EFFECT OF LITHIUM CHLORIDE AND SB216763 ON CHONDROGENIC DIFFERENTIATION OF HUMAN WHARTON'S JELLY DERIVED MESENCYMAL STEM CELLS

Prapot Tanthaisong

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ผลของลิเทียมคลอไรด์และ SB216763 ต่อการเหนี่ยวนำเซลล์ต้นกำเนิดมีเซนไคม์จากเนื้อเยื่อวาร์ตันเจลลี่ของมนุษย์เป็นเซลล์กระดูกอ่อน

นายประพจน์ ทันไธสง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ประพจน์ ทันไธสง : ผลของลิเทียมคลอไรด์และ SB216763 ต่อการเหนี่ยวนำเซลล์ต้นกำเนิดมีเซนไคม์จากเนื้อเยื่อวาร์ตันเจลลี่ของมนุษย์เป็นเซลล์กระดูกอ่อน (EFFECT OF LITHIUM CHLORIDE AND SB216763 ON CHONDROGENIC DIFFERENTIATION OF HUMAN WHARTON'S JELLY DERIVED MESENCYMAL STEM CELLS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 65หน้า

กระดูกอ่อนผิวข้อคือเนื้อเยื่อที่ไม่มีหลอดเลือด ไม่มีท่อน้ำเหลือง และไม่มีระบบประสาท จึงมีการฟื้นฟูที่ต่ำมากเนื่องจากมีข้อจำกัดในการซ่อมแซมตัวเอง เซลล์ต้นกำเนิดมีเซนไคม์ (Mesenchymal stem cells, MSCs) จึงเป็นอีกทางเลือกหนึ่งที่นำมาใช้ในการรักษาแบบเซลล์บำบัด สารยับยั้งเอนไซม์ไกลโคเจนซินเทรสไคเนส 3 (Glycogen synthase kinase 3 inhibitors, GSK-3 inhibitors) เป็นสารประกอบที่มีศักยภาพในการเหนี่ยวนำให้เกิด Wnt signaling pathway ซึ่งเกี่ยวข้องกับการเกิดเซลล์กระดูกอ่อนและการสร้างกระดูกอ่อนผู้วิจัยจึงทำการตรวจสอบอิทธิพลของ ลิเทียมคลอไรด์ (LiCl)และ SB216763ร่วมกับ Transforming growth factor - beta 3 (TGF-β3) ต่อการเหนี่ยวนำให้เป็นเซลล์กระดูกอ่อนจากเซลล์ต้นกำเนิดมีเซนไคม์จากเนื้อเยื่อวาร์ตันเจลลี่ของมนุษย์ (human Wharton's jelly derived mesenchymal stem cells, hWJ-MSCs) โดยนำเซลล์มาเลี้ยงในน้ำยาเหนี่ยวนำเซลล์กระดูกอ่อนภายใต้สภาวะการทดลองแบบพื้นผิวชั้นเดียว (monolayer) และแบบกลุ่มก้อน (pellet) โดยแบ่งกลุ่มการทดลองเป็นดังนี้ คือ 1) กลุ่มน้ำยาเหนี่ยวนำเซลล์กระดูกอ่อน (chondrogenic medium)2) กลุ่มน้ำยาเหนี่ยวนำเซลล์กระดูกอ่อนที่เติมลิเทียมคลอไรด์ (chondrogenic medium + LiCl) และ 3) กลุ่มน้ำยาเหนี่ยวนำเซลล์กระดูกอ่อนที่เติม SB216763 (chondrogenic medium + SB216763)หลังการเหนี่ยวนำในหลอดทดลอง นำเซลล์ที่เพาะเลี้ยงมาตรวจสอบการแสดงออกของยีนและโปรตีน*Sox9*,*ACAN*, *Col2a1*, และ*β-catenin* และการสะสมของไกลโคสมิโนไกลแคน(Glycosaminoglycans, GAGs) ด้วยการย้อมสี Alcain blue จากผลการทดลองแสดงให้เห็นว่า SB216763 มีประสิทธิภาพ ในการเหนี่ยวนำ เซลล์ต้นกำเนิดมีเซนไคม์จากเนื้อเยื่อวาร์ตันเจลลี่ของมนุษย์ให้เป็นเซลล์กระดูกอ่อนได้ มากกว่าลิเทียมคลอไรด์ซึ่งดูได้จากการเพิ่มขึ้นของยีนและโปรตีนเป้าหมายของกระดูกอ่อนผิวข้อ คือ *Sox9*, *ACAN*, และ*Col2a1*รวมถึงการเพิ่มขึ้นของไกลโคสมิโนไกลแคนด้วย นอกจากนี้ยังพบว่าการแสดงออกของโปรตีน collagen type II ในกลุ่มน้ำยาเหนี่ยวนำเซลล์กระดูกอ่อน ที่เติม SB216763 เห็นได้ชัดเจนหลังจากตรวจสอบโดย Western blot ทั้งสองกลุ่มการทดลองส่งผลให้เกิดการส่งสัญญานผ่าน Wnt signaling pathway โดยมีผลมาจากการเพิ่มการแสดงออกของ*β-catenin*ทั้งนี้ในทุกกลุ่มการทดลองไม่ส่งผลต่อกระบวนการทำให้เซลล์กระดูกอ่อนมีขนาดใหญ่หรือบวมขึ้น (chondrocyte hypertrophy) ซึ่งเป็นผลมาจากการ

ยับยั้งการแสดงออกของ *Col10a1*และ *Runx2*จากผลการศึกษาครั้งนี้บ่งชี้ให้เห็นว่าลิเทียมคลอไรด์และ SB216763 อาจเป็นตัวเลือกที่เหมาะสมสำหรับการนำมารักษาการฟื้นฟูกระดูกอ่อนผิวข้อในสัตว์ทดลองและมนุษย์ต่อไป

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา2559 ลายมือชื่ออาจารย์ที่ปรึกษา_____

PRAPOT TANTHAISONG : EFFECT OF LITHIUM CHLORIDE AND SB216763 ON CHONDROGENIC DIFFERENTIATION OF HUMAN WHARTON'S JELLY DERIVED MESENCYMAL STEM CELLS. THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph D., 65 PP.

MESENCHYMAL STEM CELLS/WHARTON'S JELLY TISSUE/ CHONDROCYTES/LITHIUM CHLORIDE/SB216763/GSK-3 INHIBITORS

Articular cartilage is avascular, alymphatic, and aneural system with very low regeneration due to its limited capacity for self-repairs. Mesenchymal stem cells (MSCs) are a preferred choice for cell-based therapies. Glycogen synthase kinase 3 (GSK-3) inhibitors are potential compounds to initiate Wnt signaling pathway which involved in chondrogenesis and cartilage development. Here we investigated the influence of Lithium chloride (LiCl) and SB216763synergistically with transforming growth factor - beta 3 (TGF-β3) on chondrogenic differentiation in human mesenchymal stem cells derived from Whaton's jelly tissue (hWJ-MSCs). The hWJ-MSCs were cultivated and induced chondrogenic differentiation under monolayer and pellet conditionsin chondrogenic medium, chondrogenic medium supplemented with LiCl or SB216763. After *in vitro* differentiation, cultures cells were then examined for the expression of*Sox9*, *ACAN*, *Col2a1*, and *β-catenin* markers. Glycosaminoglycans (GAGs) accumulation was also examined by Alcain blue staining. The results indicated that SB216763 was more effective inducing chondrogenic differentiation than LiCl as evidenced by their higher up-regulate of the cartilage-specific markers including *Sox9*, *ACAN*, *Col2a1* as well as GAGs accumulation.Western blot

analysisindicated thatthe collagen type II expression was also strongly observed in alls grown in the chondrogenic medium + SB216763. Both treatments appeared to mediate the Wnt signaling pathway by up-regulation of*β-catenin* gene. Further analysis showed that all treatments suppressed the progression of chondrocytes hypertrophy markers as seen by the decreasedexpressions of *Col10a1* and *Runx2*. These results indicated that LiCl and SB216763 are choice candidates for further *in vivo* therapeutic trials and would be of great importance for cartilage regeneration in animals and human.

Academic Year 2016 Advisor's Signature _____

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LIST OF ABBREVIATIONS

AP	=	alkaline phosphatase
BMPs	=	bone morphogenetic proteins
CD	=	cluster of differentiation
CD-RAP	=	cartilage-derived retinoic acid-sensitive protein
COMP	=	cartilage oligomeric protein
DAPI	=	4’,6-diamino-2phenylindole
DMSO	=	dimethyl sulfoxide
ECM	=	extracellular matrix
FBS	=	fetal bovine serum
FGF	=	fibroblast growth factor
GAGs	=	glycosaminoglycans
GSK-3	=	glycogen synthase kinase-3
hWJ-MSCs	=	human Wharton’s jelly derived mesenchymal stem cells
IBMX	=	3-isobutyl-1-methylxanthine
ITS	=	insulin-transferrin-selenium-ethanolamine
MSCs	=	mesenchymal stem cells
α-MEM	=	alpha modification of Eagle’s medium
MMP-13	=	matrix metallopeptidase 13
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OA	=	osteoarthritis

LIST OF ABBREVIATIONS(Continued)

Runx2	=	runt-domain transcription factor 2
Sox9	=	transcription factor sex-determining region Y protein (SRY)-box 9
TGF-β	=	transforming growth factor-beta
UC	=	human umbilical cord
VEGF	=	vascular endothelial growth factor
WJ	=	Wharton's jelly tissue

CHAPTER I

INTRODUCTION

1.1 Background

Articular cartilage is a highly specialized connective tissue of synovial joints. Chondrocytes are specialized cells in this tissue responsible for the generation of extracellular matrix (ECM) and maintain the tissue function. Injuries of articular cartilage are generally not able to self-repair due to the lack of vascular, lymphatic, or nervous systems (Fox et al., 2009).

Recently, an alternative approach of cartilage preservation and repair uses stem cell-based therapies such as Mesenchymal stem cells (MSCs). MSCs could be isolated from bone marrow (Friedenstein et al., 1961), adipose tissue (Zuk et al., 2001), dental pulp (Gronthos et al., 2000), umbilical cord blood (Phuc, et al., 2011), and Wharton's jelly tissue (Wang et al., 2004). Several sources of MSCs exhibited different properties of stemness, expansion capacity, and multilineage differentiation (Bonab et al., 2006; Dominici et al., 2006). Wharton's jelly tissue (WJ) is another alternative source of MSCs which show properties similar to MSCs from other source. It is a rich source of primitive cells (Wang, 2004; Troyer and Weiss, 2008). In addition, WJ-MSCs has greater proliferation rates, expansion potential as well as differentiation potential than other adult MSCs (Fong et al., 2011). Thus, WJ-MSCs have been considered asa source for candidate cellsand therapeutic potential for cartilage regeneration.

Members of transforming growth factor-beta (TGF-β) superfamily are the most crucial inducer of chondrogenic differentiation in MSCs such as transforming growth factor-beta (TGF-β), and bone morphogenetic proteins (BMPs) (Indrawattana et al., 2004). Wnt signaling pathway has also been reported to be involved in chondrogenesis and cartilage development (Chun et al., 2008). Canonical Wnt signaling is mediated by β-catenin, which accumulates in the cytoplasm and then translocates to the nucleus. β-catenin forms a complex with DNA-binding T-cell factors (TCFs) to activate the transcription of target genes. β-catenin signaling pathway often crosstalk with other signaling pathways in modulating chondrogenesis (Fischer et al., 2002; Tuli et al., 2003; Kirton et al., 2007; Yang et al., 2012; Eslaminejad et al., 2013; Narcisi et al., 2015). However, Wnt/β-catenin signaling pathway plays a crucial role in the hypertrophic maturation of chondrocytes in endochondral ossification process (Kawata et al., 2010). Another key component regulator of the Wnt signaling pathway is Glycogen synthase kinase 3 (GSK-3) enzymes that mediated β-catenin phosphorylation (MacDonald et al., 2009). Inhibition of GSK-3 enzyme has been shown to promote the accumulation of β-catenin and complex with a co-transcription factors LEFs/TCFs to promote transcription(Li and Jope, 2010; Chuang et al., 2011).

