INDUCTION OF HUMAN WHARTON'S JELLY OF UMBILICAL CORD DERIVED MESENCHYMAL STEM CELLS TO BE CHONDROCYTES AND TRANSPLANTATION IN GUINEA PIG MODEL WITH SPONTANEOUS OSTEOARTHRITIS

Gulrez Nadeem

A Thesis Submitted in Fulfillment of the Requirements for the
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การเหนี่ยวนำเซลล์ต้นกำเนิดมีเซ็นไคม์ที่แยกได้จากเนื้อเยื่อวาร์ตันเจลลี่ของสายสะดือมนุษย์ไปเป็นเซลล์กระดูกอ่อน และการปลูกถ่ายให้หนูตะเภาที่มีภาวะข้อเข่าเสื่อม

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาปรัชญาดุษฎีบัณฑิต
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INDUCTION OF HUMAN WHARTON'S JELLY OF UMBILICAL CORD DERIVED MESENCHYMAL STEM CELLS TO BE CHONDROCYTES AND TRANSPLANTATION IN GUINEA PIG MODEL WITH SPONTANEOUS OSTEOARTHRITIS

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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กุลเรซ นาดีม : การเหนี่ยวนำเซลล์ต้นกำเนิดมีเซ็นไคม์ที่แยกได้จากเนื้อเยื่อวาร์ตันเจลลี่ของสายสะดือมนุษย์ไปเป็นเซลล์กระดูกอ่อน และการปลูกถ่ายให้หนูตะเภาที่มีภาวะข้อเข่าเสื่อม (INDUCTION OF HUMAN WHARTON'S JELLY OF UMBILICAL CORD DERIVED MESENCHYMAL STEM CELLS TO BE CHONDROCYTES AND TRANSPLANTATION IN GUINEA PIGS MODEL WITH SPONTANEOUS OSTEOARTHRITIS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 87 หน้า.

คำสำคัญ: สายสะดือมนุษย์/เซลล์ต้นกำเนิดมีเซ็นไคม์/เซลล์กระดูกอ่อน/ภาวะข้อเข่าเสื่อม/การปลูกถ่าย

สายสะดือมนุษย์จำนวน 2 ตัวอย่าง จากผู้บริจาค ถูกนำมาแยกเซลล์ต้นกำเนิดมีเซ็นไคม์ได้สำเร็จ ได้เซลล์ต้นกำเนิดมีเซ็นไคม์จำนวน 2 สายพันธุ์ ครบตามที่กำหนด เซลล์ทั้งสองสายพันธุ์ถูกนำมาวิเคราะห์คุณสมบัติของเซลล์ต้นกำเนิดมีเซ็นไคม์ ได้แก่ โปรตีนที่ผิวเซลล์ การสร้างโคโลนีกลุ่มเซลล์ ระยะเวลาในการเพิ่มจำนวนเป็น 2 เท่า การเปลี่ยนแปลงไปเป็นเซลล์ชนิดอื่น (เซลล์กระดูก เซลล์ไขมัน และ เซลล์กระดูกอ่อน) พบว่ามีเซลล์สายพันธุ์เดียวที่มีคุณสมบัติของเซลล์ต้นกำเนิดมีเซ็นไคม์ที่เหมาะสมซึ่งจะถูกใช้ในการทดลองอื่นต่อไป ในการวิเคราะห์โปรตีนที่ผิวเซลล์ได้เปลี่ยนวิธีการวิเคราะห์จากการย้อมเซลล์บนจานเลี้ยงเซลล์มาเป็นวิธีโฟลไซโทเมทรี ซึ่งวิธีการนี้เป็นวิธีที่ดีกว่าเนื่องจากจะทำให้ได้ข้อมูลสัดส่วนจำนวนเซลล์ที่ติดสีย้อมต่อจำนวนเซลล์ทั้งหมดด้วย การเหนี่ยวนำเซลล์ต้นกำเนิดมีเซ็นไคม์ให้เปลี่ยนไปเป็นเซลล์กระดูกอ่อนสามารถทำได้สำเร็จ โดยใช้เวลาในการเหนี่ยวนำเป็นเวลา 28 วัน แล้ววิเคราะห์ความเป็นเซลล์กระดูกอ่อนโดยการย้อม immunofluorescence การวิเคราะห์การแสดงออกของยีนด้วยวิธี qPCR และตรวจสอบการสร้างโปรตีนด้วยวิธี immunoblot ในขั้นตอนการย้อม immunofluorescence และการตรวจสอบโปรตีนด้วย immunoblot ได้เสนอการตรวจสอบ Type II collagen เพียงอย่างเดียว แต่ได้ย้อม Sox9 Aggrecan และ Type X collagen และตรวจสอบสอบโปรตีน Type X collagen ด้วยเพื่อยืนยันผลเพิ่มเติม นอกเหนือจากนี้ยังได้มีการนำกระดูกอ่อนมนุษย์มาใช้แยกเซลล์กระดูกอ่อนเพื่อเปรียบเทียบผลการแสดงออกของยีนกับเซลล์กระดูกอ่อนที่ได้จากการเหนี่ยวนำและใช้สกัดโปรตีนเพื่อเปรียบเทียบกับโปรตีนที่ได้จากกระดูกอ่อนของหนูตะเภาหลังการปลูกถ่ายเซลล์อีกด้วย การเหนี่ยวนำเซลล์ต้นกำเนิดมีเซ็นไคม์ให้เปลี่ยนไปเป็นเซลล์กระดูกอ่อนใช้เวลาเวลา 14 วัน เพื่อให้ได้เซลล์กระดูกอ่อนระยะเริ่มต้นสำหรับฉีดเข้าข้อเข่าหนูตะเภาอายุ 7 เดือน ที่มีอาการข้อเข่าเสื่อมโดยธรรมชาติในระยะเริ่มต้น และเก็บข้อเข่ามาวิเคราะห์ผลหลังการปลูกถ่ายเป็นเวลา 5 สัปดาห์ โดยเปรียบเทียบผลกับการฉีดเซลล์ต้นกำเนิดมีเซ็นไคม์ที่ไม่ถูกเหนี่ยวนำและการฉีด Hyaluronic acid พบว่าเซลล์ที่ฉีดเข้าไปเกาะที่ผิวกระดูกอ่อนช่วยซ่อมแซมกระดูกอ่อนที่เสียหายให้กลับมาดีขึ้นได้ โดย

ให้ผลดีกว่าการฉีดเซลล์ต้นกำเนิดที่ไม่ได้เหนี่ยวนำและดีกว่าการฉีด Hyaluronic acid ทำให้เนื้อเยื่อกระดูกอ่อนสมบูรณ์ขึ้นใกล้เคียงกับเนื้อเยื่อกระดูกอ่อนของหนูตะเภาอายุ 3 เดือนที่ยังไม่มีภาวะข้อเข่าเสื่อม โดยมีคะแนนความเสียหายของเนื้อเยื่อกระดูกอ่อนโดยใช้เกณฑ์ของ Mankin ไม่แตกต่างกันทางสถิติเมื่อตรวจสอบทางเนื้อเยื่อวิทยา

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา G. Nadeem.

