetes type II effect on bone

Diabetes type II patient has increased risk of fracture despite having high bone mineral density (BMD) (Poiana and Capatina, 2019). This is possible because diabetes type II affect bone strength despite the high BMD (Poiana and Capatina, 2017). High glucose condition in diabetes type II impairs bone homeostasis process, thus, resulting in cortical porosity (Picke et al., 2019). Accumulation of cortical porosity and microcracks overtime also worsens bone strength (Oei et al., 2013). This paradox might be caused by the lower quality of bone material, thus, making the bone more fragile. In addition, disturbances of biomechanical properties, alteration of cortical, and

trabecular bone microarchitecture and lower bone strength also contribute to higher risk of fracture (Sanches et al., 2017).

Low quality of bone material leads to lower bone strength due to increase of cortical porosity and the change of collagen structure (Ferrari et al., 2018). This change can be caused by several conditions, such as high glucose level and accumulation of advanced glycation end products (AGEs) (Picke et al., 2019). High glucose conditions may decrease osteoblast differentiation by increasing dickkopt-1 (DKK1). It affects the Wingless-type MMTV integration site (Wnt) pathway as an inhibitor, thus, decrease the osteogenic differentiation of MSCs (Hie et al., 2011). Suppression of Wnt causes increase of peroxisome proliferator-activated receptor gamma (PPAR-γ) signaling, resulting in MSCs differentiation into adipocyte, thus, causing increase of adipogenicity (Napoli et al., 2014). Adipogenicity also induce inflammatory condition by secretion of TNF-α from bone marrow adipocyte (Hardouin et al., 2016). This inflammatory condition causes an increase of saturated fatty acid and induces apoptosis of the osteoblast through lipo-toxicity (Li et al., 2018). Adipocytes treated with TNF-α will secrete more osteoclast precursors called receptor activators of nuclear factor kappa-B ligand (RANKL) (Goto et al., 2011). Besides, TNF-α also working with macrophage colony-stimulating factor (MCSF) increasing osteoclastogenesis through differentiation of bone macrophage into osteoclast (Kobayashi et al., 2000). High glucose condition also induces osteocyte apoptosis that causing impaired mechanosensing properties of osteocyte. In diabetes type II AGEs levels are increased (Kilhovd et al., 1999). The AGEs have deleterious effects on collagen and laminin properties, osteoclast, osteoblast, and osteocyte response, which result in change of collagen structure causing fragility of elastic fiber and increase of cortical porosity that reduces the hardness of bone (Picke et al., 2019). Based on these facts, it is known that in high glucose and accumulation of AGEs affect bone material and strength in diabetes type II.

Interleukin-10

Interleukin-10 (IL-10) is one of anti-inflammatory cytokines produced in several different cells such as, macrophage, dendritic cell, B cell, and several T cells. This

cytokine targets are natural killer (NK) cells, macrophage, and T helper (Th) cell (Steen et al., 2020). It inhibits interferon-gamma (IFNγ) synthesis in Th1 cell (Ouyang et al., 2011) and decreases the production of pro-inflammatory cytokines (IL-1, IL 6 and TNF-α) and chemokines (CC and CXC) in monocyte and macrophage (Zhang and An, 2007; Steen et al., 2020).

Interleukin-10 role in diabetes

In diabetes type II individuals, low concentration of IL-10 has been reported to cause impaired glucose tolerance, which is one of the common pathogenesis for metabolic syndrome (Shin et al., 2013). As the IL-10 is a counter regulator for pro-inflammatory cytokines such as TNF α and IL-6, in low IL-10 condition, the pro-inflammatory cytokine will be produced thus maintain the inflammation condition (van Exel et al., 2002). Besides, inflammation is a risk factor for insulin resistance in diabetes type II which is worsened by glucotoxicity and increase of oxidative stress, this leads to β cell dysfunction. Inflammation also increases saturated fatty acid thus resulting in lipo-toxicity (Kluth et al., 2011). Therefore, inflammation is known as one of the important mechanisms of insulin resistance in diabetes type II due to its effect on β cell dysfunction (Herder et al., 2013).

Interleukin-10 role in bone homeostasis

There are two major roles of IL-10 in bone homeostasis; inhibition of osteoclastogenic, and induction of osteogenic differentiation. Research done by Xu et al. (1995) demonstrated that IL-10 is able to inhibit the formation of preosteoclast cells in the early stage of osteoclast differentiation. The inhibition of pro-inflammatory cytokine production by IL-10 is also an important factor in osteoclast differentiation as it serves as major trigger for osteoclast activation (Schett, 2011). Interleukin-10 also increases osteoprotegerin (OPG) and decrease RANKL. A decrease in RANKL, an osteoblast ligand, causes lower bone resorption and increase of OPG, an osteoblast precursor that will increase bone formation (Evans and Fox, 2007; Zhang et al., 2014). *Nuclear factor of activated T cells 1* (*Nfatc-1*) gene is also decreased by IL-10 (Evans

and Fox, 2007). This gene serves as a master transcription regulator in osteoclast differentiation (Kim and Kim, 2014). Past study by Dresner-Pollak et al. (2004) showed that the absence of IL-10 decreased the mBM-MSCs osteoblast differentiation in IL-10 KO mice. This attributable to lack of IL-10 leads to upregulation of the pro-inflammatory cytokines such as TNF-α and IL-1 that have a negative effect on osteoblast differentiation (Hughes et al., 2006).

Gut Leak

Gut leak is a condition where gut barrier integrity is disturbed (Mu et al., 2017; Sorini et al., 2019). Gut leak and diabetes both share the same pathogenesis factor such as obesity and food intake. These factors causes the change of gut microbiota composition (dysbiosis) (Winer et al., 2016) which lead to suppression of lipoprotein lipase (LPL) suppressor (Boulangé et al., 2016) and most importantly disturbing gut barrier integrity (Brandl and Schnabl, 2015). Disturbed gut barrier integrity leads to increase in gut permeability which causes the 'leakages' of bacteria, its toxin, and lipopolysaccharides (LPS) passing through the intestinal barrier (Winer et al., 2016).

Gut leak and Interleukin-10 role in type II diabetes mellitus

Not only in diabetes, IL-10 also plays important part in managing gut homeostasis. A research done by Hasnain et al. (2013) has shown that IL-10 improves mucus production through reduction of endoplasmic reticulum stress. Importance of IL-10 in gut homeostasis has been proven by transgenic IL-10 KO mice that spontaneously developed colitis (Gomes-Santos et al., 2012). Moreover, administration of IL-10 that precedes DSS induction of colitis help protect the gut from developing colitis (Cardoso et al., 2018).

Study done by Madsen et al. (2000) show that germ-free conditions and administration of antibiotics to IL-10 KO mice showed to prevent colitis condition. This shows that gut microbes also one of the factors that cause colitis in these mice. Under normal condition, there is a balance of gut microbe, thus, normal gut homeostasis can be maintained (Zhang et al., 2018). In gut leak condition, there is increase of gut permeability and changes of gut microbes composition (Fasano, 2020). These cause

the leakage of microbes, its toxin and LPS into the bloodstream (Taniki et al., 2018). LPS is known to further induce insulin resistance through inflammation (Vrieze et al., 2010), change in metabolism and regulation of bile acid profile, and enzyme secretion of the bacteria itself (Utzschneider et al., 2016). Lipopolysaccharides can induce inflammation through toll-like receptor-4 (TLR4) pathway which resulting in stimulation of monocyte to produce large number of pro-inflammatory cytokines and decrease of anti-inflammatory cytokine IL-10 production by dendritic cells. This condition cause low grade inflammation (Shukla et al., 2018). A study done by Hawkesworth et al. (2013) stated that secretion of pro-inflammatory cytokine, TNFα, can leads to diabetes by binding with insulin receptors and This binding cause inactivation of insulin receptor and lead to insulin resistance. In conclusion, gut leak in lack of IL-10 condition can lead to diabetes type II through insulin resistance.

Research done by Adachi et al. (2019) shows that diabetic type II individuals develop gut dysbiosis. In diabetic type II individuals, number of Gram-positive butyrate-producing bacteria is lower whereas Gram-negative bacteria opportunistic pathogen is higher than normal person (Allin et al., 2015). Gram-negative bacteria are known to produce more LPS (Allin et al., 2015). Therefore, high amount of LPS influx may exacerbate current inflammation conditions in diabetes type II individuals (Thaiss et al., 2018). In addition, gut dysbiosis interferes with LPL suppressor, resulting in high adiposity and FFA. These conditions lead to obesity, inflammation, and oxidative stress which subsequently result in insulin resistance and destruction of β cells (Adachi et al., 2019; Woldeamlak et al., 2019). It is known that diabetic type II individuals has low concentration of IL-10 (Shin et al., 2013). As mentioned above, IL-10 helps improving mucous production and gut leak condition resulting in lower IL-10 level, thus low IL-10 in diabetic type II might be caused by gut leak as well as exacerbate existing gut leak condition (Hasnain et al., 2013) . In conclusion both lack of IL-10 and gut leak serve as pathogenesis of each other and diabetes type II.

Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cell is a cell that has a self-renewal ability (Li and Xie, 2005). This type of cells is able to differentiate into several different lineages, such as osteogenic, chondrogenic, adipogenic, and myogenic lineage (Gazit et al., 2019). Mesenchymal stem cells can be found in several tissues, such as adipose, dental, bone marrow, blood, tendon, etc. In addition, it is safer, as it does not trigger any immune response and have immunosuppressive property. This characteristic of MSCs makes them favorable to be studied for their promising potential in stem cell therapy, tissue engineering, and *in-vitro* model of disease (Soleimani and Nadri, 2009; Huang et al., 2014; Yusop et al., 2018).

Mouse bone marrow mesenchymal stem cells (mBM-MSCs)

Morphologically, upon isolation mBM-MSCs has small round shape, then it starts to become spindle shape in the following day (Huang et al., 2014). It has an ability to differentiate into blood vessel smooth muscle which can be useful during the formation of blood vessels (Charbord, 2010). Similar to human BM-MSCs (hBM-MSCs), mBM-MSCs show several surface markers of CD44, CD90, CD105, and negative/low percentage of CD45 is depend on the passage (Huang et al., 2014; Chaudhary and Rath, 2016). Interestingly, mBM-MSCs is able to show a surface marker which is not shown in other species or several strains of mice called SCA-1/Ly-A (SCA-1), this marker is crucial for self-renewal ability (Charbord, 2010). However, the Isolation of mBM-MSCs is not easy (Caroti et al., 2017). As there is only low percentage of mBM-MSCs inside the bone marrow (Cai et al., 2015). Moreover, it is located in the heterogeneous cell population and has a slow growth, thus, making it more difficult to isolate (Hu et al., 2018). Research conducted by (Ahmadbeigi et al., 2011) showed that there are three different phases of mBM-MSCs derived from BALB/C and C57BL/6 mice. Those phases are primary phase, dormant phase, and immortal phase. In the dormant phase, mBM-MSCs stop growing, but it produces small daughter cell that is non-proliferating until the end of this phase. The dormant phase lasts for around 2 months, and this makes mBM-MSCs is difficult to grow. The properties of primary phase and immortal phase also different in terms of multilineage differentiation and surface

marker. In the immortal phase, mBM-MSCs losing the ability to do multilineage differentiation and there is some change of the MSC surface related marker expression.

Mesenchymal stem cell in diabetes

Only a few researchers have used and analyzed the MSCs derived from diabetic individuals such as Wang et al. (2020) from diabetic rats, Sun et al. (2020) from diabetic mice, and Phadnis et al. (2009) from diabetic human. In some diseases like renal failure and rheumatoid arthritis, MSCs have some alteration in their biological properties and genome (Zhao et al., 2007; Garayoa et al., 2009). Such thing is possible because its differentiation properties are affected by some factors such as its genetic, environment, and extracellular cues, like signaling cascade and soluble-insoluble factors (Li and Xie, 2005). In DM, there are some changes in the environment and extracellular cues that affect the bone cells. Nevertheless, undifferentiated cell of DM hBM-MSCs shows markers of Insulin and C-peptide (Phadnis et al., 2009). On the other hand, BM-MSCs derived from diabetic rats show expression of MSCs related surface marker CD90 and CD29, but have lesser proliferation rate compared to AD-MSCs (Wang et al., 2020).

The increase of the usage of MSCs in cell-based therapy has raised concern about the usage of allogenous stem cells. In diabetic patient where the patient condition already severe, introducing allogenous stem cell might rise the problem in immune rejection (Wang et al., 2020). Thus, autogenous stem cell surfaced as prospect due to its survival and tolerance properties (Batten et al., 2007; Wang et al., 2020). However, this raises a concern of MSCs characteristic derived from diabetic individual as it might have different properties. This is because diabetes type II has low level of IL-10 which important in bone homeostasis regulation and diabetes type II itself. Therefore, change in bone homeostasis might affect the MSCs derived from bone marrow. To sum up, understanding mBM-MSCs characteristic derived from diabetes individual is important for its utilization for cell therapy.

Gene flow of MSCs osteogenesis

Osteogenesis is process of bone formation. It started with mesenchymal stem cell which undergo several phases. These phases are characterized according to

distinct gene expression profile along the progression of osteogenic differentiation (Javed et al., 2010). The first phase is lineage commitment. In this phase commitment of mesenchymal stem cell into osteogenic lineage and formation of osteoprogenitor were mainly controlled by *Runx2* and followed by *Osterix* (*Osx*) as the downstream gene (Bruderer et al., 2014). Second phase is the proliferation of progenitor cells and extra cellular matrix (ECM) production. In this stage several growth-related gene such as *Histone*, *C-myc*, *C-fos* were expressed to support the proliferation (Choi et al., 2011). Furthermore, *Collagen 1 Alpha 1* (*Col1), Alkaline phosphatase* (*Alp*), and *Osteopontin* (*Opn*) also secreted for ECM production by mature osteoprogenitor, and pre-osteoblast (Javed et al., 2010). Once the osteoblast is formed, it enters the last phase, the mineralization phase. In addition to expressing gene in pre-osteoblast stages, osteoblast also express another gene called *Osteocalcin* (*Ocn*) (Huang et al., 2007).

Osteogenic differentiation of mesenchymal stem cells in diabetes, gut leak and lack of IL-10 condition.

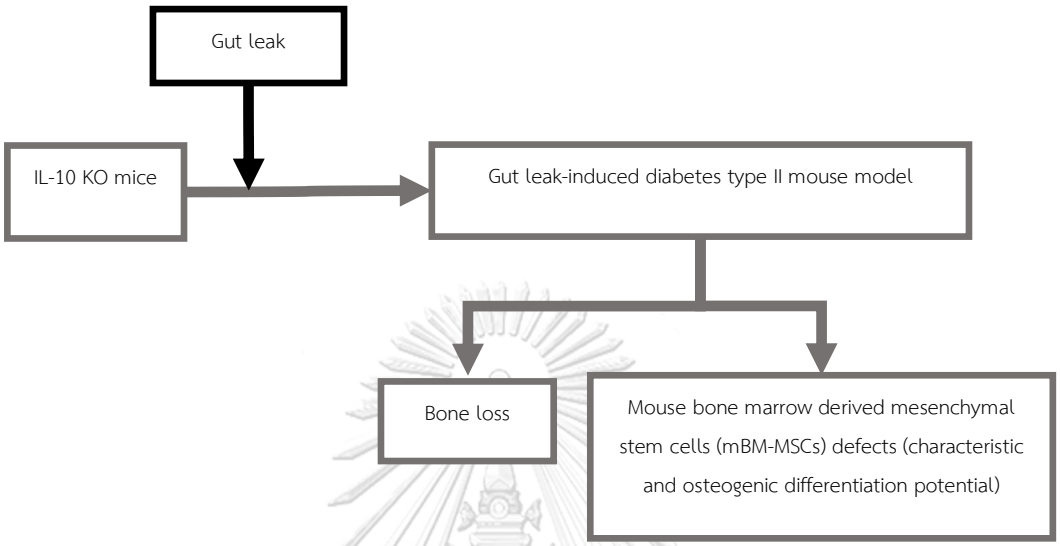
Osteogenic differentiation is essential in the application of MSCs therapy in bone, as different states of MSCs differentiation may affect the efficacy of the treatment (Oryan et al., 2017). A previous study by Marupanthorn et al. (2017) showed that in different days of hBM-MSCs osteogenic differentiation, they show different characteristic that can be seen through their alkaline phosphatase (ALP) activity and expression of osteogenic genes such as *runt-related transcription factor 2 (Runx2), osterix (Osx),* and *osteocalcin (Ocn).* In diabetes, high glucose was known to alter the osteogenic differentiation of MSCs. A study done by Deng et al. (2018) has proven that serum of diabetic individual inhibits human MSCs osteogenic differentiation to osteoblast. A similar finding was also found in mice by Al-Qarakhli et al. (2019), they demonstrated that the MSCs derived from endosteal niche lining compact bone under the hyperglycemia condition, its osteogenic differentiation was inhibited at population doubling (PD) 15, 100, and 200. Based on these facts, it is known that hyperglycemic conditions may have deleterious effect to MSCs.

The effect of gut microbes on bone using DSS colitis model have been conducted by Hamdani et al. (2008), it shown that in DSS colitis mice the bone

regulation is affected, which also might affect the mesenchymal stem cells condition. In leaky gut conditions, dysbiosis will increase the formation of short chain fatty acid (SCFAs) (Allin et al., 2015). The SCFAs trough G protein coupled receptors causing dysregulation of mitochondrial function and suppression of insulin signaling leading to damage of mitochondria, increase of reactive oxygen species (ROS) and imbalance glycolysis (Jocken et al., 2017). As a result, incorrect MSCs differentiation is increased thus, MSCs number is depleted. In addition, SCFAs also induced stem cells ageing, which shown by reduction of the MSCs differentiation capacity as exhibited by human chorion-derived MSCs (hCMSCs). In BM-MSCs ageing leads to lower osteogenic differentiation and proliferation (Tan et al., 2019). In conclusion, gut leak leads to increase of SCFAs that resulting in MSCs ageing thus affecting its osteogenic differentiation properties.