In this study, we investigated the influence of LiCl and SB216763, which act synergistically with TGF-β3 on chondrogenic differentiation in hWJ-MSCs. The results demonstrated that LiCl and SB216763 can greatly enhance the chondrogenic potential in monolayer and pellet cultures. These treatments shown to up-regulate the cartilage-specific markers including *Col2a1*, *Sox9*, *ACAN*, as well as GAGs

accumulation. Moreover, the progression of chondrocytes hypertrophy was suppressed by inhibited the expression of *Col10a1* and *Runx2* markers**.**

1.2 Research objectives

1.2.1 To establish MSCs from Wharton's jelly tissue from human umbilical cords.

1.2.2 To investigate the influence of LiCl and SB216763 synergistically with TGF-β3 on chondrogenic differentiation of hWJ-MSCs.

1.2.3 To examine the chondrocytes derived from hWJ-MSCs and the progression of chondrocytes hypertrophy.

1.3 Research hypotheses

1.3.1 MSCs could be isolated and expanded from Wharton's jelly tissue of human umbilical cord and should exhibit typical properties of MSCs, which are self-renewal, immunophenotyping, as well as their ability to multipotent differentiation in*in vitro*.

1.3.2 GSK-3 inhibitors, LiCl and SB216763 activate the Wnt signaling pathway and promote the chondrogenic differentiation of hWJ-MSCs in the present of TGF-β3.

1.3.3 The chondrocytes derived from hWJ-MSCs could suppress the progression of chondrocytes hypertrophy during the differentiation.

1.4 Scope of the study

1.4.1 MSCs were isolated and expanded from Wharton Jelly tissue on umbilical cords. Properties of MSCs derived from this tissue were examined by the expression profile of surface antigens (e.g. CD34, CD73, CD90, and CD105). The multipotent properties were verified by the differentiation of hWJ-MSCs into mesodermal lineages; chondrocytes, adipocytes and osteocytes.

1.4.2 Monolayer and Pellet cultures methods were used to differentiate toward chondrocytes by appropriate culture conditions with an addition of LiCl or SB216763. Wnt signaling pathway was demonstrated by the expression of *β-catenin* gene. GAGs accumulations were stained by Alcian blue. Immunofluorescent and Western blot analysis used to examine the expressions of ECM protein. Gene expressions of *Col2a1*, *Col10a1*, *ACAN*, *Sox9*, *β-catenin*, and *Runx2* were qualified by qPCR.

CHAPTER II

LITERATURE REVIEW

2.1 Articular cartilage

Articular cartilage is a hyaline cartilage that covers bone heads on a synovial joint (Poole et al., 2001). The principal function is to provide a smooth, lubricated surface for articulation and to facilitate the transmission of loads with a low frictional coefficient (Fox et al., 2009). Chondrocytes cells are the major component of this tissue which generated the ECM and maintenance the tissue function (Buckwalter and Mankin, 1998). Native cartilage ECM consisting of collagen fiber and abundant ground substrate rich proteoglycan more than 95% of its volumes. Articular cartilage is subject to a harsh biomechanical environment due to a lack of blood, lymphatic, and nerves system (Fox et al., 2009). Most importantly, articular cartilage has a limited capacity for self-repair.

2.1.1 Structure of articular cartilage

The classification of articular cartilage structure is based on cell morphology and organization of collagen fibrils (Fig 2.1). Three zones of articular cartilage are composed of the superficial zone, the intermediate zone (middle), and the deep zone (Säämänen et al., 2010).

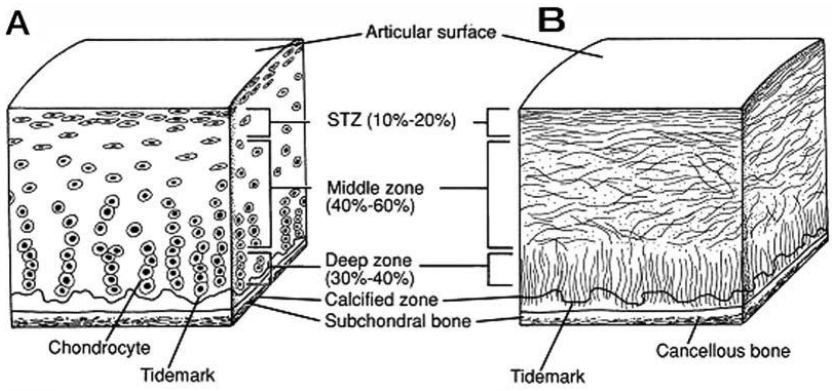

Figure 2.1 Schematic of cross-sectional diagram of healthy articular cartilage (Fox et al., 2009).

(A) Cellular organization in the zones of articular cartilage.

(B) Collagen fiber.

The superficial zone is the surface of the articular cartilage that protects the deeper layers from shear stresses. It makes up approximately 10-20% of articular cartilage thickness. This zone has high concentration of collagen fibrils and lowest concentration of proteoglycans, thus making this as the highest water content. The primarily type II and IX collagen fibrils are majors of this zone. There are packed tightly and aligned parallel to the articular surface. The parallel arrangement of the fibrils is responsible for providing the greatest tensile and shear strength by articulation (Fox et al., 2009; Säämänen et al., 2010).

The immediately zone (middle zone) represents 40-60% of the total cartilage volume. Chondrocytes are spherical and at low density. In this zone, there contains the higher proteoglycans and thicker collagen fibrils are randomly arranged (Fox et al., 2009; Säämänen et al., 2010).

The deep zone occupied up to 30% of the articular cartilage volume. This zone has the highest proteoglycan content, the largest diameter collagen fibrils, and the lowest water concentration. It is responsible for providing the greatest resistance to compressive forces (Fox et al., 2009; Säämänen et al., 2010).

The tide mark are distinguishes of the deep zone and the calcified cartilage. Cell population is scarce and chondrocytes are hypertrophic. This zone is developed to the subchondral bone.

2.1.2 Compositions of articular cartilage

2.1.2.1 Chondrocytes

Chondrocytes are the main cells type in articular cartilage and represent in 5% of the wet weight. The functional of chondrocytes are responsible for the production, organization, and maintenance of the articular cartilage ECM. Unfortunately, chondrocytes have limited potential for repair articular cartilage injury due to a factor that contributes to the limited intrinsic healing capacity. Chondrocytes survival depends on an optimal chemical and mechanical environment (Fox et al., 2009).

2.1.2.2 Extracellular matrix (ECM)

Three classes of cartilage ECM comprised of collagens (60–86% of dry weight), proteoglycans (15–40% of dry weight), and other noncollagenous proteins (Fig 2.2).

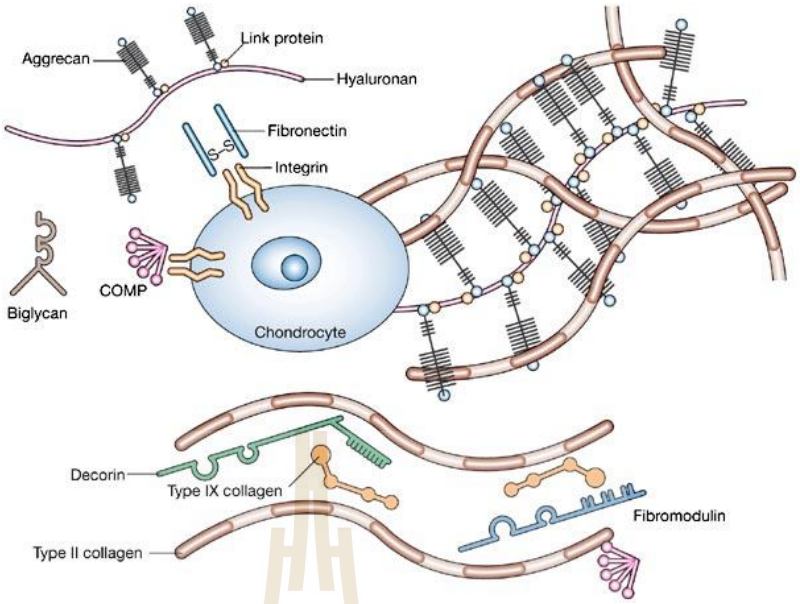

Figure 2.2 Extracellular matrix of articular cartilage. Two macromolecules: collagens (mainly, type II) and proteoglycans (notably, aggrecan) are present in articular cartilage (Chen et al, 2006).

Collagen fibrils are the most abundant structural macromolecule in ECM, their maintaining tensile resilience and strength of cartilage. The preliminary type II collagen is the major matrix protein constituting the bulk of the fibril. Type IX and XI collagen are facilitates fibril interaction with the proteoglycan molecules and regulates the fibril size (Roughley, 2006). Minor quantities of other types of collagen, including types VI, XII, and XIV, are found in the cartilage matrix (Eyre et al., 2002). Type X collagen is produced exclusively by prehypertrophic and hypertrophic chondrocytes (Schipani, 2005).

Proteoglycans represent the second largest group of macromolecules in the ECM.The most abundant of proteoglycan is aggrecan that has a large negative charge and affinity for water molecules (Roughley, 2006). Aggrecan is characterized by its ability to interaction with hyaluronan (HA) to form large

proteoglycan aggregates via link proteins (Fox et al., 2009). The subunits of proteoglycans are called glycosaminoglycans (GAGs). They contain two types, chondroitin sulfate and keratin sulfate. GAGs are bound to the protein core by means of sugar bonds, to form aggrecan molecule.Several small proteoglycans are found in cartilage ECM including decorin, biglycan and fibromodulin (Chen et al, 2006; Heinegard, 2009).

Glycoproteins are found within articular cartilage such as cartilage oligomeric matrix protein (COMP), their small glycoproteins that commonly found in hyaline cartilage. COMP is shown to express at a specific time during chondrogenesis, it can utilize as markers of cartilage turnover and degeneration (Heinegard, 2009).

2.2 Articular cartilage degeneration

Degenerative articular cartilage can be harmed in numerous ways, including paediatric growth plate disorders, trauma-induced injuries, and age-related degenerative joint disorders, such as Osteoarthritis (OA) (Song et al., 2004).

2.2.1 Osteoarthritis (OA)

Osteoarthritis (OA) is the most commonly degenerative articular disease affecting mainly the elder above 65 years of age worldwide(Frech and Clegg, 2007). The most frequently affected sites are knees, hands, and hips. OA is associated with multiples risk factor such as aging, joint trauma, metabolic, genetic, as well as obesity (Hunter and Felson, 2006;Goldring and Goldring, 2007). OA characterized by the progressive destruction of articular cartilage and eventually the entire joint. There

are associated with variable degrees of local inflammation, pain, and swelling of the damaged tissue. This tissue loosed of it stiffness and reduced mobility due to an imbalance in mechanical loading of the articular cartilage. Eventually, their development of osteophytes (bone spurs) and induced bone ends rub together due to lack of ability chondrocytes to repair tissue (Hunter and Felson, 2006; Frech and Clegg, 2007). (Fig 2.3)

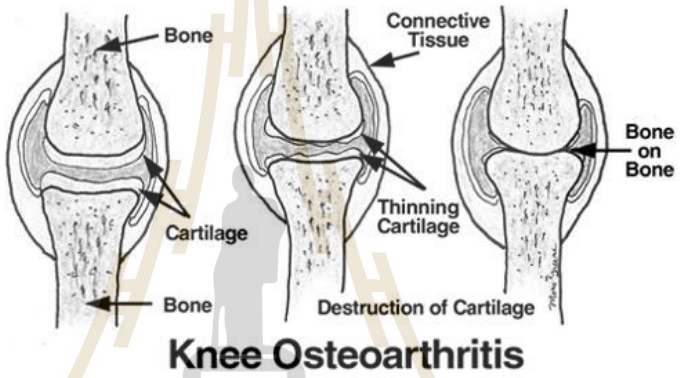

Figure 2.3 Progression of osteoarthritis in a healthy knee joint.