ปีการศึกษา 2567 ลายมือชื่ออาจารย์ที่ปรึกษา

GULREZ NADEEM : INDUCTION OF HUMAN WHARTON'S JELLY OF UMBILICAL CORD DERIVED MESENCHYMAL STEM CELLS TO BE CHONDROCYTES AND TRANSPLANTATION IN GUINEA PIG MODEL WITH SPONTANEOUS OSTEOARTHRITIS. THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 87 PP.

Keyword: UMBILICAL CORD/MESENCHYMAL STEM CELLS/CHONDROCYTES/ OSTEOARTHRITIS/TRANSPLANTATION

Mesenchymal stem cells (MSCs) were successfully isolated from the umbilical cords of two donors. Two cell lines of the MSCs were obtained and characterized. The analyzed characteristics included cell surface markers, colony forming unit, population doubling time, surface protein expression, and differentiation potencies into three cell lineages (adipogenic, chondrogenic and osteogenic). The results revealed that only one cell line exhibited appropriate MSCs characteristics and it was recruited to do in the next experiments. In the determination of cell surface markers, flow cytometry was used instead of cell staining on culture dish because it can provide quantitative values of the positive cell proportion. MSCs were induced to be chondrocytes via the induction of chondrogenic differentiation for 28 days. The obtained chondrocytes were characterized by immunofluorescent staining, qPCR, and immunoblotting. In the project proposal, only type II collagen was proposed to be determined in the immunofluorescent staining and immunoblotting. However, Sox9, Aggrecan, and type X collagen were also detected in the immunofluorescent staining, and type X collagen was further determined in the immunoblotting to strongly confirm the results. Moreover, a human cartilage was included in the research for chondrocyte isolation and protein extraction. The human cartilage-derived chondrocytes were used to compare the gene expressions with the MSC-derived chondrocytes, whereas the human cartilage protein lysate was used to compare the proteins composition with guinea pig cartilage protein lysates. Early stage chondrocytes can be obtained by chondrogenic differentiation induction of MSCs for 14 days. For transplantation, MSCs-derived early stage chondrocytes were intra-articular injected into the knee joints of 7-month old guinea pigs which have symptom of early spontaneous

osteoarthritis. After transplantation for 5 weeks, the joints were collected. The results from the injection of MSCs-derived early stage chondrocytes were compared with those from the injection of undifferentiated MSCs and the injection of Hyaluronic acid. The results revealed that the transplanted cells were integrated into the guinea pig cartilage surfaces and restored the degenerated cartilages. The injection of MSCs-derived early stage chondrocytes recovered the degenerated cartilages better than the injection of undifferentiated MSCs and the injection of Hyaluronic acid. The tissues of the recovered cartilages after the injection of MSCs-derived early stage chondrocytes resemble the cartilages of 3-month old guinea pigs, which have no symptom of osteoarthritis, with no significant difference in the Mankin's scores from the histological assessment.

School of Biotechnology
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LIST OF ABBREVIATIONS

ASC-2-P	=	Ascorbic acid 2-phosphate
BSA	=	Bovine serum albumin
cDNA	=	Complementary DNA
CFDA-SE	=	Carboxyfluorescein diacetate succinimidyl ester
CFU	=	Colony forming unit assay
CRD	=	Completely Randomized Design
DAPI	=	6-diamino-2-phenylindole
DMSO	=	Dimethyl sulfoxide
DTT	=	Dithiothreitol
ECM	=	Extracellular matrix
FBS	=	Fetal bovine serum
H&E	=	Hematoxylin and eosin
HA	=	Hyaluronic acid
HRP	=	Horseradish peroxidase
hWJ-MSCs	=	Human Wharton's jelly-derived mesenchymal stem cells
IBMX	=	Isobutyl methylxanthine
ICC	=	Immunofluorescence staining
ITS-X	=	Insulin-Transferrin-Selenium-Ethanolamine
MSCs	=	Mesenchymal stem cells
OA	=	Spontaneous osteoarthritis
OCT	=	Optimal cutting temperature compound
PDT	=	Population doubling time
PFA	=	Paraformaldehyde
PVDF	=	polyvinylidene difluoride
qPCR	=	Quantitative polymerase chain reaction
SDS-PAGE	=	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

LIST OF ABBREVIATIONS (Continued)

TBS	=	Tris-buffered saline
TBST	=	Tris-buffered saline with 0.1% Tween 20
TGF-β3	=	Transforming growth factor beta 3
α-MEM	=	Alpha modification of Eagle's medium

CHAPTER I
INTRODUCTION

1.1 Background

Osteoarthritis is prevalent among elderly individuals, obese patients, and physically active people who exert significant stress on their knee joints. The incidence of osteoarthritis increases with age and obesity. According to the World Health Organization's (WHO) Global Burden of Disease Study 2010, over 70 million Europeans suffer from osteoarthritis, which hampers normal joint function due to the low self-repairing capability of cartilage. Knee and hip osteoarthritis are also the causes of disability (Cucchiarini et al., 2016). Osteoarthritis can be triggered by a range of factors, including non-genetic factors e.g. age, sex, occupational activities, sports activity, high body mass index, obesity, diabetes mellitus, muscle weakness, mechanical instability, bone marrow lesion, and bone mineral density (Jones et al., 2019; Kong et al., 2017). Genetic factors may also play a role, such as changes in gene expression in cartilage and subchondral bone. These factors can impact joints in the body, particularly in the hands and limbs that are subjected to greater weight-bearing stress, resulting in pain and functional impairment in adults. Initial symptoms involve deterioration of articular cartilage leading to pain, bone dysfunction, and difficulties in performing daily activities. Articular cartilage is a specialized type of connective tissue composed of cartilage cells and typically found in synovial joints. These cells produce extracellular matrix (ECM) and preserve the function of the tissue. Articular cartilage does not possess self-healing ability due to the absence of blood vessels, lymphatic vessels, and the nervous system (Sophia Fox et al., 2009). Arthritic cartilage degeneration can cause various symptoms, including growth abnormalities in children, injuries caused by stress from trauma, and age-related osteoarthritis (Song et al., 2004).