Cytokines known to plays role in MSCs homeostasis. Research conducted by (Vallés et al., 2020) shown that both pro- and anti- inflammatory cytokines regulate MSCs migration, proliferation and differentiation. By using macrophages conditioned media for pro- and anti- inflammatory condition, the result have shown that IL-10 secreted by anti-inflammatory macrophages increase osteogenic differentiation in term of its ALP activity and matrix mineralization. This also support results of MSCs osteogenic differentiation research in diabetes type II condition, where they show significant decrease of osteogenic differentiation, as diabetic type II individual have low level of IL-10 that might affect MSCs homeostasis.

CONCEPTUAL FRAMEWORK



CHAPTER III

MATERIALS AND METHODS

The experiment was conducted after the protocol approval by the Chulalongkorn University-Institutional Animal Care and Use Committee (CU-IACUC) (Protocol No. 1973029). The animals will be housed at the Chulalongkorn University Laboratory Animal Center (CULAC) throughout the study period.

Animals

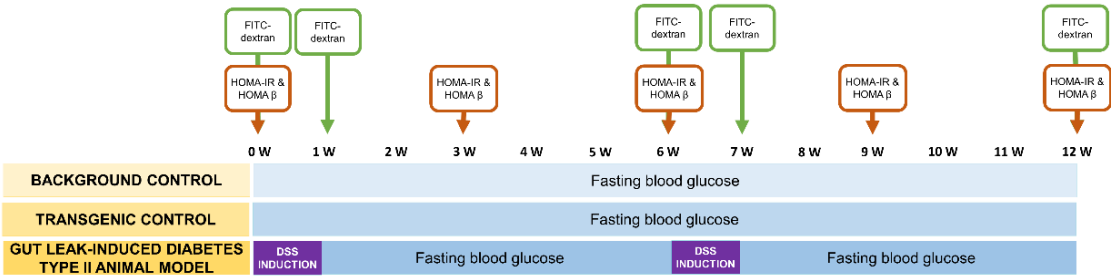
Twelve IL-10 KO: B6.129P2-IL10tm1Cgn/J mice and 6 C57BL/6J mice (Jackson Laboratories) were separated into 3 groups of 6 each, control group (C57BL/6J), transgenic control group, and gut leak-induced diabetes type II mouse model group. All mice used were at 13 weeks of age and housed in Chulalongkorn University Laboratory Animal Center (CULAC). All experimental activities have been approved by Chulalongkorn University Institutional Animal Care and Use Committee (CU-IACUC) (protocol no. 1973029). Before the experiment begins, all mice were adapted to their new environment for 2 weeks. Upon the euthanasia, femur and tibia were taken from mice by first dislocating the coxo-femoris joints and separate it from the body. All the muscle and connective tissue were removed. The femur was kept in a transport medium containing DMEM/F12 media (Thermo Fisher Scientific, USA) supplemented with 10% Fetal Bovine Serum (FBS, Thermo Fisher Scientific, USA), 3% of Antibiotics-Antimycotics (Thermo Fisher Scientific, USA), 1% sodium bicarbonate (Sigma Aldrich, USA) and 1x GlutaMax (Thermo Fisher Scientific, USA). All tibia was kept in 4% paraformaldehyde.

Gut leak induction and parameter check

To induce gut leak in mouse model, 2% of dextran sulfate sodium (DSS, MP Biomedicals, USA) was used. The induction protocol derived from previous publication

by Sorini et al. (2019), briefly 2% DSS was given as mice drinking water ad libitum in pulsative manner. The replacement of drinking water to 2% DSS started at week 0 until week 1 and repeated at week 6-7. Progression of gut leak was assessed by FITC-dextran (Sigma Aldrich, USA). This assessment was done at the week 0, week 1, and week 7. Blood was collected before giving FITC-dextran by oral gavage, and 4 hours after. The result from before and after giving the FITC-dextran were compared.

Time frame of gut leak induction of diabetes type II



Progression of diabetes type II assessment

The progression of diabetes type II was assessed by checking the fasting blood glucose level, homeostasis assessment (HOMA) IR and HOMA β. Measurement of fasting blood glucose level will be done using AlphaTrack glucose meter (Zoetis, USA). The level of fasting blood glucose level, HOMA IR and HOMA β from each group will be compared.

Histology and histomorphometry

The harvested tibia was fixed in 4% paraformaldehyde for histopathology, the femur was decalcified, then the bone sectioned and processed into paraffin block and follow further hematoxylin-eosin (HE) and modified Movat pentachrome staining process (Doello, 2014). Histomorphometry analysis were done using Fiji (Schindelin et al., 2012) software with modified Movat pentachrome staining result according to previous publication by Malhan et al. (2018). Briefly, the image was processed into stacks image by split into 4x4 individual images. Images were then saved as image sequences. Cortical bone in each sequence were then removed using polygonal tools. Trainable weka segmentations plug-ins (Arganda-Carreras et al., 2017) were used to

categorized area of the images into its representation tissues. Once the trainable weka segmentation result was obtained, images were turned into 8-bit and processed into binary image. Local thickness was used to measure the trabecular thickness of the images. In addition, trabecular space was measured by inverting the binary image and followed by measurement using local thickness. The result derived from the local thickness were then multiplied by the pixel dimensions. Result from each section was shown in mean of thickness in from each section. These were then added to provide total mean of thickness from one full image. All results were then compared among groups with 4 replications in each group.

Cell isolation and expansion

Before cell isolation, femur was washed with 70% ethanol 2 times. Thus, both of the diaphysis was cut using sterile scalpel. Two ml of culture medium consisting of DMEM/F12 media (Thermo Fisher Scientific, USA) supplemented with 10% FBS (Thermo Fisher Scientific, USA), 1% GlutaMax (Thermo Fisher Scientific, USA), 1% sodium bicarbonate (Sigma Aldrich, USA) and 1% Antibiotics-Antimycotics (Thermo Fisher Scientific, USA) were used to flush the bone marrow out of the bone using 27 G needle (Nipro, Japan) drop by drop into a 60 mm culture dish (Corning, USA). Cell suspensions then were resuspended and divided into two 60 mm dishes (Corning, USA). Next, 2 ml of culture medium was added to each dish and incubated for 72 hours in 37°C 5% CO_2. The culture medium was changed every 48 hours. After 5 days, cells were trypsinized using 0.25% Trypsin EDTA (Thermo Fisher Scientific, USA). Cells then expanded into 2 of 60 mm culture dishes (Corning, USA). Cells from passage 1 were used in this research. The characterization of the MSCs were done by checking the mRNA expression of stemness markers (*Nanog*, *Rex1* and *Oct4*), proliferative marker (*Ki67*) using RT-qPCR and MSCs-related surface markers (SCA-1, CD44, CD73, CD105) and hematopoietic marker surface marker (CD45) were analyzed using flow cytometry.

Flow cytometry

Briefly, all collected mBM-MSCs from 3 groups were washed using FACS buffer and incubated using 1:10 dilution of MSCs related surface marker antibody (CD44,

CD73, CD105, and SCA-1) as well as hemopoietic cell surface marker antibody CD45. The antibodies that used for flow cytometry were FITC anti-CD45 and Isotype antibody; Alexa Flour 488 anti-CD105, -SCA-1 and isotype antibody; PE anti-CD73, -CD44 and isotype antibody (all antibody used in this research were sourced from Bio Legend, USA). Cells will be incubated for 1 hours before analyzed using flow cytometer (BD).

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

The RNA collection was done by scraping the cell with TRIzol® reagent (Thermo Fisher Scientific, USA). RNA was then converted into cDNA using reverse transcriptase enzyme kit (Promega, USA). The mRNA expression was done by Quantitative real-time PCR (RT-qPCR) sample cDNA by FastStart ® Essential DNA Green Master® using CFX96 real-time PCR detection system (Bio-Rad, USA). This method was used for measuring stemness property and gene expression. All the primer that used for RT-qPCR listed in Table 1. The relative mRNA expression method follow the previous method done by Sawangmake et al. (2016). The result was normalized to *ribosomal protein L 13* (*Rpl.13*) expression and control into 2-**ΔΔ**Ct. The formula: **ΔΔ** Cycle threshold (**ΔΔ** Ct) = [Ct target gene – Ct *Rpl.13*] transgenic mice - [Ct target gene – Ct *Rpl.13*] control.