Pathogenesis of OA caused by multiples complex with the mechanical stresses, a limited supply of nutrients and oxygen, inadequate synthesis of ECM components, increased synthesis of matrix metalloproteinases (MMP) and aggrecanases as well as overall apoptosis of chondrocytes (Ross, 2006). Degenerative articular cartilage often leads to progressive degradation of the joint and the imbalanced homeostasis of the tissue. Therefore, articular cartilage damage need the treatment of the imbalanced homeostasis of the tissue eventually results in the development of OA (Buckwalter, 1998). The treatment strategies for degenerative articular cartilage such as OA are one of the major challenges.

2.2.2 Regenerative therapies

Degenerative articular cartilage affects the entire synovial joint. The regeneration of this tissue is a challenge due to their lacks of the ability to properly repair and regenerate tissue injuries.

Treatment mediates relieve the symptoms of joint pain and stiffness such as anti-inflammatory drugs (NSAIDs), which generally decrease pain and improve function, but have no beneficial effect on chondroprotection or OA disease prevention and modification (Cheng and Visco, 2012). Other clinical treatments for cartilage damage strategies have been developed to enhance the repair of articular cartilage such as the grafting of autologous osteochondral tissue or the transplantation of autologous chondrocyte suspensions (Hunziker et al., 2002; Redman et al., 2005). However, the biological and mechanical properties of the repair tissue formed are inferior to those of native articular cartilage. These methods do not sufficiently restore long-term function due to the restricted ability of the chondrocytes to repair the erosion of the cartilage ECM (Andriacchi et al., 2003). The challenges of regeneration have sought to improve chondrogenic recovery following expansion and find to alternative cell sources for cartilage repair.

2.3 Mesenchymal stem cells (MSCs)

Mesenchymal stem cells (MSCs) were identified firstly by Friedenstein et al., (1961) as a minor population in bone marrow stromal (Friedenstein et al., 1961). Currently, MSCs could be isolated from bone marrow (Pittenger et al., 1999), adipose tissue (Zuk et al., 2001), dental pulp (Gronthos et al., 2000), umbilical cord blood (Phuc, et al., 2011), and Wharton's jelly tissue (Wang et al., 2004). Several sources of

MSCs exhibited different properties of stemness, expansion capacity, and multilinage differentiation (Bonab et al., 2006; Dominici et al., 2006). To define MSCs properties, some minimal criteria were suggested by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) used to identify MSC (Fig 2.4). Firstly, their plastic adherent growth and subsequent expansion under standard culture conditions. Secondly, their express display a phenotype of CD105+, CD73+, CD90+, CD34-, CD45-, CD11b-, CD14b-, CD19-, CD79a-, and HLA-DR- Thirdly, multipotency differentiation, their ability to differentiate into osteoblasts, adipocytes and chondrocytes, *in vitro* (Dominici et al., 2006). The absence of tetaloma formation when transplanted *in vivo* and lack of ethical concerns make MSCs as a preferred choice for cell-based therapies (Lin et al., 2005).

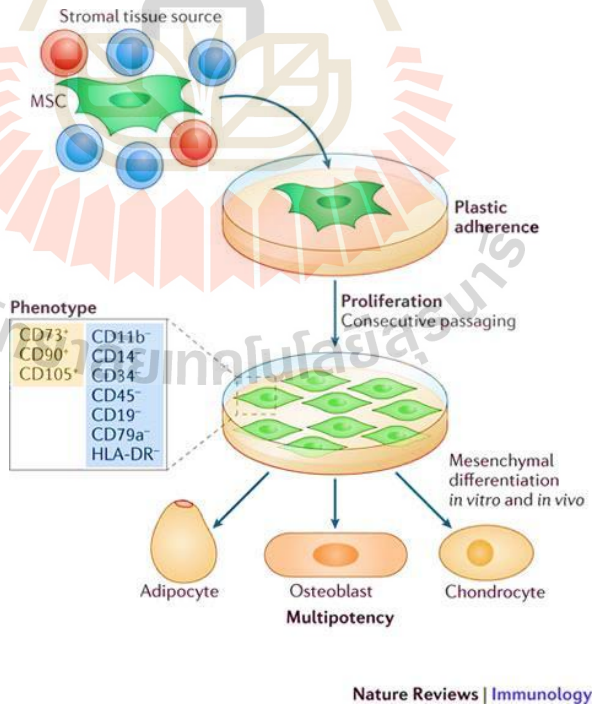

Figure 2.4 Three minimal criteria recommended for identification of MSCs (Le Blanc and Mougiakakos, 2012).

Umbilical cord (UC) has been shown as alternatives source of MSCs, there are an extraembryonic formation that constitutes the essential link between placenta and fetus during pregnancy. The structure of the umbilical cord is composed of umbilical vessels, two arteries and one vein, embedded intravascular mucous connective tissue known as Wharton's jelly (WJ) as shown in Fig 2.5 (Ishige et al., 2009). Thomas Wharton discovered this tissue in 1956. It usually produces epithelioid cell islands in a primary culture which transform into fibroblasts-like cells. WJ-MSCs show properties similar to the MSCs and represent a rich source of primitive cells (Wang, 2004; Troyer and Weiss, 2008). WJ-MSCs meet all criteria used to describe MSCs properties, as mentioned previously. In contrast to other MSC, WJ-MSCs express CD117, CD68, and CD14 (La Rocca, et al., 2009; Kita et al., 2010). Importantly, WJ-MSCs possessed some surface markers of embryonic stem cells (ESCs), Tra-160, Tra-181, SSEA-1, and SSEA-4 (Hoynowski et al., 2007). Pluripotent genomic markers also expressed at low levels such as Oct-4, Nanog, and Sox2 (Fong et al., 2007).

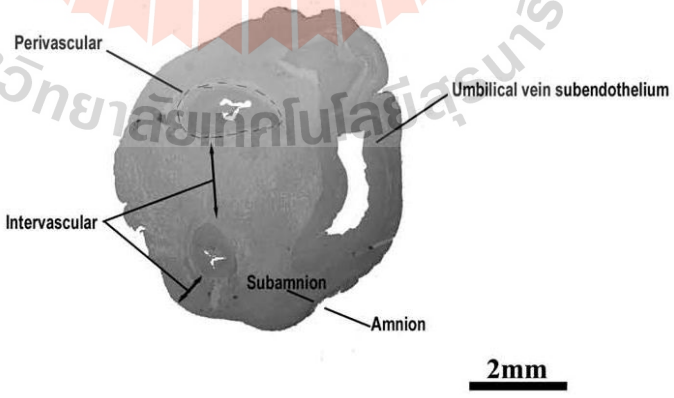

Figure 2.5 Traverse section through human umbilical cord tissue. Scale bar = 2 mm. Wharton's Jelly is the connective tissue included between the subamnion and the perivascular regions (Angelucci, 2010).

WJ-MSCs had grater faster proliferation rates and greater expansion potential than other adult MSCs (Fong et al., 2011). WJ-MSCs are defined as multipotent stem cells since they can differentiate into at least three different cellular lineages: osteoblasts, adipocytes and chondrocytes (Dominici et al., 2006). Indeed, WJ-MSCs has shown to differentiate ability goes far beyond the classic connective tissue cell types such as endothelial (Chen et al., 2009), glial cells and neurons (Mitchell et al., 2003), and insulin-producing cells (Wang et al., 2011). Such properties of WJ-MSC could alternative source of MSCs with potential for use in cell base therapies.

2.4 Chondrogenesis

MSCs are multipotent stem cells that are present in various adult tissues. There has been evidenced for regeneration and developmental of cartilage tissue. MSCs can undergo chondrogenesis and deposit an ECM in the presence of the appropriate growth factors (Erickson et al., 2002; 2009). Members of transforming growth factor-beta (TGF-β) superfamily are inducer for chondrogenesis such as TGF-β and BMPs (Indrawattana et al., 2004). The process of chondrogenesisinvolved the cells condensation followed by their differentiation into chondrocytes, chondrocyte maturation, and production of ECM (Fig 2.6).

MSCs begin with the cells condensation and aggregation in the present of a growth factor such as TGF-β3 (Roux, et al., 2013). Cells were expressed the adhesion molecules during condensation process including N-cadherin (Ncad),neural cell adhesion molecule (NCAM), and versican, involving the chondrogenesis (Zuscik et al., 2008).The levels of adhesion molecules dropped in the aggregation process, and possibly as a result of the assembly of pre-cartilage ECM between differentiating

cells. As differentiation proceeds, cells matrix proteins are expressed collagen type II and aggrecan as the main components of ECM (Bobick et al., 2009; Sundelacruz and Kaplan, 2009). Transcription factor Sox9 is an essential regulator for the initiation of chondrogenesis andmaintenance of chondrogenic differentiation.Sox9 belongs to the SRY (sex-determining region on the Y chromosome) family and contains the HMG (high mobility group) box DNA binding domain (Akiyama et al., 2002). In addition, other Sox family has many roles of chondrogenesis and chondrocyte differentiation such as Sox5 and Sox6 (Akiyama et al., 2002). There acts as a specific consensus motif in the minor groove of DNA and induces DNA bending (DeLise et al., 2000). During chondrocyte differentiation, Sox9 and Sox5/Sox6 cooperate to activate the expression of cartilage-specific ECM (Zuscik et al., 2008). Furthermore, Sox9 expression is down-regulated and Runt-domain transcription factor (Runx2) is up-regulated involving chondrocyte hypertrophy(Hinoi et al., 2006). Cells proliferated rapidly and enlarged the cartilage templates that perform individual skeletal elements (Zuscik et al., 2008). Runx2 stimulates chondrocyte maturation and the expression of hypertrophy markers like collagen type X, MMP-13, and AP (Hinoi et al., 2006). Cells developed to the terminal differentiation, mineralization, and ultimately apoptosis (Zuscik et al., 2008).