Current OA treatments are commonly determined based on disease severity, and the physician's recommendations including pharmacological and non-

pharmacological therapies. The first-line pharmacologic treatment is acetaminophen to cure mild and intermittent symptoms, and then followed by non-steroidal anti-inflammatory drugs (NSAIDs) when acetaminophen is ineffective to alleviate pain. However, NSAIDs prescription should be considered due to their gastric ulcer complication, and cardiovascular risk (Yusuf, 2016). Combination of pharmacological and non-pharmacologic treatment, namely diet and weight loss, physical therapy and exercise, and nutritional supplements (glucosamine and chondroitin sulfate) is a common advice for OA treatment to reduce symptoms and improve functional performance of the joint. Surgery is an invasive procedure that should be conducted when the combined therapy is unsuccessful to produce desired outcomes (Yusuf, 2016). Additionally, there is a possibility of recurrence and complications after surgery in many patients. Consequently, a novel and more effective procedure for osteoarthritis is indispensable, like application of cartilage cells. However, extraction of these cells from a human requires invasive surgery, which is complicated and expensive (Ebihara et al., 2012; Wong et al., 2020). Research is currently underway to explore the potential use of mesenchymal stem cells (MSCs) in treating osteoarthritis. The stem cells possess the unique ability to stimulate the growth of cartilage cells and other types of cells. MSCs are utilized in treating various disorders and can be sourced from several different locations, including bone marrow, blood, adipose tissue, and dental pulp. They can be isolated and cultured with a high level of proliferation activity. Previous studies have identified Wharton's jelly, found in the umbilical cord of humans, as a common source of MSCs. This tissue can be collected from pregnant women following childbirth, without complex collection process required (Troyer et al., 2008). As a result, MSCs isolated from the Wharton's jelly of human umbilical cords are a promising area of interest for future clinical trials.

The use of Dunkin Hartley guinea pigs as an animal model for studying spontaneous cartilage degeneration in the knee joint, which is similar to osteoarthritis in humans, has been well established (Tessier et al., 2003; Yan et al., 2014). Researchers reported that the knee joint of guinea pigs closely resembles that of humans affected by osteoarthritis (Fernandez et al., 1997; Kraus et al., 2010). Moreover, spontaneous cartilage degeneration in the Dunkin-Hartley guinea pigs are used for study (Bendele and Hulman, 1988). Previous studies demonstrated that

injecting mesenchymal stem cells (MSCs) with hyaluronic acid (HA) into the articular cartilage of guinea pigs with osteoarthritis led to recovery (Sato et al., 2012). HA-based formulations are currently delivered into the joint to relieve pain and improve joint mobility of OA patients by partial restoration of the rheological properties of the synovial fluid (La Gatta et al., 2021).

In this study, we isolated MSCs from human Wharton's jelly of the umbilical cord and induced them into cartilage cells. We then transplanted the early chondrogenic differentiated MSCs into the guinea pigs which have osteoarthritis and monitored their progress to evaluate the effectiveness of the treatment. The results demonstrated promising outcomes in the experimental animals, suggesting that this treatment approach using early chondrogenic differentiated MSCs could be developed.

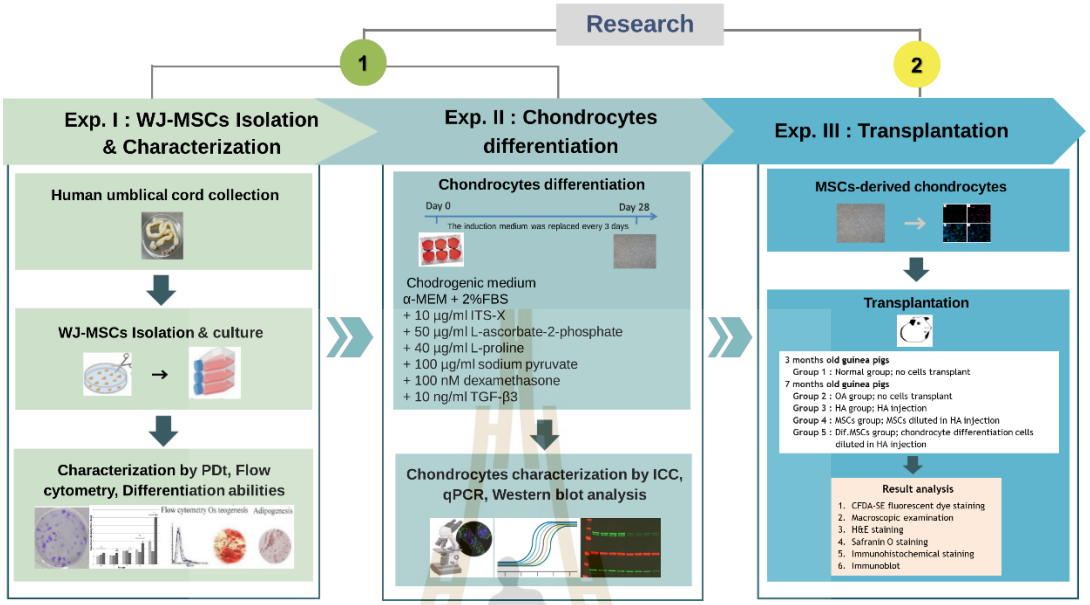
1.2 Research objectives

1.2.1 To isolate and characterize mesenchymal stem cells (MSCs) derived from the Wharton’s jelly of the human umbilical cord.

1.2.2 To investigate the potential of WJ-MSCs for differentiating into chondrocytes.

1.2.3 To investigate the potential therapeutic effects of chondrocytes derived from Wharton’s Jelly mesenchymal stem cells (WJ-MSCs) in the treatment of osteoarthritis using an animal model.

1.3 Experimental design



1.4 References

Bendele, A. M., & Hulman, J. F. (1988). Spontaneous cartilage degeneration in guinea pigs. **Arthritis Rheum.** 31(4): 561-565.

Cucchiarini, M., de Girolamo, L., Filardo, G., Oliveira, J. M., Orth, P., Pape, D., & Reboul, P. (2016). Basic science of osteoarthritis. **J. Exp. Orthop.** 3(1): 22.

Ebihara, G., Sato, M., Yamato, M., Mitani, G., Kutsuna, T., Nagai, T., Ito, S., Ukai, T., Kobayashi, M., Kokubo, M., Okano, T., & Mochida, J. (2012). Cartilage repair in transplanted scaffold-free chondrocyte sheets using a minipig model. **Biomaterials.** 33(15): 3846-3851.

Fernandez, M. L., Vergara-Jimenez, M., Conde, K., Behr, T., & Abdel-Fattah, G. (1997). Regulation of apolipoprotein B-containing lipoproteins by dietary soluble fiber in guinea pigs. **Am. J. Clin. Nutr.** 65(3): 814-822.

Jones, I. A., Togashi, R., Wilson, M. L., Heckmann, N., & Vangsness, C. T. (2019). Intra-articular treatment options for knee osteoarthritis. **Nat. Rev. Rheumatol.** 15(2): 77-90.

Kong, L., Zheng, L-Z., Qin, L., & Ho, K. K. W. (2017). Role of mesenchymal stem cells in osteoarthritis treatment. **J. Orthop. Translat.** 9: 89-103.

Kraus, V. B., Huebner, J. L., DeGroot, J., & Bendele, A. (2010). The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the guinea pig. **Osteoarthritis Cartilage.** 18 Suppl 3(Suppl 3): S35-52.

La Gatta, A., Stellavato, A., Vassallo, V., Di Meo, C., Toro, G., Iolascon, G., & Schiraldi, C. (2021). Hyaluronan and derivatives: an in vitro multilevel assessment of their potential in viscosupplementation. **Polymers.** 13: 3208.