Table 1 Primer list

Gene	Accession mRNA	Probe	Primer sequence	Tm	Lenght
Rpl.13.a	NM_009438.5	F	TGAATACCAACCCCTCCCGA	60.25	99
		R	CTCTCTTGGTCTTGTGGGGC	60.32	
Nanog	NM_001289828.1	F	GGTGTCTTGCTCTTTCTGTGG	59.12	172
		R	TGTCAGTGTGATGGCGAGG	59.71	
Ssea-1	NM_010242.3	F	ACGTGTCTGTGGACGTGTTT	62.18	134
		R	ACGTGCCGTGAGTTCTCAAA	60.48	
Ki67	NM_001081117.2	F	TCAGTTCTACCAATCCAACTCAAG	58.45	181
		R	CGCCTTGATGGTTCCTTTCC	59.19	
Alp	NM_001287172.1	F	CCCAGACACAAGCATTCCCA	60.25	149
		R	GAGAGCGAAGGGTCAGTCAG	59.83	

Runx2	NM_001145920.2	F	ATCCCCATCCATCCACTCCA	60.03	168
		R	AGTTCTGAAGCACCTGCCTG	60.25	
Opn	NM_001204201.1	F	TCCTCCCTCCCGGTGAAAGT	62.4	111
		R	CTTCTGAGATGGGTCAGGCAC	60.41	
Ocn	NM_007541.3	F	TTCTGCTCACTCTGCTGACC	59.68	154
		R	GGGACTGAGGCTCCAAGGTA	60.62	
Osx	NM_130458.4	F	GTCCTCTCTGCTTGAGGAAGAA	59.44	131
		R	TCTTTGTGCCTCCTTTCCCC	59.89	
Col1a1	NM_007742.4	F	CTGGCGGTTCAGGTCCAAT	60	190
		R	TCCAAACCACTGAAGCCTCG	60.25	

Proliferation assay

Proliferation assay was done using Alamar Blue reagent (Thermo Fisher Scientific, USA) by following the manufacturer instruction with some modification. Five thousand cells were seeded in 24 well plate (Corning, USA) and cultured in 37°C 5% CO_2 for 1,5, and 7 days. At day 1,5, and 7, the culture medium was removed and changed with 5% Alamar Blue (Thermo Fisher Scientific, USA) and incubated for 3 hours and 30 minutes alongside negative control (5% Alamar Blue) (Thermo Fisher Scientific, USA), blank (culture medium), and culture medium for dilution. Next, all 5% Alamar blue were diluted 1:2 with culture medium. Absorbance for both reduction and oxidation was read at 570 and 600 nm respectively.

Colony-forming unit assay

Colony-forming assay protocol was modified from (Hu et al., 2018). Ten thousand cells were seeded into 60 mm culture dish (Corning, USA) with culture medium and incubated 37°C 5% CO_2 for 14 days. Culture medium was changed every 2 days. Upon 14 days, medium was removed and washed using phosphate buffered saline (PBS, Sigma Aldrich, USA) followed by fixation using cold methanol. Culture dish then stained with crystal violet for 5 minutes and washed with PBS (Sigma Aldrich,

USA). Colony count was based on Cai et al. (2015) which cell cluster with ≥ 5 cells was counted as one colony .

Osteogenic induction and characterization

Induction of osteogenic differentiation was done by seeding the cell into 24 well plate (Corning, USA) with osteogenic differentiation medium for 21 days. Osteogenic differentiation medium consists of DMEM/F12 (Thermo Fisher Scientific, USA) supplemented with 10% FBS (Thermo Fisher Scientific, USA), 1% Antibiotic Antimycotic (Thermo Fisher Scientific, USA), 1x GlutaMax (Thermo Fisher Scientific, USA), 1% sodium bicarbonate (Sigma Aldrich, USA), 0.1 µM dexamethasone (Sigma Aldrich, USA), 10 mM β-glycerol phosphate (Sigma Aldrich, USA), 50 L-ascorbic acid 2-phosphate (Sigma Aldrich, USA). The osteogenic medium was changed every 2 days. After 21 days mRNA expression of *Alp, Runx2, Opn, Col1a1, Ocn, Osx* genes from the cells were examined using RT-qPCR method.

Alkaline phosphatase (ALP) activity

Alkaline phosphatase activity assay was done as previously demonstrated by Sawangmake et al. (2016). Upon last day of osteogenic differentiation, the media was removed, and cells were lysed using alkaline lysis buffer. The suspension then was mixed with 1 mg of *p*-nitrophenyl phosphate (Thermo Fisher Scientific, USA) and 100µl 0.1 M 2-amino-2-methyl-1,3-propanediol (Sigma Aldrich, USA) and incubated 37°C 5% CO_2 30 minutes. After the incubation, reaction was stopped by adding 0.1 M NaOH (Thermo Fisher Scientific, USA) then the absorbance of ALP was read at 410 nm.

Alizarin red S staining

Mineralization of the osteogenic differentiated cells was assayed using Alizarin red S (Sigma Aldrich, USA). Upon the last day of the osteogenic differentiated induction, cells on the well were washed with PBS (Sigma Aldrich, USA) followed by fixation using cold methanol (Thermo Fisher Scientific, USA) and washed with DI pH 4.2. Then, 300 µl 2% Alizarin red S (Sigma Aldrich, USA) were added into each well and incubated at

room temperature for 5 minutes. After incubation, the well was washed with DI pH 4.2 two times and let dry at room temperature overnight (Sawangmake et al., 2016).

Von Kossa staining

Osteogenic differentiated cells produced minerals. *Von Kossa* staining was also used to identify mineral deposits. On the 21st day of the induction, the osteogenic media was removed. Filtered PBS (Sigma Aldrich, USA) was used to wash cells and fixated with cold methanol (Thermo Fisher Scientific, USA). After fixation, cells were washed with DDI water two times before 5% of silver nitrate (Sigma Aldrich, USA) added. The plate then was put under UV light and washed with DDI. To remove the excess silver nitrate that did not bind to the mineral. Next, sodium thiosulfate (Sigma Aldrich, USA) was added then followed by washing with DDI. The plate then was let dry at room temperature overnight before observation.

Statistical analysis

Four replicates of mBM-MSCs from each group were used in this study. Dot plots from GraphPad Prism program (GraphPad, USA) were used to generate graph in this study. The statistical analysis was done using SPSS statistic software (IBM Corporation, USA). Mean and standard deviation from all three independent groups were analyzed using analysis of variance (ANOVA) with Dunnett as post hoc analysis. The result is significantly different if the p-value <0.05.

CHAPTER IV

RESULT

Effect of gut leak-induced diabetes type II on mice body weight and bone gross morphology

The induction of gut leak into IL-10 KO mouse resulting in weight loss throughout the duration of the study compared to both control group as illustrated in Figure 1A. In contrary, on gross morphology observation there is no significant difference of tibia length among group. Nonetheless, there is change in color of gut leak-induced mouse model tibia as seen in Figure 1B. It shows paler color compared to both controls.

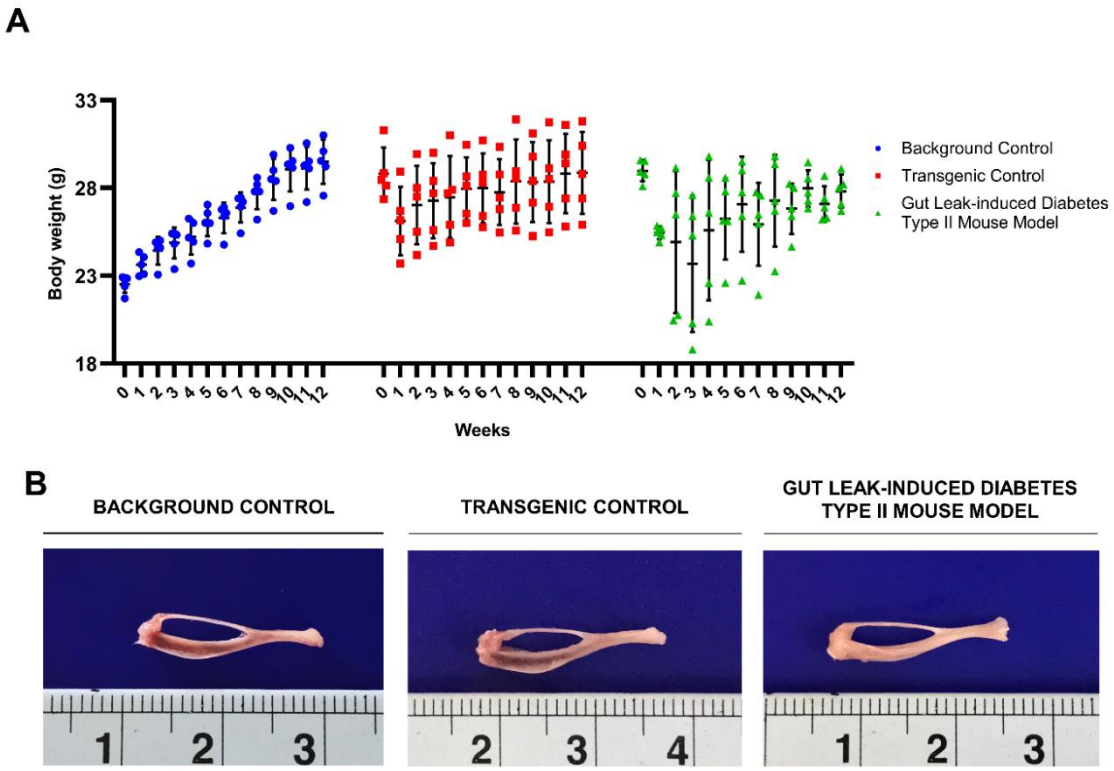


Figure 1. Body weight and gross morphology of mice tibia. Body weight of the mice was measured every week throughout the study (A). The harvested tibia from each group was observed for its gross morphology, scale in cm (B).