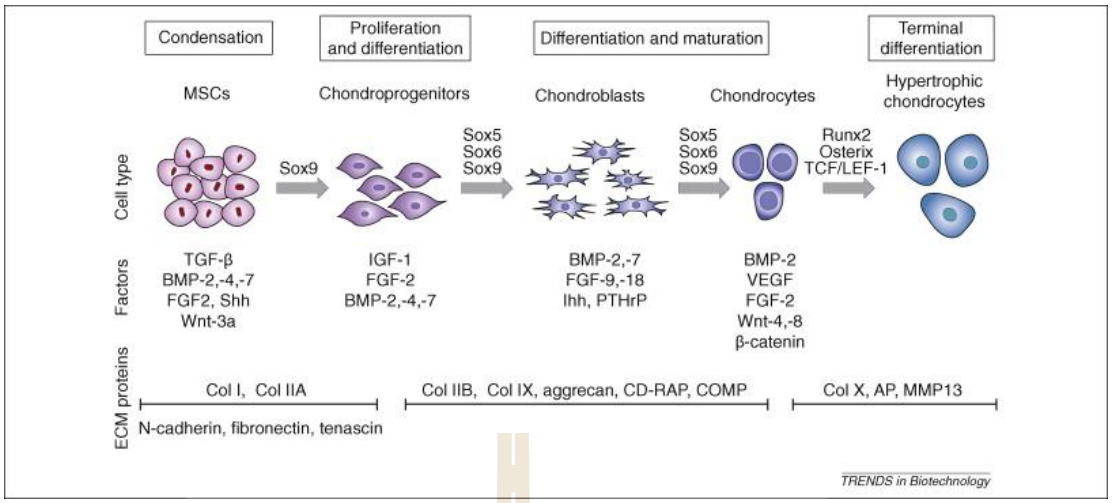

Figure 2.6 The stages of chondrogenesis. The main ECM proteins are expressed at different stages throughout for the various stages. Abbreviations: AP, alkaline phosphatase; CD-RAP, cartilage-derived retinoic acid-sensitive protein; Col, collagen; COMP, cartilage oligomeric protein; MMP, matrix metalloprotease; VEGF, vascular endothelial growth factor (Vinatier et al., 2009).

2.5 Signaling of chondrogenesis

2.5.1 Transforming growth factor (TGF-β)superfamilies

Transforming growth factor (TGF-β) superfamilies are inducers of skeletal development in MSCs. MSCs begin to condense and differentiate into osteochondroprogenitor cells that are the precursors for cartilage and bone tissue (Kronenberg, 2003). Member of TGF-β superfamilies including TGF-β, BMPs, activins, and inhibins, they initiate signal from the cell surface by interacting with type I and type II receptors (Vinatier et al., 2009). After BMP and TGF-β activation, the type II receptor activates the type I receptor, which phosphorylates the downstream mediators: Smads1/5/8 or Smads2/3, respectively (Vinatier et al., 2009).

The phosphorylated Smads are complex with Smad4 and translocate to the nucleus, where they participate in gene transcription.Both BMP and TGF-β signaling promote chondrogenic differentiation but have opposing effects on later differentiation steps during skeletal development. TGF-β signaling stimulates the early chondrocytes and inhibits chondrocyte hypertrophy while BMP signaling promotes chondrocytes hypertrophy.

2.5.1.1 TGF-β signaling pathway

TGF-β signaling pathway has been shown to stimulate chondrogenesis in skeletal development. TGF-β induces downstream phosphorylation of Smad2/3 pathway, leading to being potent stimulators of chondrogenic differentiation of MSCs, while inhibits chondrocyte maturation and hypertrophy *in vitro* and *in vivo* (Furumatsu et al., 2009; Amano et al., 2011). Sox9 are complexes with Smad2/3 to stabilize and maintain the chondrocytes phenotype (Furumatsu et al., 2009; Amano et al., 2011). Cells generated the main ECM production, proteoglycans and primarily type II collagen (Vinatier et al., 2009). Member of TGF-β includes four members (TGF-β1, 2, 3, and 5), which are potent inducers of chondrogenesis *in vitro* (Weiss et al., 2010). TGF-β3 has strong positive effect on chondrogenic differentiation in MSCs than the TGF-β1, which a critical step in the progression of chondrogenesis *in vitro* and cartilage development *in vivo* while TGF-β2 mediates hypertrophic differentiation (Gooch et al., 2001). TGF-β3 can induce the chondral differentiation of MSCs to form ectopic cartilage and was able to repair a full-thickness cartilage defect by improving chondrocyte integration into the endogenous tissue (Fan et al., 2006). In contrast, TGF-β3 negatively regulates chondrocyte

maturation by slowing their rate of maturation and inhibiting hypertrophy in cell culture, and in chick embryonic limb development (Ferguson and O'Kane, 2004).

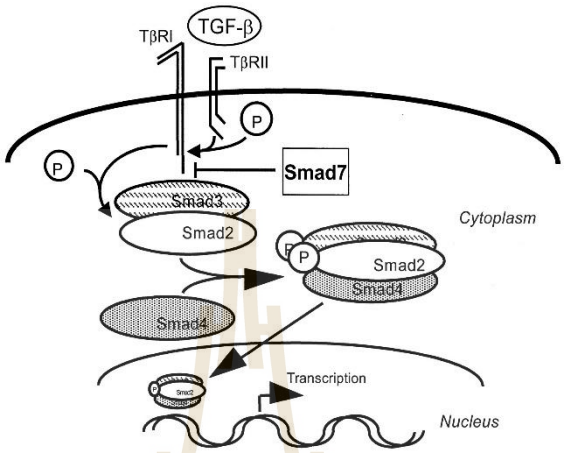

Figure2.7 Schematic diagram of transforming growth factor–β (TGF-β) signal transduction. TGF-β binds to its receptor, type II receptor is activated and phosphorylates the type I receptor. The R-Smads, Smad2/3 (BMP family, the R-Smads would be Smad1/5/8) interact with the receptor and are phosphorylated. They are complex with Smad4 and form a transcription regulating complex in the nucleus (Schnaper, et al., 2002).

2.5.1.2 Bone morphogenetic proteins (BMPs)

BMPs are growth and differentiation factors of the TGF-β superfamily, their originally ability to induce the formation of both cartilage and bone when implanted at ectopic sites (Urist, 1965). BMPs bind to transmembrane cell-surface receptors consisting of heterodimers of type I (BMPR1) and type II (BMPR2) subunits (Attisano and Wrana, 2000). Auto-phosphorylation of BMPs leads to activation of Smad or p38 mitogen-activated protein kinase (MAPK) pathways (Nohe et al., 2004). BMPR1 protein kinase phosphorylates receptor activated R-Smads

(Smads1/5/8). Smad4 complex with R-Smads translocates to the nucleus and from a complex other factor regulating target gene (Nohe et al., 2004; Zhou et al., 2010). BMPs constitute a large sub-class of 20 polypeptides that have essential roles in skeletal development. Several BMPs, including BMP-2, 4, 6, 7, 13 and 14, can stimulate the chondrogenic differentiation of MSCs and enhance the synthesis of collagen type II and aggrecan *in vitro* (Gründer et al., 2004; Roux et al., 2013). Overexpress BMP-4 had been retrovirally transduced ex vivo to enhanced chondrogenesis and significantly improved articular cartilage repair in rats (Kuroda et al., 2006). However, when implanted in ectopic localizations, BMPs led to bone formation.

2.5.2 Wnt signaling pathway

Wnt signaling pathway is involved in verities cellular activities of cell fate determination, proliferation, migration, and differentiation (Moon et al., 2002). During the skeletal development, Wnt signaling pathway is essential for the regulation of both chondrocytes and osteoblast differentiation (Day and Yang, 2008).

Canonical Wnt signaling also known as Wnt/β-catenin signaling has been implicated in the regulation of chondrocyte hypertrophy during endochondral ossification. Wnt signaling simultaneously to the Frizzled (Fz) transmembrane receptors and their low-density lipoprotein receptor-related protein (LRP) co-receptors triggers activation of β-catenin proteins. Wnt signal activated Fz receptors that recruiting Disheveled (Dish) proteins to the plasma membrane (Angers and Moon, 2009). Dish proteins directly interact with Axin, APC, and possibly together with GSK-3 enzymes (Song et al., 2014). Signal did not promote GSK-3 enzyme mediated β-catenin phosphorylation (MacDonald et al., 2009). β-catenin releases and

thus translocates to the nucleus, which complex as a co-activator of DNA-binding T-cell factors (TCFs) to promote transcription (Moon et al., 2002; MacDonald et al., 2009). While, in the absence of Wnt signal, APC cooperates with Axin to promote the phosphorylation of β-catenin by GSK-3 enzymes. The resulted is β-catenin was degradation by the ubiquitin/proteasome pathway (MacDonald et al., 2009) as shown in Fig 2.8.

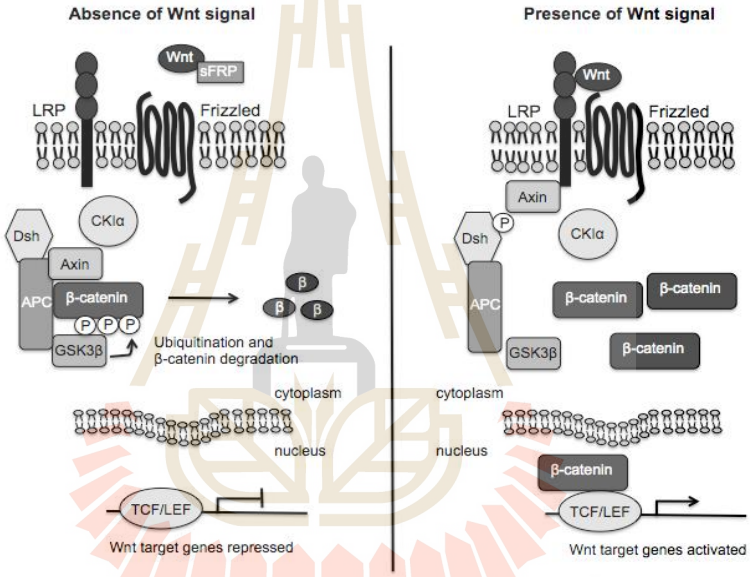

Figure2.8 The canonical Wnt signaling pathway. The action of the pathway when absence of a Wnt signal, and in the presence of an active Wnt signal.

Wnt signaling pathway is a key regulator of mesenchymal progenitor differentiation into various lineages. The activated Wnt signaling promotes osteogenesis by the stimulation of Runx2 (Gaur et al., 2005) while inhibits adipogenesis through an inhibition of adipogenic transcription factors CCAAT/enhancing binding protein (C/EBPα) and peroxisome proliferator activated receptor gamma (PPARγ) (Ross et al., 2000). Canonical Wnt/β-catenin signaling has

been associated with chondrogenesis and cartilage development. β-catenin induced transcriptional activity was shown to promote chondrocyte differentiation in a Sox9-dependent manner (Yano et al., 2005). In addition, β-catenin signaling has been shown to often crosstalk with other signaling pathways in modulating chondrogenesis (Fischer et al., 2002; Tuli et al., 2003; Yang et al., 2012; Eslaminejad et al., 2013; Narcisi et al., 2015). Fischer et al., (2002) has been reported that wnt3A enhances BMP2-mediated chondrogenesis of murine mesenchymal cells (Fischer et al., 2002). Narcisi et al., (2015) also reported wnt3A combination with FGF2 supported the long-term expansion and enhanced chondrogenic potential in MSCs (Narcisi et al., 2015). In adult human marrow stromal cells, the co-activation of β-catenin and TGF-β signaling enhanced chondrogenic differentiation (Tuli et al., 2003; Yang et al., 2012; Eslaminejad et al., 2013). However, the Wnt/β-catenin signaling pathway plays a crucial role in the hypertrophic maturation of chondrocytes in the endochondral ossification process (Kawata et al., 2010).

2.6 GSK-3 inhibitors

Glycogen synthase kinase-3 (GSK-3) enzymes is a key component of Wnt signaling that mediated β-catenin phosphorylation (MacDonald et al., 2009). GSK-3 enzymes was first initially characterized as a kinase involved in the regulation of metabolism by Embi, in 1980. They have been demonstrated that GSK-3 negatively regulates glycogen synthesis by phosphorylating and inactivating glycogen synthase (Embi et al., 1980). Currently, GSK-3 is known involved in various signaling pathways controlling metabolism, differentiation and immunity, as well as cell death and survival (Meijer et al., 2004; Metcalfe and Bienz, 2011). The phosphorylation of

GSK-3 is an important signal for degradation of β-catenin proteins, in the Wnt pathway (Aberle et al., 1997). Besides its early-identified role in metabolism, GSK-3 has a number of additional biological functions, including in stem cell renewal (Ying et al., 2008) and embryonic development (Dominguez et al., 1995).