Sato, M., Uchida, K., Nakajima, H., Miyazaki, T., Guerrero, A. R., Watanabe, S., Roberts, S., & Baba, H. (2012). Direct transplantation of mesenchymal stem cells into the knee joints of Hartley strain guinea pigs with spontaneous osteoarthritis. **Arthritis Res. Ther.** 14(1): R31.

Song, L., Baksh, D., & Tuan, R. S. (2004). Mesenchymal stem cell-based cartilage tissue engineering: cells, scaffold and biology. **Cytotherapy.** 6(6): 596-601.

Sophia Fox, A. J., Bedi, A., & Rodeo, S. A. (2009). The basic science of articular cartilage: structure, composition, and function. **Sports Health.** 1(6): 461-468.

Tessier, J. J., Bowyer, J., Brownrigg, N. J., Peers, I. S., Westwood, F. R., Waterton, J. C., & Maciewicz, R. A. (2003). Characterisation of the guinea pig model of osteoarthritis by in vivo three-dimensional magnetic resonance imaging. **Osteoarthritis Cartilage.** 11(12): 845-853.

Troyer, D. L., & Weiss, M. L. (2008). Wharton's jelly-derived cells are a primitive stromal cell population. **Stem Cells.** 26(3): 591-599.

Wong, C-C., Ou, K-L., Lin, Y-H., Lin, M-F., Yang, T-L., Chen, C-H., & Chan, W. P. (2020). Platelet-Rich Fibrin Facilitates One-Stage Cartilage Repair by Promoting Chondrocytes Viability, Migration, and Matrix Synthesis. **Int. J. Mol. Sci.** 21(2): 577.

Yan, J-Y., Tian, F-M., Wang, W-Y., Cheng, Y., Xu, H-F., Song, H-P., Zhang, Y-Z., & Zhang, L. (2014). Age dependent changes in cartilage matrix, subchondral bone mass, and estradiol levels in blood serum, in naturally occurring osteoarthritis in Guinea pigs. **Int. J. Mol. Sci.** 15(8): 13578-13595.

Yusuf, E. (2016). Pharmacologic and non-pharmacologic treatment of osteoarthritis. **Curr. Treat. Opt. Rheum.** 2: 111-25.

CHAPTER II

LITERATURE REVIEW

2.1 Osteoarthritis (OA)

OA is one of the significant contributors to years lived with disability among the musculoskeletal conditions. As OA is more prevalent in older people, global prevalence is expected to increase with the ageing of populations. OA is a degenerative joint condition. It causes pain, swelling of the damage tissue that lost it's stiffness, affecting a person's ability to move freely. Symptoms of OA include pain, swelling, stiffness and trouble moving the affected joint. As a consequence of reduced movement, muscles often lose strength and people become less able to perform physical activities. OA can affect any joint but is most common in the knees, hips, spine and small joints in the hands. Muscles and tissue around the joint are often affected. Osteophyte development and induced bone ends eventually rub against one another because chondrocytes are unable to repair tissue (Hunter & Felson, 2006; Frech & Clegg, 2007). Mainly characterized by articular cartilage degradation, OA is a heterogeneous disease that impacts all component tissues of the articular joint organ (He et al., 2020).

2.1.1 Articular cartilage

Normal adult articular cartilage is made up of extracellular matrix: ECM (water, collagen, proteoglycans and a very small component of calcium salt) and chondrocytes (Golding & Marcu, 2009). While, proteoglycan turnover is rapid collagen turnover is relatively slow. (Mow, Yong GU, & Hui Chen, 2005). The normal turnover of matrix components is regulated by chondrocytes, which produce both the components and the proteolytic enzymes responsible for their degradation. In turn, chondrocytes are influenced by several factors, such as polypeptide growth factors, cytokines, structural and physical stimuli, and even the matrix components themselves (Wise, 2010). OA occurs due to the failure of chondrocytes to maintain the balance between synthesis and degradation of ECM components (Heijink, Gomall, & Madry, 2012). In osteoarthritic cartilage, both anabolic and catabolic

activities increase. Initially, compensatory mechanisms, such as elevated synthesis of matrix molecules like collagen, proteoglycans, and hyaluronate (Goldring & Goldring, 2007), along with the proliferation of chondrocytes in the deeper cartilage layers, help preserve the integrity of the articular cartilage. However, over time, the loss of chondrocytes and alterations in the ECM become dominant, leading to the progression of osteoarthritic changes (Man & Mologhianu, 2014).

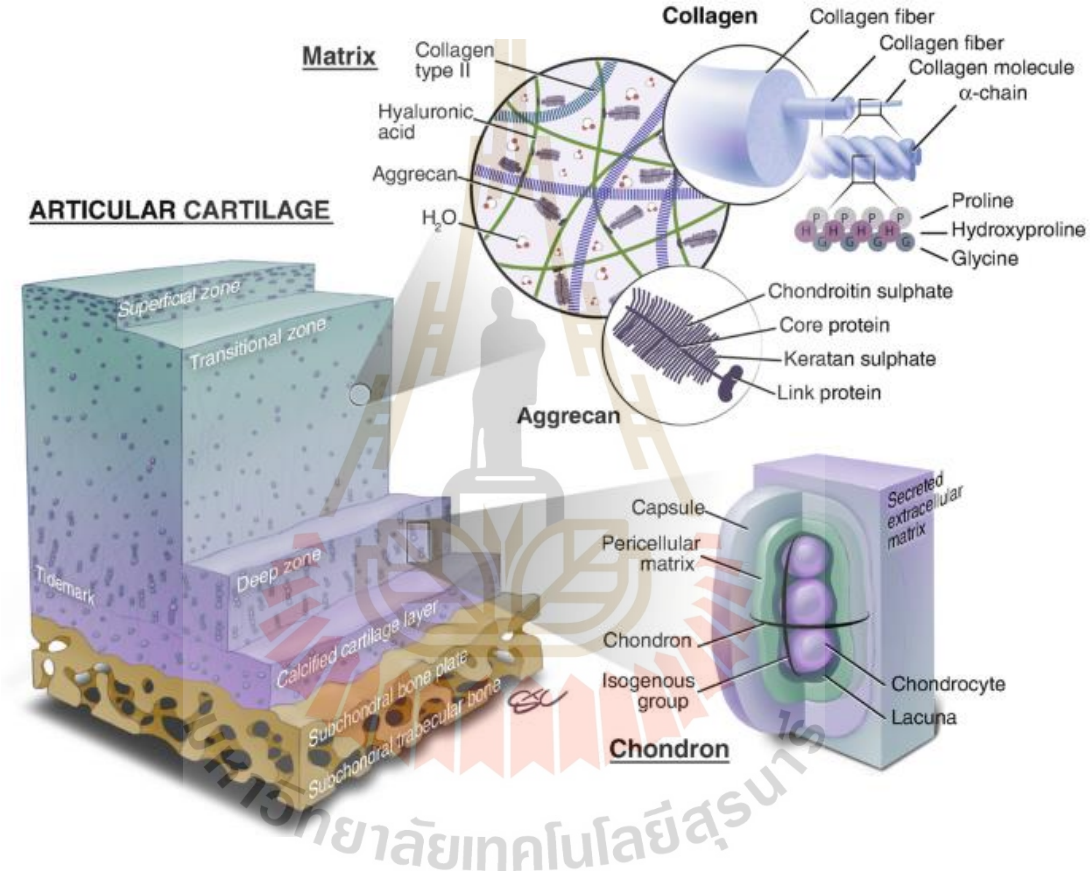


Figure 2.1 Structure of articular cartilage. (Baumann et al., 2019).