Effect of gut leak-induced diabetes type II mouse model on bone loss

Assessment of the bone loss were confirmed by histopathology and histomorphometry of the tibia. The results showed that the trabecular bone in proximal of tibia of gut leak-induced diabetes type II mouse model were lesser than the trabecular bone from transgenic control group (Figure 2A and B). In addition, the trabecular bone from transgenic control group also shows lesser trabecular bone compared to the control group. In line with histology assessment, bone morphometry analysis on modified Movat pentachrome staining showed significant lower number of trabecular thickness in gut leak-induce diabetes type II mouse model and transgenic control group compared to the control (Figure 2C). In contrast, the trabecular space among group is not statistically significant different, but it can be seen that the trabecular space trend is increasing in gut leak-induced diabetes type II mouse model compared to both controls (Figure 2D).

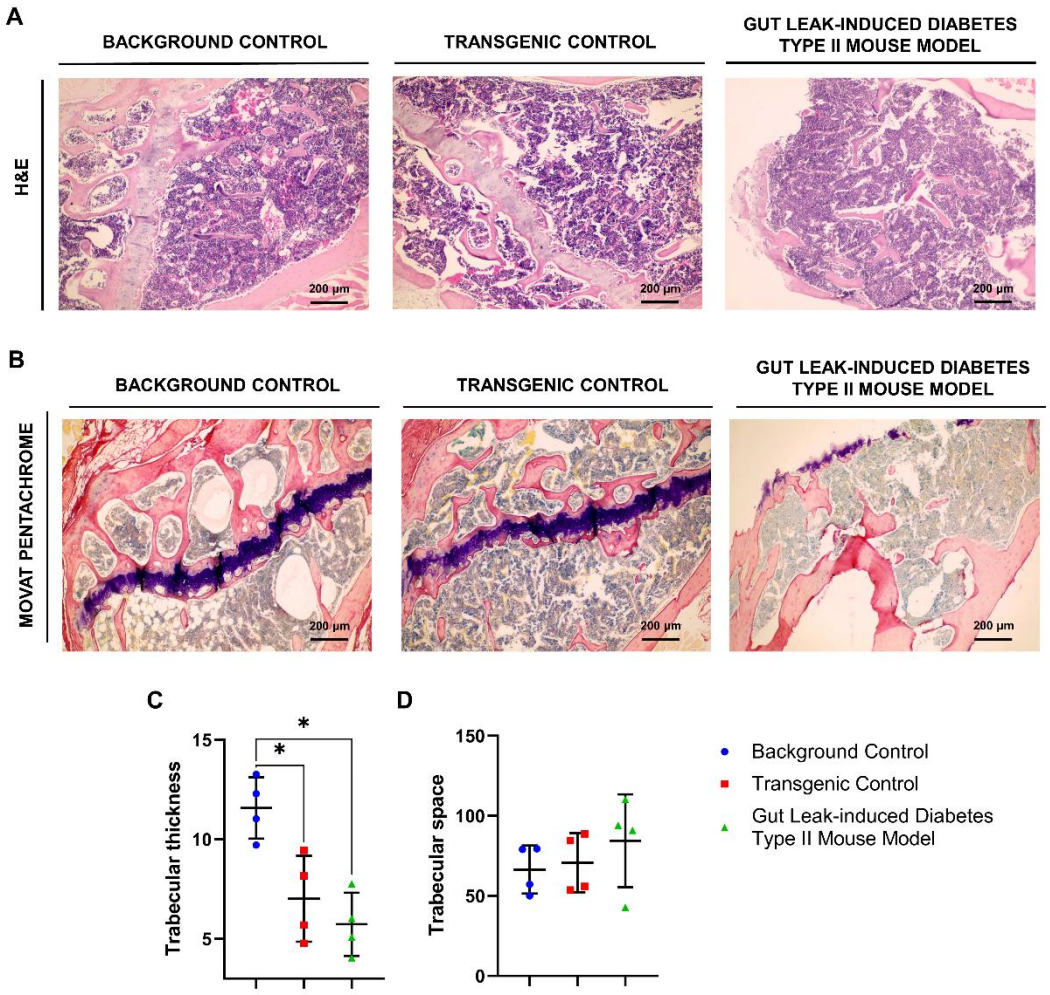


Figure 2. Tibia histology and morphometry. The harvested tibia was subjected to histology examination with Hematoxylin-Eosin stain (B) and modified Movat pentachrome stain (C), as well as morphometry analysis of proximal tibia trabecular bone from modified Movat pentachrome staining (D). Scale bar 200 µm.

Effect of Gut Leak-induced Diabetes Type II Animal Model on mBM-MSCs morphology and MSCs related surface marker.

After the isolation, mBM-MSCs morphology were observed. All mBM-MSCs derived from gut leak-induced diabetes type II, transgenic control and background control group have similar morphology (Figure 3A). Characterization of mBM-MSCs were done by flow cytometry of MSCs related surface markers, mRNA expression of stemness gene marker and proliferation marker, Alamar Blue proliferation assay, and colony forming unit assessment. Flow cytometry showed cell from background control group express lower level of CD44, compared to transgenic group and gut leak-induced diabetes type II mouse model group. In the other hand, CD73 for these group is lower compared to background control group. Expression of CD105 and SCA-1 show the highest in gut leak-induced diabetes type II mouse model group, followed by background control group and least is transgenic control group. All group showed low level of CD45 as hematopoietic cell marker (Figure 3B).

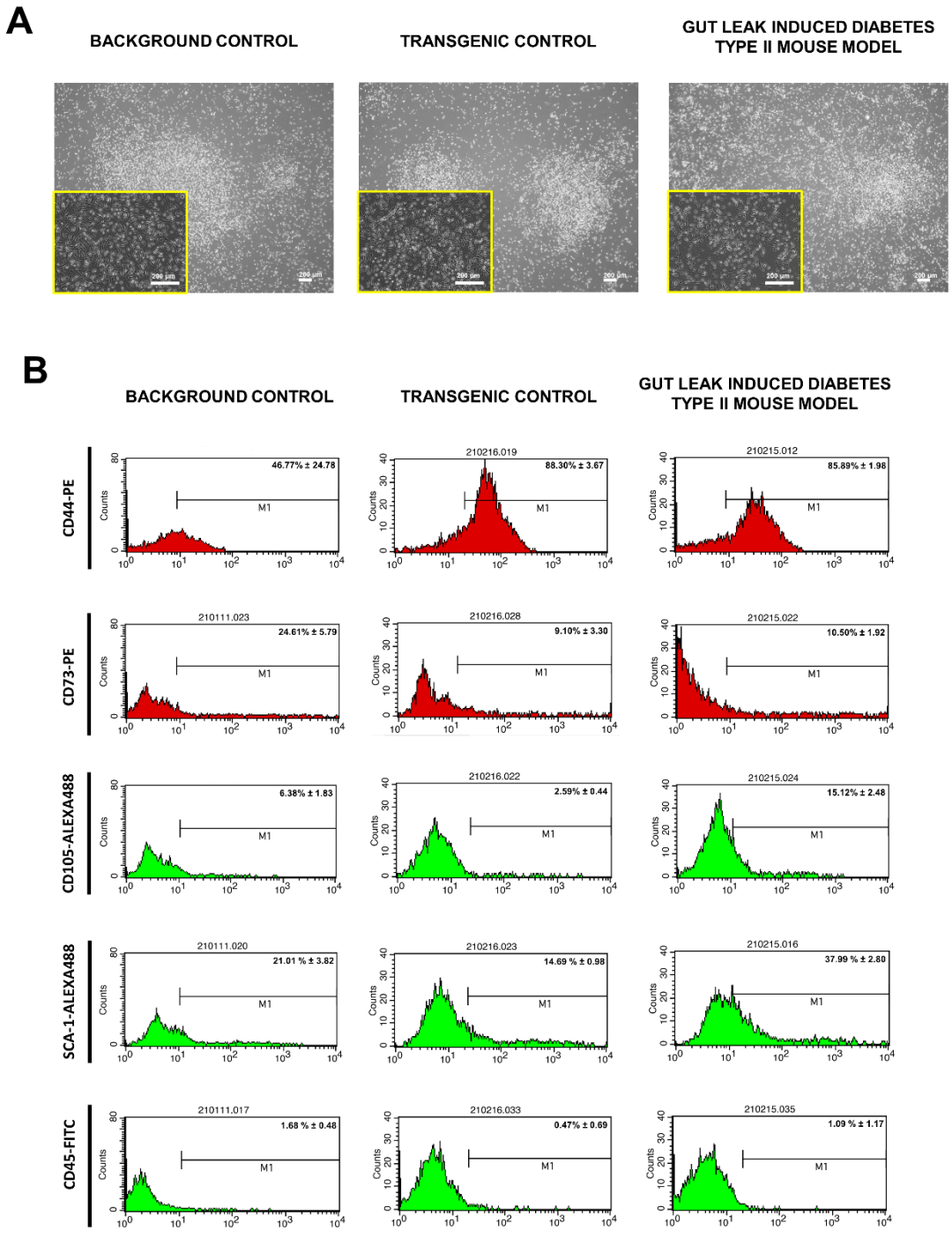


Figure 3. mBM-MSCs cell morphology and surface marker expression. Day 5 after isolation morphology of cell from all group were assessed, scale bar 200 µm (A) and MSCs related surface marker expression were analyzed using flow cytometry, data in mean and standard deviation from n=4 (B).