Inhibition of GSK-3 has been demonstrated in the wnt signaling by promoted the accumulation of β-catenin and complex with a co-transcription factors LEFs/TCFs to promote transcription (Li and Jope, 2010; Chuang et al., 2011;Zhu et al., 2014). Several Small-Molecules have potential inhibitors of GSK-3 enzymes such as Lithium chloride (LiCl) has alternative targets (Klein and Melton, 1996; Meijer et al., 2004; Zhu et al., 2014), SB216763 (Coghlan et al., 2000). LiCl was the first GSK-3 inhibitor to be discovered and has been used in the treatment of bipolar disorder (Klein and Melton, 1996). SB216763 is a synthetic small molecule that has been identified and characterized as potential of GSK-3 inhibitor (Coghlan et al., 2000). SB216763 stimulate glycogen synthesis in human liver cells and induce expression of a β-catenin-LEF/TCF-regulated reporter gene in HEK293 cells similar to the compounds LiCl (Coghlan et al., 2000).

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals and Reagents

All chemicals and reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA), unless otherwise indicated.

3.2 Human articular cartilage preparation

This study was approved by The Ethics Committee for Researches Involving Human Subjects, Suranaree University of Technology. Human articular cartilage (n=1) was obtained from patient's undergoing total knee replacement for osteoarthritis at the Suranaree University of Technology Hospital, Nakhon Ratchasima, Thailand with patient's informed consent. Human articular cartilage was used as a control in the experiments.

3.3 Isolation and expansion of hWJ-MSCs

Human umbilical cords (n=1) was collected from Suranaree University of Technology Hospital, Nakhon Ratchasima with patient's informed consent. MSCs were isolated and carried out from umbilical cord using tissue explants procedure as previously described (Petsa et al., 2009). Briefly, the umbilical cord was washed in

sterile PBS and cut lengthwise to open the gelatinous (Wharton's jelly; WJ) tissue. The vessels were excised and diced into small fragments (about 3x3 mm). Then, WJ tissues were plated onto 6-well tissue culture plate (SPL life sciences, Gyeonggi-do, Korea) and then carefully covered with 1 ml of culture medium comprised of alpha modification of Eagle's medium (α-MEM) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 10% of fetal bovine serum (FBS, Life Technologies Inc. Gibgo-BRL Division, Grand Island, NY, USA). Culture cells were then incubated at 37ºC in a humidified atmosphere of 5% CO_2 in air for 7-10 days. Mediums were replaced every 2 days and when visible fibroblasts like cells were observed, then tissue explants were removed. The cells were expanded until passage 3 (P3), then the cells were experimented either directly or cryopreserved with 10% dimethyl sulfoxide (DMSO, Calbiochem, San Diego, CA, USA) and stored in liquid nitrogen.

3.4 Characterization of hWJ-MSCs

3.4.1 Immunophenotyping

Passage 5 of hWJ-MSCs were cultured onto 4-well tissue culture dish (Nunc, Roskilde, Denmark) in the growth medium until reaching 70% confluence. Cells were fixed by 4% paraformaldehydes (PFA) for 30 min. Nonspecific binding was blocked by 10% normal goat serum. Primary antibodies were raise against CD34(BD biosciences,San Jose, CA, USA), CD73 (Millipore, Massachusetts, USA), CD90 (Santa CruzBiotechnology, Texas, USA), and CD105 (Santa Cruz Biotechnology) at 4°C overnight. Cells were incubated with secondary antibodies, Alexa fluor® 488 goats anti-mouse IgG (Invitrogen, USA) or Alexa fluor® 488 goats anti-rabbit IgG (Invitrogen). Nucleus were stained with 4, 6-diamino-2-phenylindole

(DAPI, Millipore) and observed under a fluorescent microscope (Nikon Eclipse Ti-S, Japan).

3.4.2 Multipotency assays

hWJ-MSCs were cultured at the final density of approximately $2x10^4$ cells/cm^2 in 6-well culture plate coated with 0.1% gelatin.

hWJ-MSCs were induced to osteogenic differentiation by cultivated in the culture medium with reduced FBS to 5% and supplemented with 100 nM dexamethasone, 0.2 mM L-ascorbate-2-phosphate, and 10 mM β-glycerophosphate. Medium was subsequently replaced every 2 days for 3 weeks. After that, calcium deposits of the cells were visualized by Alizarin Red staining.

To induce adipogenic differentiation, hWJ-MSCs were cultured in the culture medium with reduced FBS to 5% and supplemented with 10 µg/ml insulin, 100 µM indomethacin, 1 µM dexamethasone, 0.5 mM isobutyl methylxanthine (IBMX). IBMX was removed from this medium after 1 week of culture. Medium was subsequently replaced every 2 days for 3 weeks. Cells were then stained with Oil Red O to check oil droplet.

To induce chondrogenic differentiation, hWJ-MSCs were cultured in a completed chondrogenic medium consisting of culture medium with reduced FBS to 2% and supplemented with 1% Insulin-Transferrin-Selenium-Ethanolamine (ITS-X, Invitrogen), 50 µg/ml ascorbate-2-posphate, 40 µg/ml L-proline, 100 µg/ml sodium pyruvate, 100 nM dexamethasone, and 10 ng/ml of TGF-β3 (Prospec, NJ, USA). Medium was replaced every 2 days for 3 weeks. Cells were detected for GAGs production by Alcian blue 8x staining.

3.5 Cytotoxicity test

One thousand hWJ-MSCs were re-plated and cultivated in 96-well culture plates (SPL life sciences) in the culture medium for 6 hr to allow attachment. After that, the cytotoxicity of chemicals were test by adding either LiCl or SB216763 to the culture medium at the concentrations of 0, 5, 10, and 20 mM, and 0, 1, 2.5, and 5 µM, respectively. All cultures were maintained for 72 hr at 37ºC in a humidified atmosphere of 5% CO_2 in air. The effects of the LiCl and SB216763 on cell viability were quantified by the MTT assay. Briefly, culture medium were replaced by 5 mg/ml MTT solutions (Invitrogen) in culture medium and incubated for 2 hr. DMSO (Calbiochem) were then replaced and incubated at 37 ^{o}C for 10 min. The absorbance was measured at 540 nm. (Micro plate reader Sunrise, TECAN, Austria)

3.6 Chemicals induction of chondrogenic differentiation

For monolayer cultivations, hWJ-MSCs were cultivated and induced chondrogenic differentiations as mentioned previously. The experiments were done by divided the cells into 4 groups. Each group was cultivated in culture medium with reduced FBS to 2% (Control), chondrogenic medium, chondrogenic medium supplementation with 5 mM LiCl, or 1 µM SB216763. For pellet cultivations, $2.5x10^5$ cells of hWJ-MSCs were centrifuged at 3000 rpm for 5 min in 15 ml conical tube (Corning, NY, USA) to form pellets [24] and then incubated at 37ºC in a humidified atmosphere of 5% CO_2 in air. After 24 hr, the pellets were cultural in 4 difference medium as mentioned above. The tubes were incubated with loosened cap at 37ºC in a humidified atmosphere of 5% CO_2 in air. Medium were changed every 3 days.

3.7 Characterizations of chondrocytes derived from hWJ-MSCs

3.7.1 GAGs analysis

Monolayer and pellets differentiation experiments were fixed with 4% PFA for 30 min at room temperature. Cell pellets were embedded in Cryostat embedding medium (Pink, Killik, Italy) and frozen on dry ice. The embedded samples were cut in 10 μm thickness with cryostat microtome (CM2850, Hestion, Australia) and placed in the center of a coated slide. Monolayer expanded cells and Pellet sections sample were examined for GAGs accumulation by staining with alcian blue 8x for 30 min.

3.7.2 Immunofluorescent staining

Monolayer expanded cells and pellet sections were blocked and permeabilized for 1 hr at 37°C with 5% bovine serum albumin (BSA) , 5% normal goat serum and 0.1% triton-X100. For collagen type II and X staining, samples were predigested with 1 mg/ml pepsin enzymes and for 0.2% hyaluronidase enzymes, respectively. Anti-human type IICollagen (clone 6B3, Chemicon) and anti-human type X Collagen (Calbiochem) primary antibodies were incubated overnight. Samples were incubated for 2 hr with the respective secondary antibodies. Then, samples were stained with DAPI and observed under a fluorescent microscope.

3.7.3 Gene expressions analysis

After 28 days of induction, total RNA were isolated from the cells by total RNA extraction kit (RBC Real Genomics, RBC Bioscience, Taiwan) according to the manufacturers. Then, RNA was reverse-transcribed in the presence of oligo-dT primer for complementary DNA (cDNA) synthesis by iScript™ Reverse

Transcription Supermix for RT-qPCR (Bio-Rad, California, USA). The expressions of several genes were qualified by using Light Cycler® 480 (Roche Diagnostics, Basel, Switzerland) and KAPA SYBR-Green PCR Master mix (Applied Biosystems, California, USA). The primers used are shown in table 1. Melting curve analysis was undertaken to determine the specificity of the PCR products. Gene expressions were normalized to the reference gene *GAPDH* and calculated by relative expression compared to control cells. The qPCR were performed in three times.

Table 3.1 Primers used for qPCR analysis.

Gene	Primer Sequences (5'→3')	Annealing Temperature	Product Size (bp)	References
ACAN	F: 5' ACTTCCGCTGGTCAGATGGA 3' R: 5 'TCTCGTGCCAGATCATCACC 3'	63	111	(Peran et al., 2013)
Sox9	F: 5' GGCAAGCTCTGGAGACTTCTG 3' R: 5' CTGCAGCGCCTTGAAGATG 3'	59	207	(Peran et al., 2013)
Col2a1	F: 5′ GAGACAGCATGACGCCGAG 3′ R: 5′ GCGGATGCTCTCAATCTGGT 3′	62	67	This study
Col10a1	F: 5' CCCTCTTGTTAGTGCCAACC 3' R: 5' AGATTCCAGTCCTTGGGTCA 3'	62	155	(Karl, et al., 2014)
Runx2	F: 5' ATACCGAGTGACTTTAGGGATGC 3' R: 5' AGTGAGGGTGGAGGGAAGAAG 3'	62	131	(Karl, et al., 2014)
β-catenin	F: 5' AATGCTTGGTTCACCAGTG 3' R: 5' GGCAGTCTGTCGTAATAGCC 3'	62	176	(Eslaminejad , et al., 2013)
GAPDH	F: 5' TGCCCCCGACCGTCTAC 3' R: 5' ATGCGGTTCCAGCCTATCTG 3'	60	110	(Hasuike, et al., 2005)

3.7.4 Western blot analysis

Total protein was extracted from the sample after 4 weeks post induction by lysis buffer containing 10%sodium dodecyl sulfate (SDS, Affymetrix Inc, Santa Clara, CA, USA), 0.1M dithiothreitol (DTT, Invitrogen), 1% glyceral, 1.2% urea, and 1M Tris-HCl pH 7.4 and completed proteinase inhibitor. The total

protein was determined concentration according to the Bradford assay [27]. Twenty micrograms of total protein was separated on 10% SDS-PAGE and followed by electro-transfer to nitrocellulose membrane (Bio-rad). The membranes were exposed to blocking buffer (5% skim milk in PBS with 0.1%Tween-20 (PBST)) and then incubated with either anti-human type II collagen (dilution 1:1,000,clone 6B3, Chemicon) or anti-human β-actin (dilution 1:1,000, Millipore). Membranes were incubated with (goat anti-rabbit or -mouse) secondary antibody conjugated to alkaline phosphatases (dilution 1:20,000) and were then developed using 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium (SIGMA FAST™ BCIP/NBT).