2.1.1.1 ECM: The dense ECM primarily consists of collagen type II alpha 1 chain (Col2a1) and the sulfated proteoglycan: aggrecan (ACAN) (Naba et al., 2015; Ariosa-Morejon et al., 2021). This matrix is critical for the biomechanical properties of cartilage, providing essential elastic support to distribute pressure and shear stress during joint movement. The ECM is produced by chondrocytes, specialized cells responsible for maintaining cartilage homeostasis by balancing

anabolic and catabolic activities (Wieland et al., 2005). Type II collagen, a fibrillar collagen, is secreted as triple-helical homotrimers of Col2a1, which associate with collagen type XI alpha 1 chain (Col11a1) and collagen type IX alpha 1 chain (Col9a1) IX collagens to form heterotypic fibrils within the cartilage tissue (Zhang, Hu, & Athanasiou, 2009; Bacenkova et al., 2023). ACAN, another major ECM component, has a long core protein with approximately 200 glycosaminoglycan (GAG) chains, primarily composed of dermatan and chondroitin sulfate (Cortes-Medina et al., 2023; Shen et al., 2023). These GAG chains carry a high negative charge, attracting cations (mainly sodium) and water, which are essential for maintaining the tissue's hydration and mechanical properties.

2.1.1.2 Chondrocytes: Chondrocytes, derived primarily from the mesoderm, are the sole cellular components of normal cartilage. Their terminal differentiation defines the type of cartilage they produce-whether hyaline, fibrous, or elastic (Heinegard & Saxne, 2011; McNulty & Guilak, 2015; Nimeskern et al. 2015; Nahian & Sapra, 2023). In articular cartilage, chondrocytes typically persist without further division once skeletal maturity is reached. However, in the epiphyseal growth plate, these cells differentiate to support endochondral ossification, after which they either undergo apoptosis or transform into osteoblasts (Shum & Nuckolls, 2001; Goldring, 2007). Chondrocytes are essential for both the synthesis and degradation of ECM components, including collagen and proteoglycans, as well as matrix-degrading enzymes like collagenase, neutral proteinases, and cathepsins (Green & Lund, 2005; Lu et al., 2011; Troeberg & Nagase, 2012). This dual role positions chondrocytes as key regulators of cartilage homeostasis (Goldring, 2007; Teng et al., 2017; Nahian & Sapra, 2023), balancing synthesis with breakdown. The pericellular matrix surrounding each chondrocyte contains type VI collagen, along with proteoglycans such as decorin and ACAN (Fujii et al., 2022). During chondrogenesis, chondrocytes actively synthesize the ECM, and they continue to regulate matrix turnover in mature cartilage to ensure the tissue's proper function. The organization and renewal of ECM components are critical to maintaining normal cartilage properties (Gentili & Cancedda, 2009; Sophia Fox, Bedi, & Rodeo, 2009; Michelacci, Baccarin, & Rodrigues, 2023). Proliferating chondrocytes exhibit strong expression of essential ECM genes, including Acan, Col2a1, Col9a1, and Col11a1. In contrast, hypertrophic chondrocytes

predominantly express collagen type X alpha 1 chain (Col10a1), osteopontin, and matrix metalloproteinases-9 (MMP-9) and metalloproteinases-13 (MMP-13). These gene expression patterns are regulated by master transcription factors, which orchestrate the differentiation of MSCs into chondrocytes and subsequently guide the transition from proliferating chondrocytes to hypertrophic chondrocytes (de Crombrugghe et al., 2000; Leung et al., 2011; Liu et al., 2017; Michelacci, Baccarin, & Rodrigues, 2023).

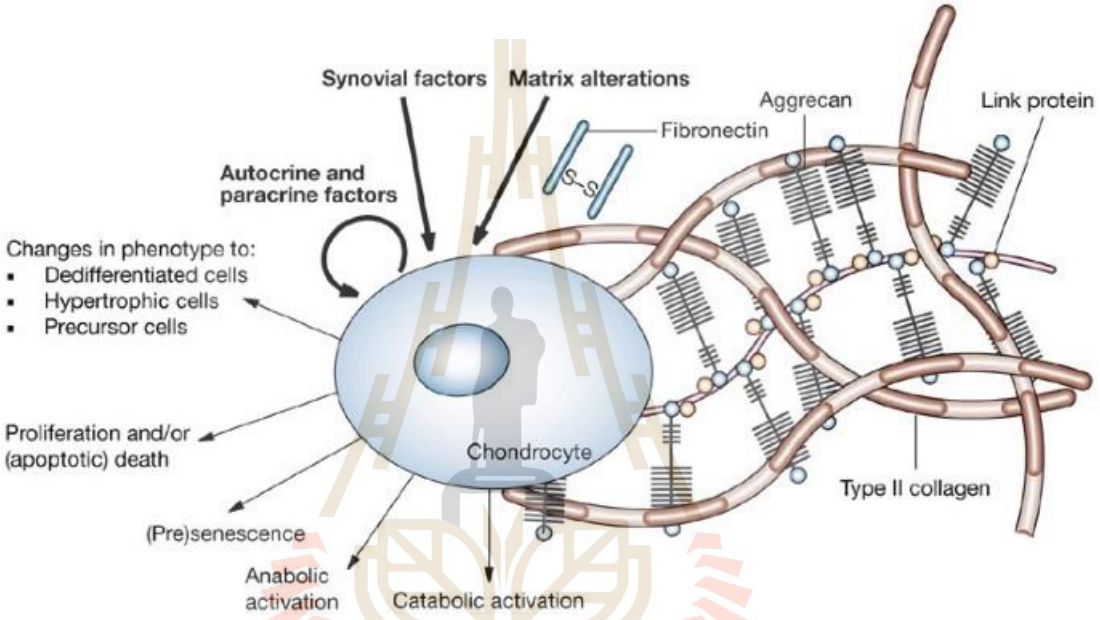


Figure 2.2 Chondrocytes in the pathogenesis of OA. (Aigner et al., 2007).