Effect of Gut Leak-induced Diabetes Type II Animal Model on mBM-MSCs Stemness and proliferation

Expression of stemness gene marker result were normalized to the house keeping gene *Rpl.13.*a. the outcome shows that, transgenic control group had down regulated mRNA expression of stemness gene *Nanog* and *Ssea-1* compared to background control and gut leak-induced diabetes type II mouse model group, whereas, compared to background control group, gut leak-induced diabetes type II mouse model group express lower *Ssea-1 and* higher *Nanog* expression (Figure 4A). The mRNA expression trend for proliferation marker *Ki67* is similar to *Ssea-1* where background control group show superior expression followed by gut leak-induced diabetes type II mouse model group and last is transgenic control group (Figure 4B).

In Alamar blue proliferation assay, the proliferation was measured based on the % reduction of Alamar blue in the culture media. Background control group showed significant increase of Alamar blue % reduction between day 1,5, and 7, however, there is no significant difference for transgenic control group and gut leak-induced diabetes type II group between day 1, 5, and 7. At day 1 % of Alamar blue reduction for both transgenic control group and gut leak-induced diabetes type II group exhibited significant increase compared to background control group. Even though it is not statistically different compared to background control group, after day 1, transgenic control group showed trends of decreasing upon day 5 and increasing again in day 7. These were in contradiction with gut leak-induced diabetes type II mouse model group compared with background control group, it showed significant increase in day 5, while in day 7 it doesn't show significant difference. Nevertheless, it still shows decreasing trends of the Alamar Blue % of reduction compared to previous day (Figure 4C).

Figure 4D showed all groups were able to form colony during the colony formation unit assessment despite the differences in colony number. Transgenic control group colony count was significantly lesser compared to the background control group. In contrast, there is no significant difference in colony count between gut leak-induced diabetes type II group with the background control group (Figure 4E).

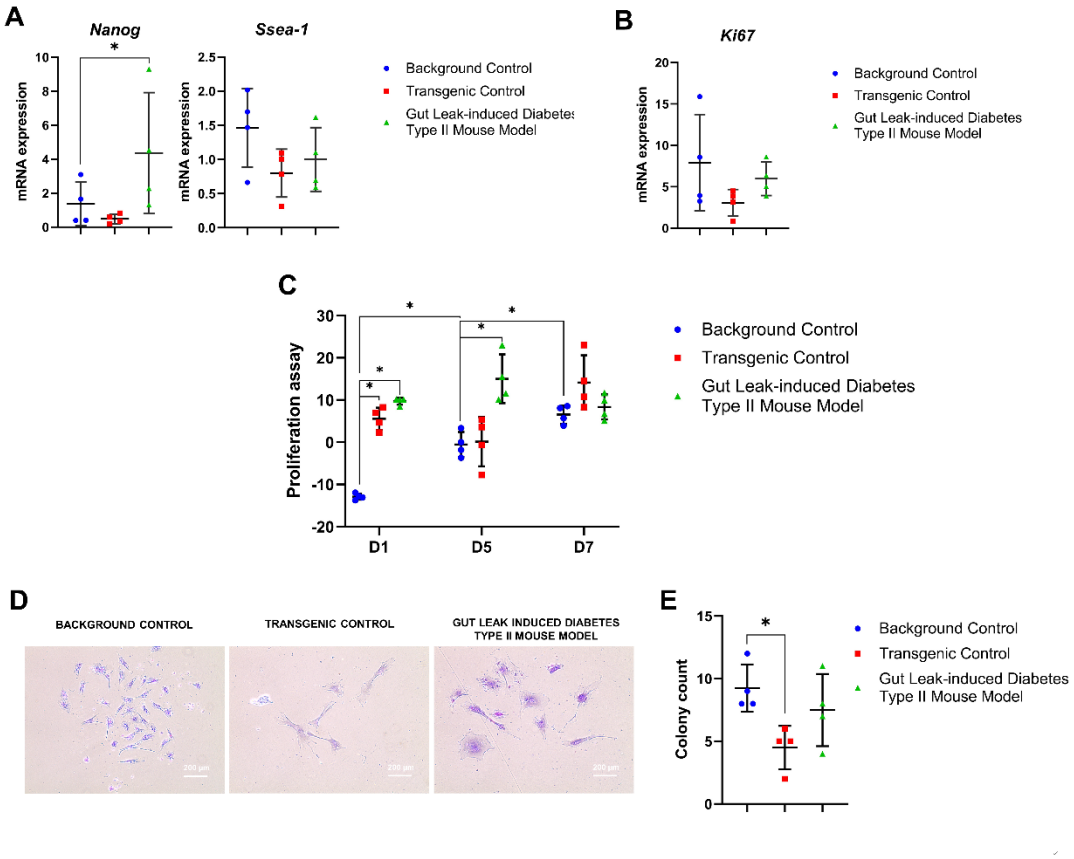


Figure 4. Stemness and Proliferation of mBM-MSCs. Isolated mBM-MSCs were analyzed for its stemness gene marker, Nanog and Ssea-1 (A) and proliferation gene marker, Ki67 (B). Cells from passage 1 also subjected into proliferation assay using Alamar Blue (C) and colony forming unit morphology, scale 200 µm (D) and colony count (E).

Effect of gut Leak-induced diabetes type II animal model on mBM-MSCs Osteogenic gene expression

Upon 21 days of induction with osteogenic differentiation medium, cells were assessed for osteogenic gene expression. Osteogenic genes which were measured are *Runx2*, *Col1a1*, *Alp*, *Opn*, *Ocn*, and *Osx*. Similar findings were found in both control groups for *Runx2* and *Ocn* expression as seen in Figure 5 A and E, they demonstrate significant upregulated *Runx2* and *Ocn* expression. The differences are in gut leak-induced diabetes type II mouse model where *Runx2* were significantly downregulated but *Ocn* were significantly upregulated. The *Col1a1* result expression showed that it is not upregulated in both control groups. However, gut leak-induced diabetes type II mouse model shows significantly downregulated expression compared to background control group (Figure 5B). The *Alp* gene expression in Figure 5C illustrated significant decrease of alp gene in osteogenic induced cell not only in gut leak-induced diabetes type II mouse model but also in transgenic control group compared to background control. Additionally, *Opn* result shows there is no significant different between osteogenic induced cell and controls, as well as osteogenic induced cells among groups (Figure 5D). *Osx* gene expression result also shows no difference of osteogenic induced cells among groups. Nevertheless, it shows significant increase of *Osx* expression in osteogenic induced cells of background control group compared to the control cells (Figure 5F).