3.8 Statistical analysis

All experiments were repeated three times. Statistical analyses were performed using SPSS 17.0 (SPSS, Inc., USA). Data was represented as mean ± standard deviation of independent experiments. Statistical difference was analyzed using one-way analysis of variance (ANOVA) with Tukey's HSD Post Hoc Test. *P-values* < *0.05*.

CHAPTER IV

RESULTS

4.1 Isolation and characterization of hWJ-MSCs

MSCs were isolated from WJ tissue of human umbilical cord. The morphologies of primary MSCs derived from WJ tissue were adherent, spindle shape and fibroblast-like cells. The outgrowths of cell were observed during 5-7 days of culture (Fig. 1A). After 80% confluences (Fig. 1B), the cells were harvested and expanded for further usage. Based on the MSCs properties using standard criteria prescribed for identification of MSCs [28], we found that the hWJ-MSCs were positive for the MSCs markers, CD73, CD90, and CD105, and were negative for hematopoietic marker, CD34 (Fig. 2A). In addition, the hWJ-MSCs were induced toward adipocytes, osteocytes and chondrocytes to confirm their capacity for MSCs differentiation. As shown in Fig. 2B, the lipid droplets formation was demonstrated by Oil Red O staining. Calcium deposit was shown with Alizarin Red. Moreover, the accumulation of GAGs was examined with Alcian blue staining.

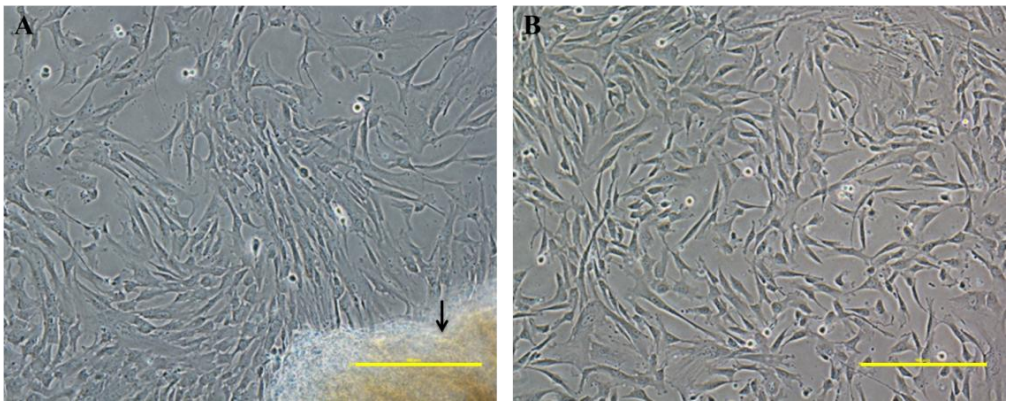

Figure 4.1 **Morphology of hWJ-MSCs with a typical fibroblast-like morphology.(A)** Phase contrast images of hWJ-MSCs expanded from Wharton's jelly tissue (arrow) and **(B)** hWJ-MSCs at 80% confluences. Scale bar = 4 µm.

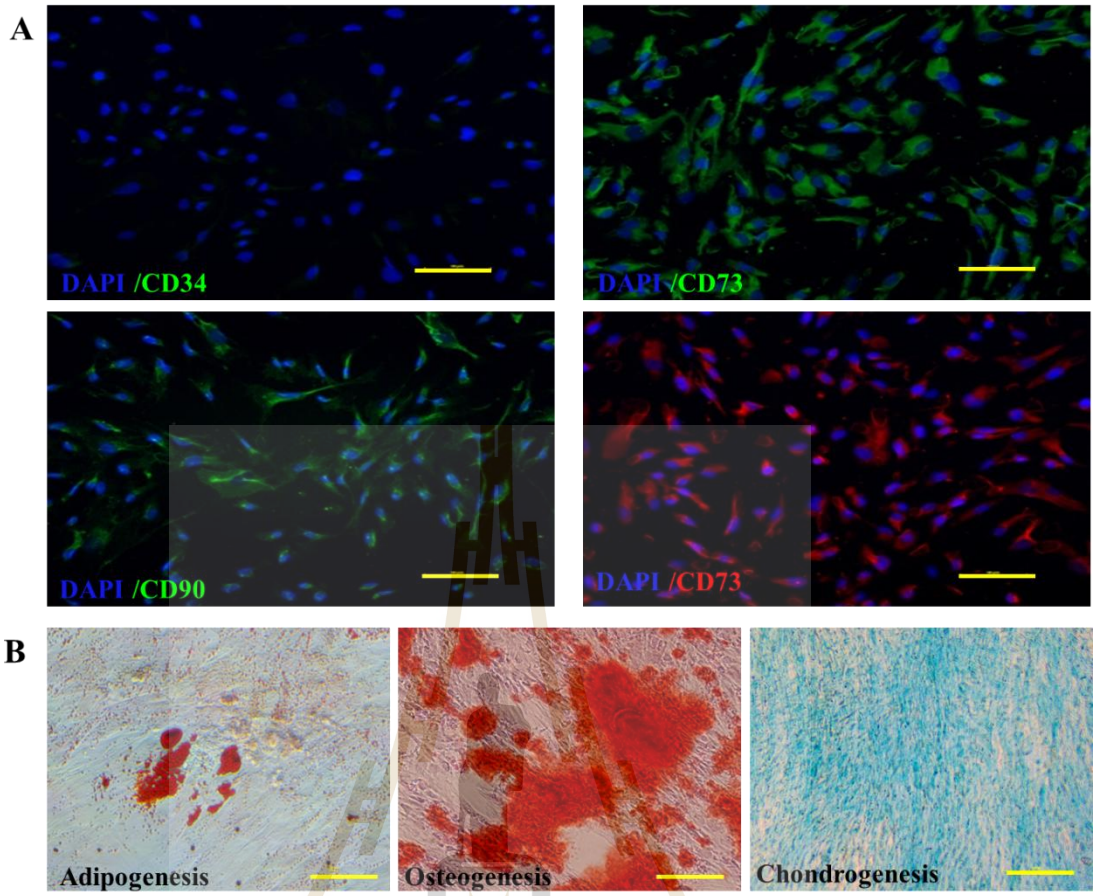

Figure 4.2 **Characterization of hWJ-MSCs. (A)** Immunophenotype of MSCs, immunofluorescent micrographs staining expression of MSCs markers (CD73, 90, and 105), Nuclei were counterstains with DAPI (blue). Cells were negative for hematopoietic marker (CD34). Scale bar = 20 µm. **(B)** Differentiation of hWJ-MSCs to mesodermal linage cells. The cells were induced to undergo adipogenic, osteogenic, and condrogenic differentiation.

4.2 Effect of LiCl and SB216763 on hWJ-MSCs viability

The viability of hWJ-MSCs was determined after culture in different concentration of LiCl and SB216763 for 3 days using MTT assay. Culture medium without supplemented with LiCl and SB216763 was used as control. Data were expressed as percent (%) normalized to the control condition without chemical supplemented. For the vehicle control, the cells were treated with DMSO (0.1-0.5%). The results indicated that culture cells in the present of DMSO did not decrease hWJ-MSCs viability as shown in Fig. 3. However, toxicity effect of LiCl and SB216763 were observed. In the present of LiCl the viability of hWJ-MSCs were reduced in a dose-dependent manner. We found that at dose 5 mM LiCl, the cell viability was 100.3%±2.73% which was significantly higher than the 10 and 20 mM LiCl (79.17%±7.00%, 73.96%±8.5%, respectively) treatments. These results indicated that high LiCl concentration 10 and 20 mM could induce hWJ-MSCs death. In the present of SB216763, the cell viability was significant difference in a dose-dependent manner. At the dose of 1 µM SB216763, the cell viability was 109.38%±2.36% which was significantly higher than the 2.5 and 5 µM SB216763 (95.64%±1.79%, 84.52%±5.14%, respectively) treatments (Fig. 3B). Hence, for further experimental, 5 mM of LiCl and 1 µM SB216763 were selected to study the effect of chondrogenic differentiation.

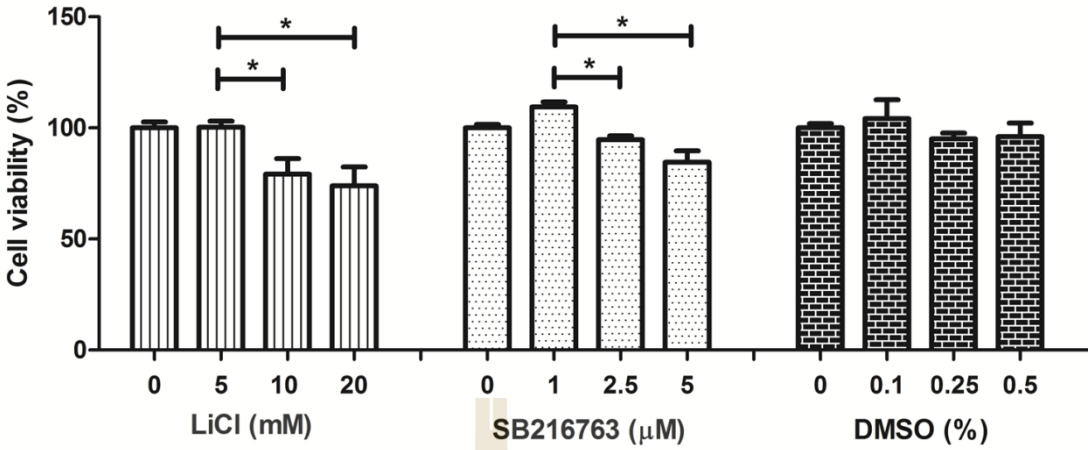

Figure 4.3 **The toxicity effect of LiCl and SB216763 on hWJ-MSCs viability.** hWJ-MSCs were cultured with 0-20 mM LiCl **(A),** 0-5 µM SB216763 **(B),** or 0-0.5% DMSO **(C),** for 72 hrs in 96-well plate. Then, the viability was detected by MTT assay. **DMSO without chemical was use as vehicle control. **Data were exposed as mean ± SD. *$P<0.05$.

4.3 Effect of LiCl and SB216763 on chondrogenic differentiation

The chondrogenic differentiation of hWJ-MSCs was confirmed by alcian blue staining for GAGs matrix synthesis that is important ECM component of the cartilage tissue. hWJ-MSCs were cultured in difference culture medium that containing chondrogenic medium, chondrogenic medium supplemented with LiCl or SB216763. Monolayer and pellet cultures were grown in the medium. After 2 weeks of monolayer experiment, positive staining with alcian blue was identified in all treatments except for the control group (Fig. 4A-D). Strong staining was observed in chondrogenic medium + SB216763 and less blue staining was seen in chondrogenic

medium + LiCl and chondrogenic medium alone, respectively. For pellet experiment, the morphology after 4 weeks was achieved as a cartilage-like appearance with white shiny (Fig. 4I). We further stained the pellet section with alcian blue (Fig. 4E-H). The positively alcian blue staining GAGs syntheses were observed in the all treatments except for the control group.