2.2 Regenerative therapy for OA

Treatments for OA are typically determined by the severity of the disease and guided by a physician's recommendations, which may include medication, physical therapy, or surgery. However, many patients experience recurrence or complications following treatment. Therefore, novel and more effective procedures, such as cartilage cell application, are essential. Unfortunately, obtaining these cells involves invasive surgery, making the process both complicated and expensive (Ebihara et al., 2012; Wong et al., 2020; Chen et al., 2022; Cong et al., 2023; Wang et al., 2024). Stem cell technology is currently regarded as a promising tool for cell-based therapy. Mesenchymal stromal cells (MSCs) have gained significant attention due to their potential for transdifferentiation

from one phenotype to another, offering exciting possibilities for cellular therapies (Talèns-Visconti et al., 2006; Musial-Wysocka, Kot, & Majka, 2019; Zhuang et al., 2021). Ongoing research is investigating the potential of MSCs for treating OA. These cells exhibit several desirable properties, including tri-mesodermal differentiation into osteoblasts, chondrocytes, and adipocytes, plastic adherence, self-renewal capabilities, and the expression of unrestricted MSC markers (Baddoo et al., 2003; Dominici et al., 2006; Ambrosi et al., 2017; Wolock et al., 2019; Azadniv et al., 2020). For numerous of these reasons, MSCs are currently an excellent candidate for therapeutic purpose in clinical applications. MSCs possess the unique ability to stimulate the growth of cartilage cells and other types of cells.

2.3 Mesenchymal stem cells (MSCs)

MSCs, are multipotent adult stem cells with the ability to differentiate into multiple specialized cell types and to self-renew, at least in mesodermal lineages of cells. (Morrison et al., 1997; Urbani et al., 2006; Guadix, Zugaza, & Gálvez-Martin, 2017). MSCs were first isolated from mouse bone marrow in 1976 by Friedenstein and colleagues. (Friedenstein, Gorskaja, & Kulagina, 1976). MSCs have the ability to adhere to plastic culture dishes and resemble fibroblasts in that they are spindle-shaped cells. Additionally, the adherent cells were defined as colony-forming unit fibroblasts due to their clonogenic feature or clonal density form (Friedenstein, Chailakhjan, & Lalykina. 1970). Moreover, the cells in this group have been defined as multipotent stromal precursor cells. Subsequent research over the years has revealed that these cells have the ability to develop *in vitro* into mesodermal lineages cells like oseteoblasts, adipocytes, and chondrocytes (Ashton et al., 1980; Castro-Malaspina et al., 1980; Bab et al., 1986; Pittenger et al., 1999). MSCs are extensively interesting in their biology, phenotypes, and features ever since Friedenstein and colleagues conducted pilot research on them in 1976. According to additional research, MSCs can be isolated from the bone marrow of rodents as well as from monkey, goat, sheep, dog, pig, and human (Rozemuller et al., 2010).

2.3.1 Sources and characteristics of MSCs

Although bone marrow was the original source of MSCs, other adult connective tissues have also been identified as potential sources of MSCs, including adipose tissue (Zuk et al., 2001; Romanov et al., 2005; Gruber et al., 2010), muscle (Asakura, Komaki, & Rudnicki, 2001), dental pulp (Perry et al., 2008; Ponnaiyan et al., 2012), and peripheral blood (Tondreau et al., 2005; Chong et al., 2012). Furthermore, MSCs can be obtained from tissues of fetal origin, including placenta (In't Anker et al., 2004), amniotic fluid (Tsai et al., 2004), umbilical cord blood (Lee et al., 2004), and Wharton's jelly (Troyer & Weiss, 2008; Ishige et al., 2009; Witkowska-Zimny & Wrobel, 2011; Mishra et al., 2020; Mebarki et al., 2021; Yea et al., 2023). Because MSCs have been investigated in laboratories using varying sources and culture techniques, several studies have reported various immunophenotype of MSCs. According to these findings, MSCs have absence of unique specific antigens but slight different expression of cell surface antigens depending on their origins (Hass et al., 2011). The International Society for Cellular Therapy (ISCT) established minimal criteria for characterizing MSCs in 2006 in order to clarify any confusion (Dominici et al., 2006). The following is a minimum definition of MSC characteristics. Firstly, after being cultured under standard culture conditions, MSCs must adhere to plastic culture flasks. Secondly, they must be negative stained for human haematopoietic stem cells surface antigens such as CD34 and CD45 and positive stained for some surface antigens such as CD44, CD73, CD90, and CD105. They also need to be negative stained for human leukocytes surface antigens such as CD11b, CD14, CD19, and CD79α and for human major histocompatibility complex (MHC) class II antigen or HLA-DR. Thirdly, *in vitro* differentiation into mesodermal-lineage cells such as osteoblasts, adipocytes, and chondroblasts must be possible.

In general, MSCs that have been isolated from various tissues have similar characteristics. There have been some differences observed in the proliferation and differentiation capability of adult and fetal MSCs *in vitro*. The proliferation rate of human first-trimester fetal MSCs was higher than that of adult MSCs. This finding can be explained that fetal MSCs have longer telomeres than adult MSCs, which relates to their more primitive origins (Campagnoli et al., 2001; Guillot et al., 2007). Furthermore, MSCs isolated from bone marrow and adipose tissue shown a higher

capacity for adipogenic differentiation than MSCs obtained from umbilical cord blood (Kern et al., 2006; Rebelatto et al., 2008). Based on the fundamental standards for MSC characterization, MSCs from various origins nevertheless have certain characteristics in common even though they have certain distinct properties.

2.3.1.1 Wharton's jelly mesenchymal stem cells (WJ-MSCs)

WJ-MSCs represent a primitive stromal population with several key characteristics defined by the International Society for Cellular Therapy. They exhibit mesenchymal morphology, self-renewal capabilities, and the ability to differentiate into various cell types, including bone, cartilage, and muscle. WJ-MSCs also support hematopoietic stem cell expansion and are well-tolerated by the immune system, with the ability to home to tumors. Compared to bone marrow-derived MSCs, WJ-MSCs have superior expansion potential, faster *in vitro* growth, and a unique cytokine synthesis profile. Preclinical studies show their therapeutic efficacy in models of neurodegenerative diseases (Staff, Jones, & Singer, 2019; Mattei, & Delle Monache, 2024), cancer (Lin et al., 2019; Lim, & Khoo, 2021; Vicinanza et al., 2022), heart disease (Bagno et al., 2018; Correia et al., 2023), and OA (Wang et al., 2022; Lv et al., 2023; Copp, Robb, & Viswanathan, 2023), primarily through trophic rescue and immune modulation. WJ-MSCs fulfill the criteria for MSCs and demonstrate promising therapeutic potential due to their enhanced expansion capabilities. Further research is necessary to ascertain their long-term engraftment and multipotency *in vivo*, confirming whether they constitute a true stem cell population (Taghizadeh, Cetrulo, & Cetrulo, 2011; Marino et al., 2019; Kamal & Kassem, 2020; Drobiova et al., 2023).

WJ-MSCs were isolated from Wharton's jelly, a gelatinous substance found within the umbilical cord. Umbilical cord-derived MSCs are valuable tools in stem cell research due to their easy isolation, high proliferation rates, multi-lineage differentiation potential, and immunomodulatory properties, along with the absence of ethical concerns. Among the various compartments of the umbilical cord, Wharton's jelly has recently been identified as the best source of MSCs (Davies, Walker, & Keating, 2017; Bharti et al., 2018; Drobiova et al., 2023).