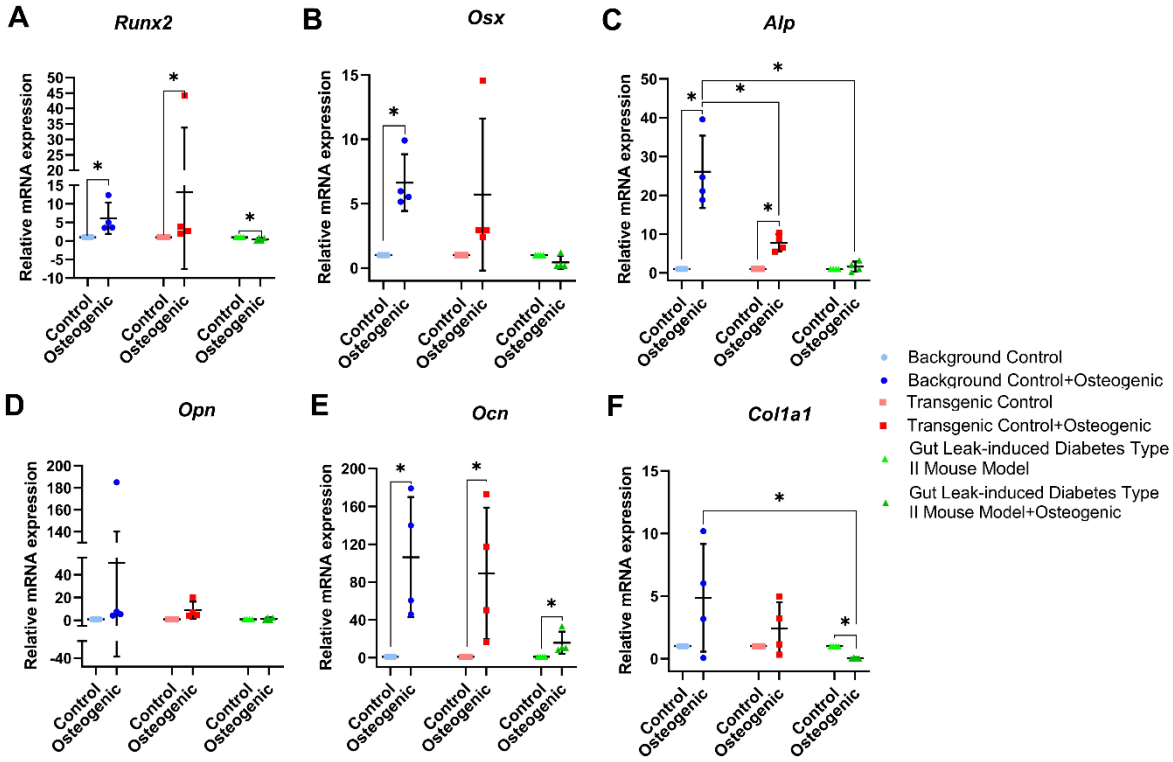


Figure 5. Osteogenic gene expression. After 21 day of induction mRNA from mBM-MSCs were collected and assessed for expression of osteogenic gene Runx2 (A), Col1a1 (B), Alp (C), Opn (D), Ocn (E), and Osx (F), the result was normalized to the house keeping gene Rpl.13. a

Effect of gut Leak-induced diabetes type II animal model on mBM-MSCs ALP activity and mineralization

Analysis of ALP activity was done by comparation of control and osteogenic induction of the same group, and osteogenic induction among groups. As shown in Figure 6A, osteogenic induction from all group shows higher ALP activity of compared to the control. In addition, osteogenic induction of gut leak-induced diabetes type II mouse model group showed significant lower ALP activity compared to osteogenic induction of transgenic and background controls. Moreover, there is no significant different of osteogenic induction ALP activity between transgenic and background control group.

Evaluation of mineralization was done by staining the cell with Alizarin red S and *Von Kossa* staining. From Figure 6B, it is evident that all osteogenic induction cell shows mineralization compared to the control cell. With Alizarin red S staining the mineral were stained red and in *Von Kossa* staining it's stained black.

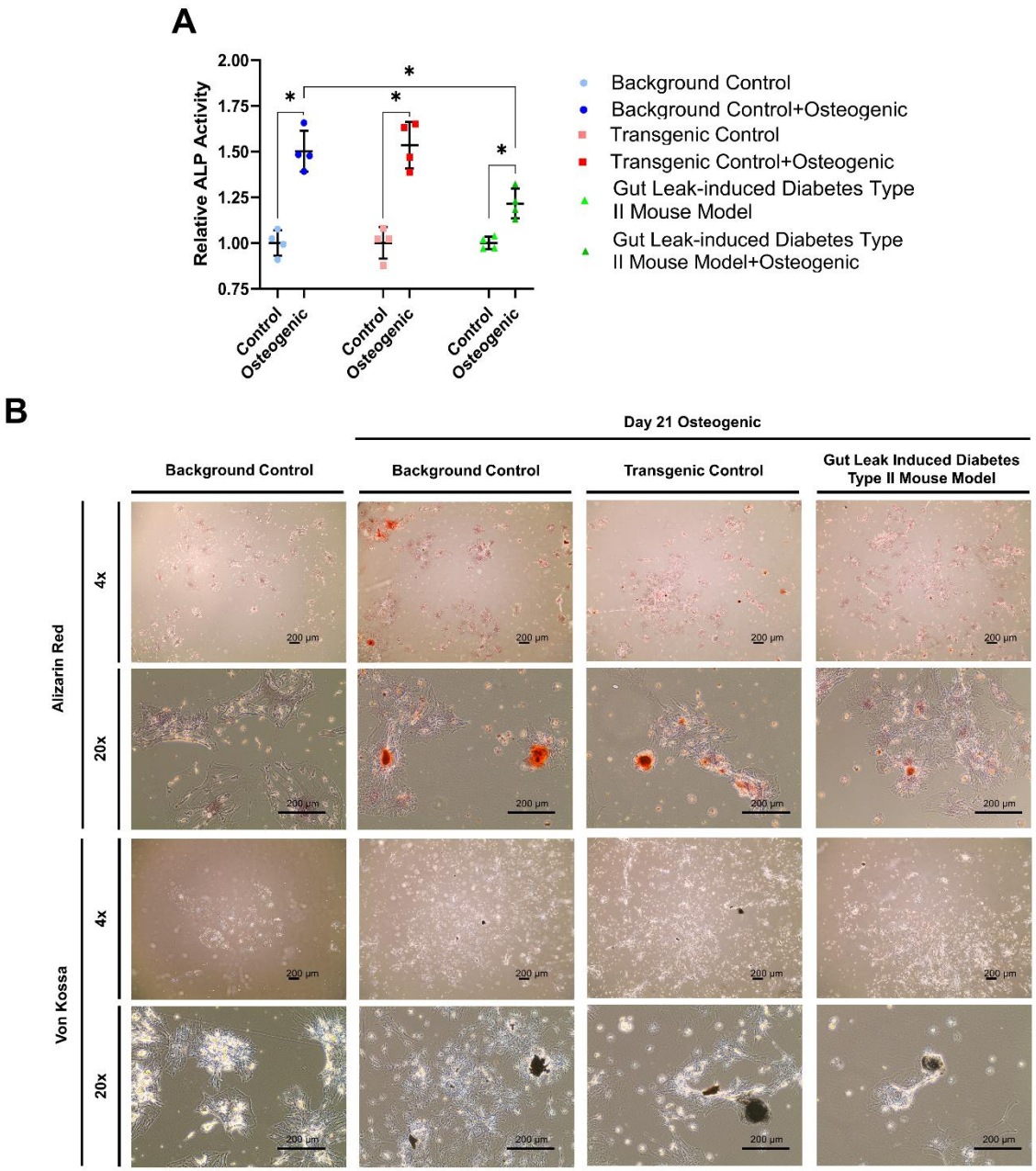


Figure 6. ALP activity and mineralization of osteogenic induced mBM-MSCs. Upon Day 21 ALP activity were measured (A) and cell mineralizationn was observed using Alizarin red S and Von Kossa staining (B).

DISCUSSION

Diabetes type II mouse model in this study has loss of body weight and color in tibia macroscopic assessment. Similar to our finding significant weight loss were seen in diabetes induced by DSS (Sorini et al., 2019). Moreover, Sikka et al. (2013) study show that there is no significant difference between C57BL/6NJcl and B6.129-IL10tm1Cgn/J. In gut leak-induced animal model despite the apparent weight loss, there were no changes in tibial size, but there was change in tibia color in this group which might be indicative of the changes in bone homeostasis and bone microscopic structure. Further histological and histomorphometry analysis result confirm that gut leak-induced diabetes type II displays loss of trabecular bone in proximal tibia. These changes were in line with research done in gut-leak induced diabetes in mice (Sorini et al., 2019) as well as other genetically modified diabetic mice models as they exhibit decrease of trabecular bone mass and thickness (Fu et al., 2015; Tanaka et al., 2018; Ham et al., 2019; Rios-Arce et al., 2020).

Isolated mBM-MSCs from all group exhibited similar spindle shape morphology at day 5, matching with Huang et al. (2014) mBM-MSCs cell's morphology. According to International Society for Cell and Gene Therapy (ICST), there are several markers including CD44, CD73, CD90 and CD105 referring MSCs characteristic. Moreover, it should be negative of CD45 and other hematopoietic cell markers. However, difference with human, mice MSCs absent of CD90 (Hu et al., 2018). Furthermore, MSCs surface marker in mice also differs among strain. The SCA-1 is a surface marker known to present in C57BL/6NJcl but absent in BALB/C mice (Peister et al., 2004). In this regard, our study demonstrated MSCs surface markers of the isolated cells using CD44, CD73, CD105 and SCA-1.