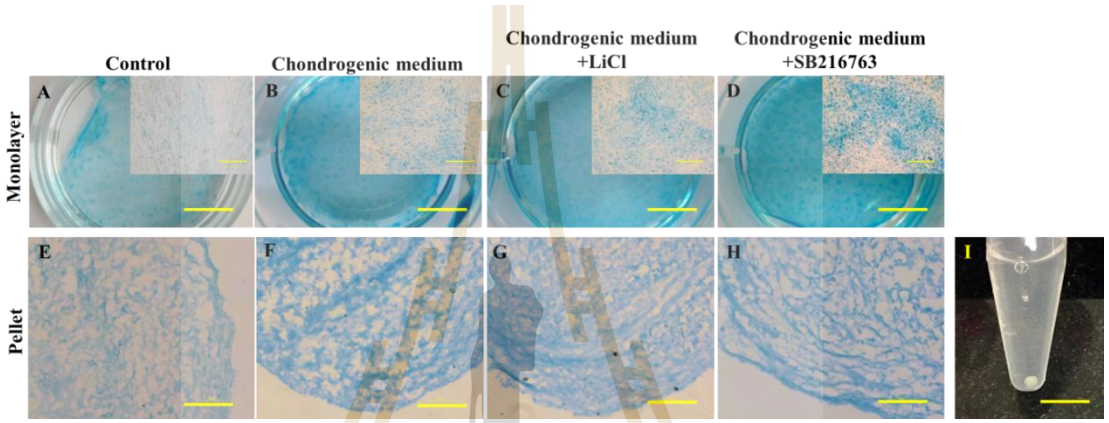

Figure 4.4 **Accumulation of GAGs was stained by Alcian blue.(A-D)** Photographs of Monolayer expanded cells cultured for 2 weeks. Scale bar=10 µm. **(B-H)** Pellets culture 4 weeks after differentiation. Scale bar=20 µm. **(I)** Morphology of pellet experiment on 4 weeks differentiation.

Tofurtheranalysischondrogenicdifferentiation,weperformedtheimmunofluoresc ent staining of cartilage specific, collagen type II marker (Fig. 5). In monolayer experiment, we clearly observed an increase in the expression of collagen type II in the treatment groups which develop a dense filamentous matrix network deposited which was not seen in the control group (Fig. 5A). We also observed a substantial number of collagen type II positive marker in the pellet experiment (Fig. 5B).

Interestingly, the localization of collagen type II expression was clearly increased in the treatment groups. However, in the control group no tissue-like morphology was observed and was negative for the collagen type II marker. The collagen type II expression was confirmed by western blot analysis. As shown in Fig. 6, the chondrogenic medium + SB216763 treatment was strongly observed the collagen type II expression when compare to the chondrogenic medium + LiCl and chondrogenic medium alone.

In addition, the chondrogenic gene expressions (*Col2a1*, *ACAN*, and *Sox9*) were investigated. Gene expression was normalized to coresponding *GAPDH* and calculated by relative expression compared to the control group. For the *Col2a1* expression, the chondrogenic medium + SB216763 (12-fold) and chondrogenic medium + LiCl (10-fold) groups showed significantly higher than the chondrogenic medium alone (4-fold) (Fig. 7A). The chondrogenic medium + SB216763 group was not significant difference when compared to the chondrogenic medium + LiCl group. The expression of *ACAN* was increased in both groups (chondrogenic medium + SB216763and chondrogenic medium + LiCl) and reached 30-and 31-fold of the control group (Fig. 7B), respectively. However, no significant difference when compared with the chondrogenic medium alone (18-fold). The chondrogenic medium + SB216763 group showed the *Sox9*expression reached a 5-fold and significantly difference with the chondrogenic medium (2-fold) alone group while chondrogenic medium + LiCl was no significant difference which showned as 4-fold of the control group (Fig. 7C).

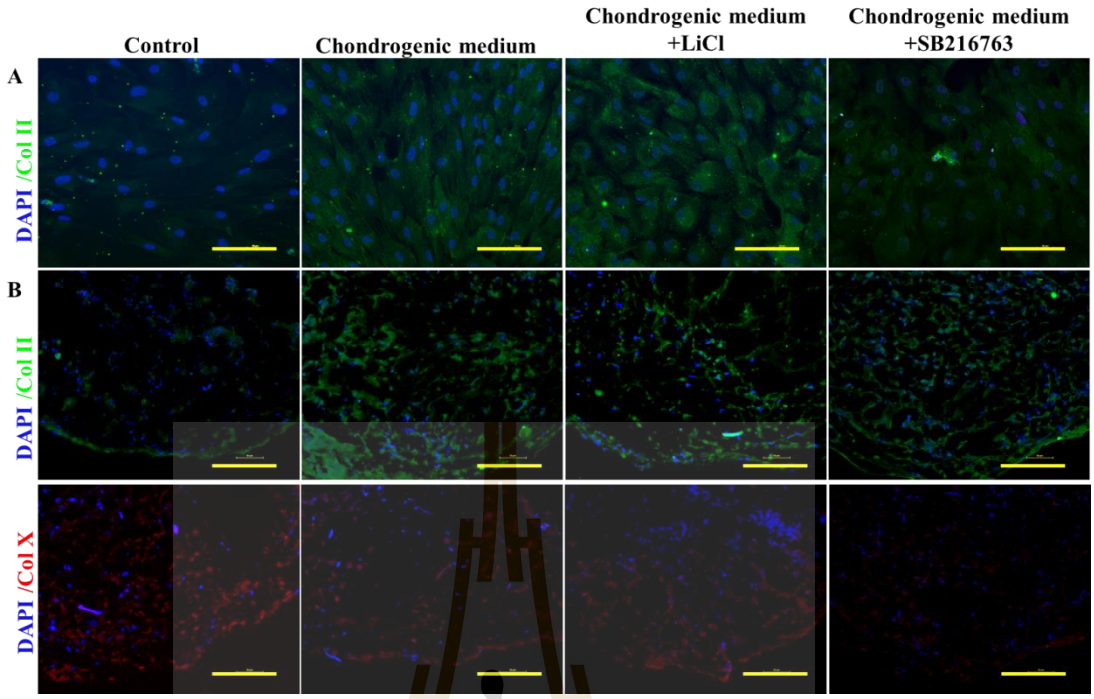

Figure 4.5 **Immunofluorescent staining of cartilage specific type collagen.(A)** immunofluorescent staining for collagen type II in monolayer expanded cultured on 3 weeks after differentiation. Scale bar = 20 µm. **(B)** Co

llagen type II and X expressions in pellet experiment on 4 weeks after differentiation. Scale bar = 20 µm.

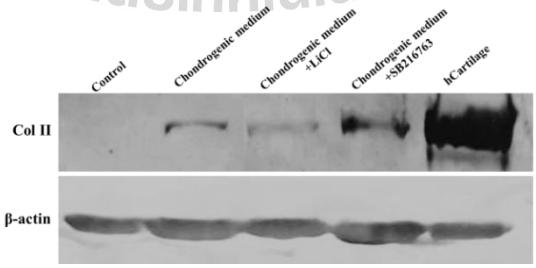

Figure 4.6 **The collagen type II protein after 4 weeks of inductions examined by Western blot analysis.** β-actin was used as an internal control.

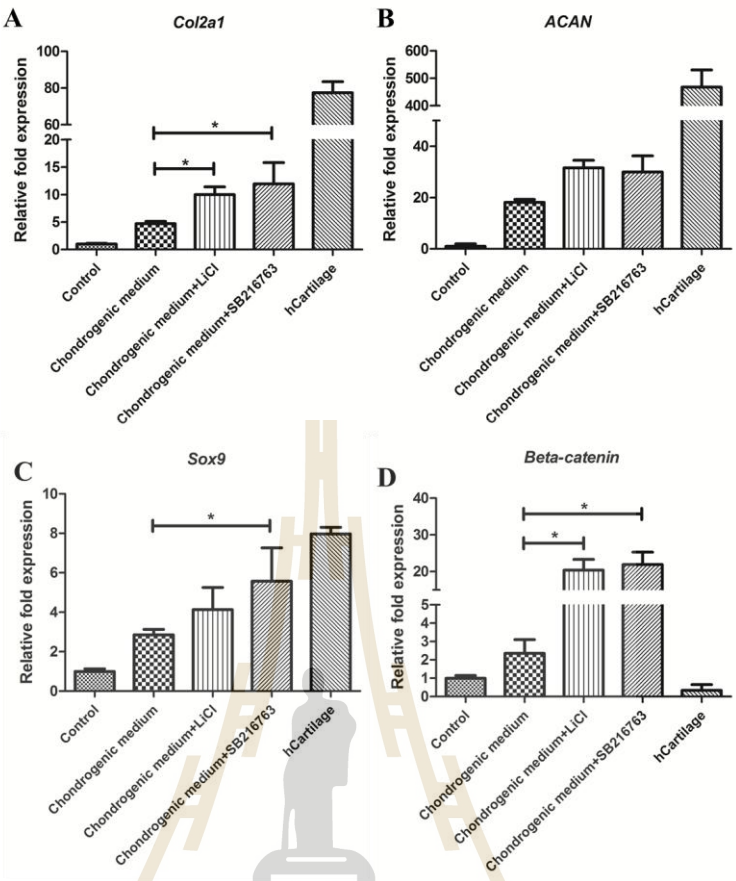

Figure 4.7 **qPCR analysis for chondrogenic gene expressions after 4 weeks of inductions (A)***Col2a1*, **(B)** *ACAN*, **(C)***Sox9* and **(D)** *β-catenin*. Gene expression was normalized to coresponding *GAPDH* and calculated by relative expression compared to control cells. The experiments were perfromed three times.**Data were expressed as mean±SD, *$P<0.05$.

4.4 Effect of LiCl and SB216763 on Wnt signaling pathway

We next examined the expression profiled of Wnt/β-catenin signaling pathway. The *β-catenin* expression increased in the chondrogenic medium + SB216763 and chondrogenic medium + LiCl groups, reaches 22-and 20-fold of the

control group, respectively (Fig. 7D). In both groups the *β-catenin* expression were significantly higher than the chondrogenic medium alone (2-fold). This resultes indicated that LiCl and SB216763 are potent GSK-3 inhibitors as they are able to progress the Wnt signaling pathway by activating the *β-catenin* expression.

4.5 LiCl and SB216763 treatments suppressed the progression of chondrocyte hypertrophy

We also demonstrated the effect of LiCl and SB216763 on the chondrocyte hypertrophy markers. The pellet sections were stained with anti-human collagen type X, a marker of hypertrophic chondrocyte developed to the osteogenic lineage. The control group developed normally to osteogenic linage as evidence by strong collagen type X expression. However, the treatment groups did not or only slightly observed positive staining in the pellet sections (Fig 5B).

In addition, qPCR were used to examine the development of chondrocytes derived from hWJ-MSCs to the hypertrophic state. We collected samples at different time points of 2, 3, and 4 weeks after differentiation to evaluate the *Runx2*, and *Col10a1* expressions. We observed a modest and transient enhancement of the *Col10a1* and *Runx2* mRNA levels, which was more evident in chondrogenic medium + SB216763 and chondrogenic medium + LiCl groups after 2 weeks of inductions. In the later time-points of differentiation (4 weeks of inductions), the *Col10a1* and *Runx2* expressions were decreased in all treatments (Fig. 8A and B). These results indicated that LiCl and SB216763 did not develop the hWJ-MSCs hypertrophic differentiation concomitantly with promote chondrogenesis.