2.3.2 Differentiation abilities

MSCs are multipotent progenitor cells that can differentiate into several lineages, including bone, cartilage, fat, and muscle (Fig 2.3). MSCs are known for their

ability to differentiate into various cell types, particularly those within the mesodermal lineage. Key differentiation pathways including osteogenic differentiation, chondrogenic differentiation, and adipogenic differentiation (Benayahu, 2022). Osteogenic differentiation: MSCs can become osteoblasts, which are responsible for bone formation (Pittenger et al., 2019). Chondrogenic differentiation: MSCs can differentiate into chondrocytes, which form cartilage, making them ideal for cartilage repair therapies (Augello & Bari, 2010). Adipogenic differentiation: MSCs are also capable of transforming into adipocytes, which store energy as fat (Tivig et al., 2024). These cells retain high plasticity and exhibit regenerative potential by responding to specific environmental cues *in vitro* or *in vivo*. This versatility positions them as promising candidates for various therapeutic applications (Yang et al., 2018).

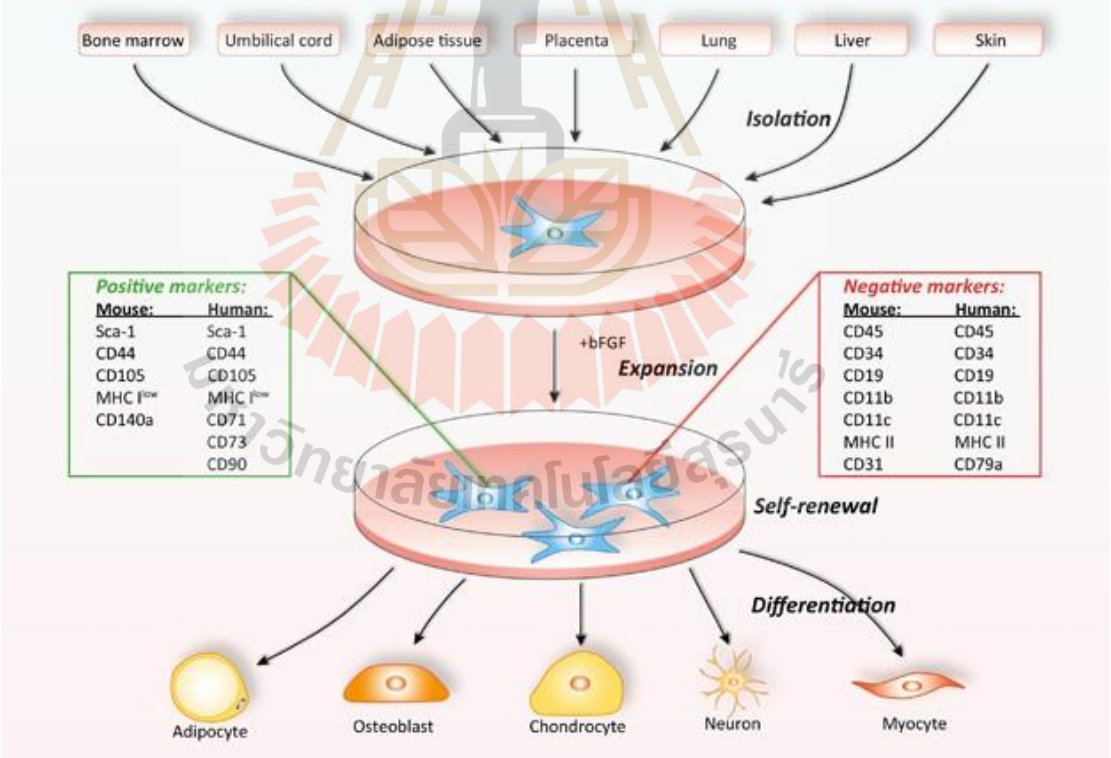


Figure 2.3 Isolation, expansion, and differentiation of MSCs (Chen et al., 2016).

MSCs are a major focus in regenerative therapies due to their robust differentiation abilities. MSCs can differentiate into mesodermal lineages, including osteoblasts (bone cells), chondrocytes (cartilage cells), and adipocytes (fat cells). This makes them essential for bone and cartilage repair (Pittenger et al., 2019; Saeedi, Halabian, & Imani Fooladi, 2019). MSCs can release bioactive molecules that promote the growth and differentiation of other stem cells, enhancing tissue regeneration (Vasanthan et al., 2020; Margiana et al., 2022). Under controlled conditions, MSCs display regenerative potential both *in vitro* and after implantation *in vivo*. This dual capability supports their therapeutic use in treating degenerative diseases, such as OA (Pittenger et al., 2019; Maldonado et al., 2023). MSCs exhibit self-renewal properties and can modulate immune responses, making them ideal for reducing inflammation and promoting tissue repair (Maldonado et al., 2023).

WJ-MSCs show high potential for chondrogenic differentiation, which is critical for cartilage repair. WJ-MSCs grown on silk scaffolds and supplemented with L-ascorbic acid (LAA) or platelet-rich plasma (PRP) demonstrated enhanced chondrogenic differentiation. These scaffolds provide a suitable 3D environment that mimics the natural ECM for cartilage formation (Barlian et al., 2020). WJ-MSCs have superior chondrogenic potential compared to bone marrow-derived MSCs. This makes WJ-MSCs preferable for therapeutic applications aimed at cartilage regeneration (Liau et al., 2019; Pelusi et al., 2021). WJ-MSCs retain their hypoimmunogenic nature during differentiation, making them suitable for allogeneic transplants without significant immune rejection risks (Voisin et al., 2020). These characteristics make WJ-MSCs promising candidates for future applications in regenerative medicine, especially for treating joint disorders and cartilage injuries.

2.4 Chondrogenesis

Chondrogenesis (Fig 2.4) refers to the process by which chondrocytes and cartilage tissues are formed. It begins with the recruitment and migration of MSCs, followed by the condensation of progenitor cells. This process then proceeds through chondrocyte differentiation and concludes with the maturation of these cells (Barna, & Niswander, 2007; Zuscik et al., 2008; Chen et al., 2009; Goldring, 2012; Yang et al., 2022). The regulation of chondrogenic differentiation in MSCs involves

intracellular proteins, receptor ligands, and transcription factors. Any disruption in these signaling pathways can lead to defective chondrocyte production (Green et al., 2015; Yang et al., 2022). The most important biological regulators of chondrogenesis and their interactions involve multiple signaling pathways and transcriptional regulation. Key signaling pathways include fibroblast growth factors (FGFs), transforming growth factor-β (TGF-β)/bone morphogenic proteins (BMPs), and the Wnt/β-catenin pathway (Mariani, Pulsatelli, & Facchini, 2014; Green et al., 2015). The transcriptional regulation of chondrogenesis is crucial, involving key factors such as the SRY-related high-mobility group-box gene 9 (Sox9) and Runt-related transcription factors (Runx) (Zhou, Zheng, & Engin, 2006; Ohba et al., 2015; Nagata et al., 2022).