In this study the isolated mBM-MSCs from control group show 46.77% expression of CD44 which lower than transgenic control group and gut leak-induced diabetes type II mouse model. Other study using similar passage number also found similar finding for their mBM-MSCs which showed weak expression of CD44. This CD44 expression were later increase in further subculture (Baustian et al., 2015). The overall

CD73 expression from isolated mBM-MSCs for all group is low. This phenomenon caused from isolated cells were still in passage 1. In agreement with Baustian et al. (2015), the result from the isolated MSCs of passage 1 showed low level of CD73 expression, while CD73 expression increased in passage 2 and 3. In this study, CD73 expression in background control group was higher compared to other groups. Our result showed the similarity to Al-Qarakhli et al. (2019) that despite in high glucose condition rat MSCs were able to expressed several marker of MSCs such as CD73 and CD105. The overall expression of CD105 in this study were low, whereas SCA-1 were weakly expressed. Nevertheless, the expression of CD105 and SCA-1 demonstrated the same trend in gut leak-induced diabetes type II mouse model group which presented the highest expression of CD105 and SCA-1 followed by background control and transgenic control, respectively. CD105 is known to express with low level in early culture of murine MSCs (Baustian et al., 2015). However, SCA-1 expression showed high throughout the culture period. The disagreement of SCA-1 expression with other research might be due to different culture condition. Previous study illustrated that 90% SCA-1^{-} cells in first passage was able to turn into SCA-1^{+} cell in passage 2. Moreover, SCA-1- cells also still demonstrated the ability to differentiate into osteogenic, adipogenic, and chondrogenic lineage (Baustian et al., 2015). Next, the low level of CD45 is achievable in this study despite in early passage due to repetitive washing to wash out the non-attached hematopoietic cells. From the previous published method, the isolated protocol suggested to wash the culture dish with PBS to separate between the hematopoietic cells and MSCs derived from bone marrow Liu et al. (2018). Therefore, all mBM-MSCs isolated in this study expressed extremely low level of hematopoietic marker, CD45.

To further characterize the isolated mBM-MSCs, cells were analyzed for stemness gene marker including *Nanog* and *Ssea-1*. The result illustrated that mBM-MSCs from all group expressed both *Nanog* and *Ssea-1*. In addition, gut leak-induced diabetes type II mouse model showed the increase of mRNA expression compared to background control group. Despite not statistically significant difference in *Ssea-1* expression, a trend of background control illustrated more upregulation of *Ssea-1* gene compared to transgenic control and gut leak-induced diabetes type II mouse model is

evident. *Nanog* is well-known as stemness gene marker for pluripotency (Silva et al., 2009) and *Ssea-1* is known as embryonic stem cells (ESCs) marker (Calloni et al., 2013). Research done by Anjos-Afonso and Bonnet (2006) presented that *Ssea-1* positive mBM-MSCs has extensive differentiation potential proven by its superior capacity to differentiate not only into common MSCs lineage *in vivo* but also other lineages including hepatocyte-, astrocyte-and endothelial- like *in vitro*.. Therefore, following the result, isolated cells in this experiment were suggested as actual MSCs with multilineage differentiation capability.

To evaluate the proliferation of MSCs, gene level in this study was assessed the proliferation gene marker *Ki67*. Moreover, in functional level, Alamar Blue reagent was utilized. From gene level result, all groups showed the expression of the *Ki67*. Based on Alamar blue proliferation result in time series basis, the proliferation level of background control group was significantly increased over the course of a week, whereas the proliferation level of gut leak-induced diabetes type II mouse model was declined over the last day and transgenic group showed to decrease the proliferation rate at the middle of the week. The result suggested that both transgenic group and gut leak-induced diabetes type II group illustrated less proliferation rate in the long-cultured period. The absence of IL-10 known to induce inflammation (van Exel et al., 2002). Inflammation condition known to cause MSCs ageing that can lead to declining of MSCs proliferation (Lepperdinger, 2011). Gut leak-induced diabetes type II group result is in alignment with Sun et al. (2020), AD-MSCs derived from diabetic type II individual showed less proliferation level compared to control cells. In this regard, type II diabetes have effect on murine MSCs proliferation. Next, the CFU assay result demonstrated that isolated cells from all group were able to form as colony. The colony definition was done according to previous research done by Cai et al. (2015) which cell cluster with ≥5 was counted as 1 colony. In addition, the number of colonies in background control group was significantly higher than transgenic control group. However, it is not significantly different of colony count compared to gut leak-induced diabetes type II mouse model. This trend is in line with the *Ki67* and proliferation assay, which is possible as the CFU assay were done for 14 days. Therefore, isolated cells with low proliferation rate produced small number of cells in the colony.

Osteogenic differentiation was done by treat the mBM-MScs with osteogenic differentiation medium for 21 day. The properties of osteogenic differentiated cells were assessed by measuring the osteogenic mRNA expression, ALP activity and extracellular matrix mineralization. Total of 6 specific genes referring osteogenic differentiation (*Runx2*, *Osx*, *Alp*, *Opn*, *Ocn*, and *Col1a1*) were analyzed using RT-q PCR. Based on result illustrated in Figure 6, gut leak-induced diabetes type II mouse model expressed the downregulation of osteogenic gene (*Runx2* and *Col1a1),* while *Ocn* showed upregulated expression. On the other hand, background control group and transgenic control group expressed upregulation of *Runx2*, *Alp*, *Ocn*, and *Col1a1*. However, none of the group presented upregulation level of *Opn* compared to control. Focusing on *Osx,* only background control group showed significant increase. Previous study reviewed that *Runx2* and *Col1a1* play crucial role in promoting proliferation and differentiation to the osteogenic lineage (Dai et al., 2019). In this study, we found the downregulated expression of *Runx2* and *Col1a1* in gut leak-induced diabetes type II mouse model compared to both background control and transgenic control group. Moreover, even though MSCs also known to secrete collagen for its ECM under undifferentiated state (Novoseletskaya et al., 2020), in gut leak-induced diabetes type II group Col1a1 gene expression from the differentiated cell were significantly lower compared to the undifferentiated control. It could be assumed that isolated cells from background control and transgenic control group have superior osteogenic differentiation properties compared to gut leak-induced diabetes type II mouse model. This is supported by Kim et al. (2013) result as osteogenic gene marker *Runx2, Osx, Alp* were down regulated in high glucose condition. In contrast, because *Opn* expressed in the middle stage of osteogenic induction marker (Kusuyama et al., 2019). In the late stage of osteogenic induction at day 21, *Opn* presented the low expression. Moreover, previous study also stated that *Opn* is not essential for normal osteogenesis in mice supported by Kusuyama et al. (2017). The findings presented *Opn* exhibits the suppressive effect to the osteoblast. Though *Ocn* is also known as middle ostegenic marker (Liu et al., 2013), *Ocn* expression pattern demonstrated the distinction with *Opn.* The result showed the high level of *Ocn* expression at day 21 post-induction.

This is possible because in mice *Ocn* is expressed later than *Opn* (Kusuyama et al., 2019).

The ALP activity result from all groups illustrated considerably increase between osteogenic induced cells and control cells. However, gut leak-induced diabetes type II mouse model presented lower ALP activity than other groups. In the same agreement with ALP gene expression, gut leak-induced diabetes type II mouse model demonstrated significantly lower mRNA expression compared to the background control group. Because ALP is an osteoblast marker and indicator of bone synthesis (Baharara et al., 2014; Blair et al., 2017; Westhauser et al., 2019), gut leak-induced diabetes type II mouse model may relate inferior properties of osteogenic lineage compared to control group.

To ensure isolated cells differentiated into osteogenic lineage, mineralization assay 21-day post-induction was investigated. From fundamental knowledge, a mature stage osteoblast become to be an osteocyte surrounded by extracellular matrix that mineralized (Florencio-Silva et al., 2015). To assess the mineralize calcification, Alizarin red S staining were considered to identify mineralized calcium and *Von Kossa* staining were employed to assess the calcium-phosphate deposition (Jeon et al., 2018). All groups exhibited mineralize formation which shown in red area using Alizarin red S and brown-black clump in color using *Von Kossa* staining. In agreement with gene expression and ALP activity result, both Alizarin red S and *Von Kossa* staining from background control group and transgenic control group showed superior osteogenic properties to the gut leak-induced diabetes type II mouse model. Our result presented the similarity to Wang et al. (2013) research which demonstrated the high glucose condition caused increase the growth of MSCs but decrease MSCs mineralization on mBM-MSCs derived from C57BL/6 mice. Moreover, Ribot et al. (2021) study of rat BM-MSCs derived from diabetes type II rat model, the normoglycemic condition showed that despite cultured in normoglycemic condition, rat BM-MSCs still expressed elevated reactive oxygen species (ROS) level due to diabetes type II environment permanently affected cellular ROS production. In addition, elevated level of ROS in MSCs were known to inhibit the osteogenic differentiation (Chen et al., 2008; Denu and

Hematti, 2016). Therefore, gut leak-induced diabetes type II condition affects the osteogenic differentiation potential in mBM-MSCs.

CHAPTER V

CONCLUSION

In conclusion, our study assessed the effect of gut leak-induced diabetes type II mouse to bone loss, mBM-MSCs characterization and osteogenic differentiation properties. The result of this study indicated gut leak-induced diabetes type II mouse model exhibited bone loss, the isolated mBM-MScs presented characteristics of mesenchymal stem cells and lack of osteogenic differentiation properties. These findings revealed that diabetes type II affects bone in term of bone loss and the osteogenic differentiation properties of MSCs derived from bone marrow. The outcome from this study is crucial as further suggestion and base line for exploration, utilization, and application of BM-MSCs to and from diabetic individual.

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