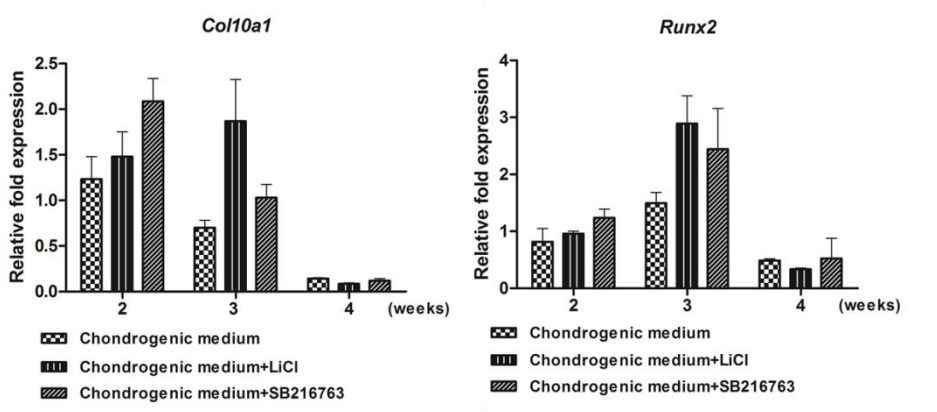

Figure 4.8 **Expression level of hypertrophic marker genes(A)***Col10a1*, and **(B)***Runx2* was quantified by qPCR after 2, 3, and 4 weeks of inductions. Gene expression was normalized to coresponding *GAPDH* and calculated by relative expression compared to control cells. Data were expressed as mean±SD, The expperiments were perfromed three times.

CHAPTER V

DISCUSSIONS AND CONCLUSIONS

Degenerative articular cartilage remains one of the major problems worldwide, especially the elder. The regeneration required chondrocytes to repair the erosion of ECM and improved cartilage repair. The used of chondrogenically differentiated MSCs could represent a therapeutic potential for cartilage regeneration as proposed (Chung, and Burdick, 2008). Here, we investigated the influence of LiCl and SB216763 synergistically with TGF-β3 on chondrogenic differentiation in hWJ-MSCs.

Wnt signaling pathway modulates the chondrogenesis and cartilage development (Chun et al., 2008), by regulates the chondrocyte proliferation, differentiation, and maintain the cell phenotype (Yasuhara et al., 2011). Inhibition of GSK-3 enzyme by the LiCl or SB216763 has been demonstrated to promote β-catenin accumulation and initiate Wnt signaling pathways (Klein and Melton, 1996; Coghlan et al., 2000). Accumulation of β-catenin activates transcription in conjunction with co-transcription factors LEFs/TCFs (Moon et al., 2002).In previously reported the LiCl (5-10 mM) have been shown to be effective in inducing the canonical Wnt signaling pathway and mediated cell differentiation in MSCs (Yang et al., 2012; Eslaminejad et al., 2013;Zhu et al., 2014)and articular chondrocytes (Krase et al., 2014).SB216763 has been shown to regulate the cells proliferation, cells survival, and induce transcription of the β-catenin dependent gene(Coghlan et al., 2000).

Several studies reported that the crosstalk between Wnt signaling and other signaling pathways has potential in modulating chondrogenesis. Fischer and colleague used the Wnt3A combination with BMP-2 can enhances chondrogenesis in mMSCs (Fischer et al., 2002). The study of Narcisi and colleague also reported that Wnt3A in combination with FGF-2 supported the long-term expansion and enhanced chondrogenic potential in MSCs (Narcisi et al., 2015). The co-activation of TGF-β and LiCl or SB216763 in the chondrogenic differentiation was also reported by Eslaminejad and colleague that the expressions of *Sox9*, *ACAN*, and *Col2a1* were increased. They also showed the proteoglycans levels were evaluated during chondrogenic differentiation in MSCs from bone marrow (Eslaminejad et. al., 2013). Our results showed that when treated the LiCl or SB216763 synergistically with TGF-β3 in the chondrogenic differentiation of WJ-MSCs, both treatments were similar up-regulated the expression of cartilage-specific markers including *ACAN*, *Col2a1*, and *Sox9* as well as GAGs accumulation in the monolayer and pellet experiments. Western blot analysis revealed that the production of collagen type II expression was increased. This study reveals the mechanisms by which TGF-β3 affects Wnt/β-catenin signaling pathways promoted the chondrogenic differentiation of hWJ-MSCs. TGF-β3 has been known as major inducer promoting chondrogenic differentiation by induces downstream phosphorylation of Smad2/3 pathway, which directly leading to induction of chondrogenesis due to stabilization of the Sox9 transcription complex by Smad2/3 (Furumatsu et al., 2009; Amano et al., 2011). TGF-β can independently or cooperatively regulate LEF/TCF target genes in the Wnt signaling pathway and these pathways can synergistically activate target genes (Labbé et al., 2000). Another reported showed that, β-catenin signaling induced transcriptional activity and promote

chondrogenic differentiation in a Sox9-dependent manner(Yano et. al., 2005). The Sox9 is an important role directly regulates the expression of the mainly cartilage genes *Col2a1* and *ACAN* during chondrogenesis (Akiyama et al., 2002).

Wnt/β-catenin signaling pathway plays a crucial role in the progression of chondrocyte hypertrophy. Our results have shown the treatments of LiCl or SB216763 suppressed the progression of chondrocyte hypertrophy as evidence by decreased the expression of *Col10a1* and *Runx2* markers.This results agree with the study from Yang and colleague that the continuous co-activation of two signaling pathways inhibited the chondrocytes hypertrophy by suppressed the expression of *Col10a1*, *Runx2*, and Alkaline phosphatase markers, and did not lead to ossified tissue *in vivo* (Yang et. al., 2012). However, the study from Kawata and colleague shown the activation of Wnt/β-catenin signaling pathway in chondrocytes cells by Wnt3a or SB216763 inhibits the phosphorylation of GSK-3 and decreased the expressions of *ACAN* and *Col2a1*, whereas increased the *Col10a1* and *MMP-13* (Kawata et al., 2010). This study was not cultured within the any TGF-β supplements. For the chondrogenic differentiation, TGF-β3 has strong positive effectively, which is a critical step for the progression of chondrogenesis and directly leads to the inhibition of chondrocyte hypertrophy (Gooch et al., 2001).

Both LiCl and SB216763 have been known to act as the GSK-3 inhibitors, thus initiating the Wnt signaling pathway. LiCl is a chemical compound that has already been approved to be a drug and used for the treatment of bipolar disorder patients (Dubovsky et al., 2005). Our results showed that the *Col2a1* expression was strongly observed in the chondrogenic medium + SB216763 as evidence by Western blot analysis. It also showed to up-regulate several gene expressions including *ACAN*,

Col2a1, and *Sox9*. We indicated that SB216763 was more effective than LiCl treatment similar as previously reported (Eslaminejad et. al., 2013). SB216763 is a synthetic small molecule that can rapidly diffuse across cell membranes, reach intracellular sites of action, and specifically target the signaling pathway (Imai et al., 2006).

In conclusion, GSK-3 inhibitors (LiCl and SB216763) are able to progress the Wnt signaling pathway and promoted chondrogenic differentiation of hWJ-MSCs in the present of TGF-β3, but did not develop to the chondrocytes hypertrophy. These results indicated thatLiCl and SB216763 are choice candidates for further *in vivo* therapeutic trials and would be of great importance for cartilage regeneration.

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APPENDICES

APPENDIX A

Table 1 Relative fold expression compared to control at 4 weeks of inductions (N=3).

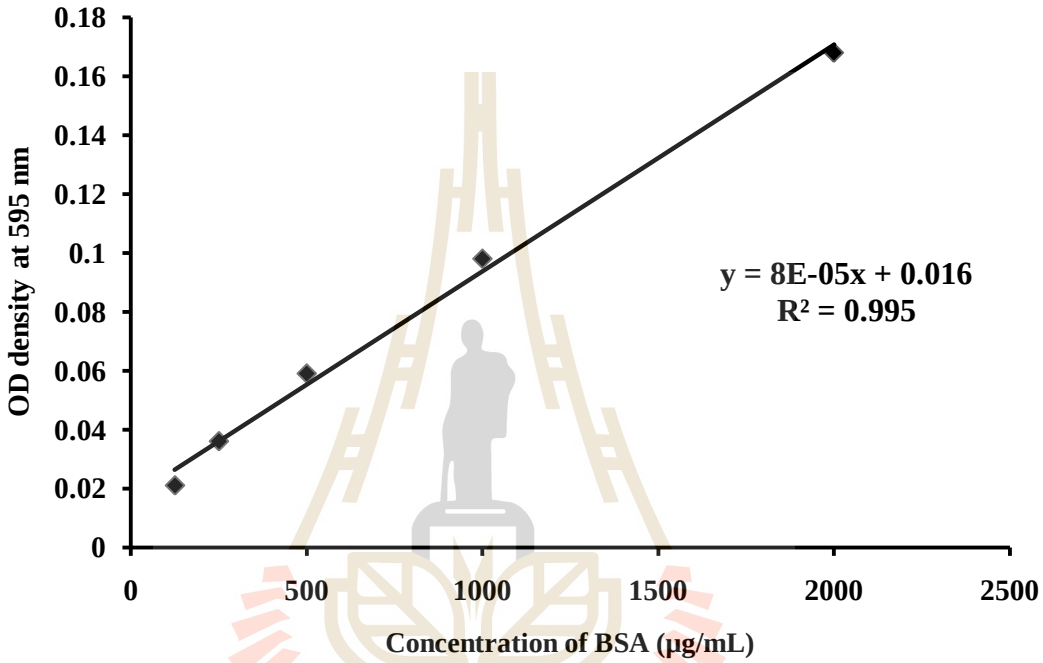
Genes / Conditions	*Col2a1*	*Sox9*	*ACAN*	*Beta-catenin*
Negative Control	1±0.13	1±0.13	1±0.99	1±0.15
Chondrogenic medium	4.69±0.43	2.85±0.28	18.11±1.15	2.36±0.75
Chondrogenic medium +LiCl	9.99±1.39	4.13±1.13	31.57±2.95	20.38±2.94
Chondrogenic medium +SB216763	11.94±3.8	5.57±1.69	29.98±6.28	21.92±3.38
hCartilage	77.43±6.0	7.97±0.33	467.29±62.87	0.34±0.31

Table 2 Relative fold expression compared to control at 2, 3 and 4 weeks of inductions (N=3)**.**

Genes / **Conditions**	***Col2a1***			***Runx2***		
	2 weeks	**3 weeks**	**4 weeks**	**2 weeks**	**3 weeks**	**4 weeks**
Negative Control	1±0.33	1±0.08	1±0.04	1±0.03	1±0.28	1±0.26
Chondrogenic medium	1.23±0.25	0.70±0.08	0.14±0.01	0.82±0.23	1.5±0.93	0.49±0.14
Chondrogenic medium +LiCl	1.48±0.27	1.87±0.46	0.09±0.01	0.96±0.04	2.89±1.14	0.33±0.09
Chondrogenic medium +SB216763	2.09±0.25	1.03±0.14	0.12±0.02	1.24±0.15	2.44±1.62	0.52±0.27

APPENDIX B

Figure 1. BSA standard curve at 595 nm by Bradford assay**.**



BIOGRAPHY

Prapot Tanthaisong was born in Sakaeo, Thailand, on March 7th, 1991. He graduated high school from Wangnamyen Wittayakom School in Sakaeo. In 2012, he received his Bachelor of Summary Degree (Medicine Science) from the Faculty of Allied Health Science, Burapha University, Chon Buri, Thailand. Then he continued his Master degree in the field of Stem cell research at the School of Biotechnology, Institute of Agricultural technology, Suranaree University of Technology with Assoc. Prof. Dr. Rangsun Parnpai. During his study, he received a scholarship from the Bangkok Stem Cell Co., Ltd., Nakhon Pathom, Thailand.