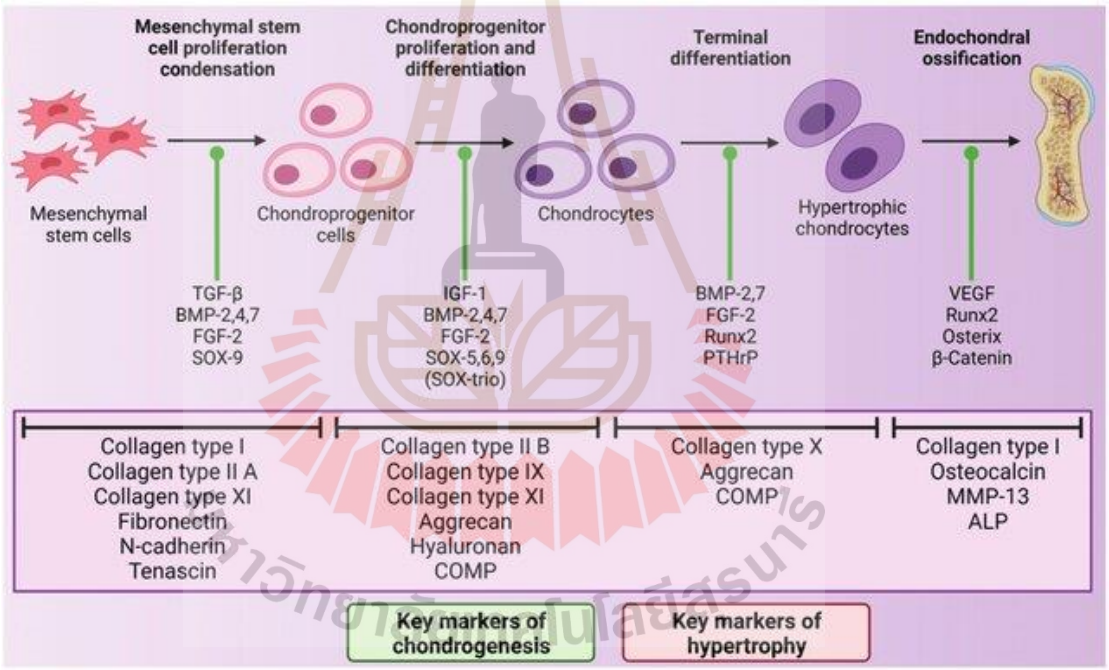


Figure 2.4 A schematic of the process of chondrogenesis (O'Shea, Curtin, & O'Brien, 2022).

2.4.1 Signaling pathways

2.4.1.1 WNT signaling

Wnt signaling can both promote and suppress the chondrogenic differentiation of progenitor cells in both embryonic and adult stages (Hartmann, & Tabin, 2000; Fischer, Boland, & Tuan, 2002; Yano, Kugimiya, & Ohba, 2005; Leijten et al.,

2012; Cheng et al., 2022; Liu et al., 2022). The antagonistic effects may result from various factors, such as the target cells' differentiation phase, the level of Wnt activity, the specific type of Wnt signal, and crosstalk with other signaling pathways like the TGF-ß/BMP pathway (Fischer, Boland, & Tuan, 2002; Zhou, Eid, & Glowacki, 2004; Hartmann, 2006; Wang et al., 2014; Vlashi et al., 2023; Wu et al., 2024). Although the role of Wnt signaling in the early phase of chondrogenesis remains poorly understood, studies on specific Wnt proteins indicate that Wnt plays a crucial role in promoting the migration and condensation of chondrogenic cells (Timothy et al., 2005; Bradley, & Driss, 2011; Kamel, Hoyos, & Rochard, 2013; Green et al., 2015; Hu et al., 2024). Wnt5b has been shown to regulate chondroprogenitor cell migration by activating the planar cell polarity pathway. It also reduces cell adhesion by suppressing the expression of a destabilizing cadherin receptor and modulates the activity of various other cadherins (Lee, Kim, & Cho, 2008; Bradley & Drissi, 2011; Green et al., 2015; Suthon et al., 2021). Wnt proteins such as Wnt1, Wnt3a, Wnt4, Wnt5b, and Wnt8 have been demonstrated to activate the canonical pathway *in vitro* (Hartmann, & Tabin, 2000; Maiese et al., 2008; Zhang et al., 2021; Qin et al., 2023). Wnt3a and Wnt5b promote chondrocyte differentiation and suppress chondrocyte hypertrophy, a critical step in the later stages of chondrogenesis (Veeman, Axelrod, & Moon, 2003; Hartmann, 2007; Green et al., 2015). In contrast, Wnt1, Wnt4, and Wnt8 have been reported to inhibit chondrocyte differentiation while promoting hypertrophy.

2.4.1.2 FGF signaling

FGF are a family of heparin-binding growth factors which play a role in the proliferation and differentiation of a variety of tissues. In general, FGFs signaling are known to be positive regulators of chondrogenic differentiation and hypertrophy (Ornitz & Marie, 2015; Green et al., 2015). The development of chondrocyte differentiation and maturation has been demonstrated to be affected by the sequential production of three FGFs: FGF2, FGF9, and FGF18 (Hellingman, Koevoet, & Kops, 2010; Oseni et al., 2011; Correa, Somoza, & Lin, 2015). FGF2 has been shown to effectively enhance chondrocyte proliferation and ready chondroprogenitor cells for terminal differentiation. However, until recently, the mechanisms through which FGF2 produces these effects have remained largely unknown (Oseni et al., 2011). FGF2 can trigger a sequence of intracellular signals, such as MAPK/ERK, that promote cell cycle

progression, thus accelerating chondrocyte proliferation. Additionally, FGF2 appears to modulate the activity of other growth factors like TGF- ß, enhance TGF-ß1 expression (Stevens, Marini, & Martin, 2004; Solchaga et al., 2009; Handorf, & Li, 2011; Green et al., 2015; Chen et al., 2021) which typically counterbalances proliferation to favor differentiation. FGF2 has been shown to downregulate TGF-ß2 expression, which contributes to FGF2-induced chondrocyte differentiation (Ito et al., 2008). Recent findings indicate that FGF2 can promote both chondrocyte differentiation and proliferation when TGF-ß3 is present, though it concurrently inhibits chondrocyte hypertrophy (Richter et al., 2009). Furthermore, FGF2 regulates chondrogenic differentiation via MAPK and Wnt signaling pathways (Solchaga et al., 2005).

2.4.1.3 TGF-ß /BMP signaling

The TGF-ß superfamily, in particular TGF-ß and BMP, are essential for multiple stages of embryonic chondrogenesis (Goldring, Tsuchimochi, & Ijiri, 2006; Cleary, van Osch, & Brama, 2015). This pathway, particularly the TGF-ß/BMP signaling pathway, is frequently used to promote chondrocyte differentiation in both MSCs and expanded chondrocyte populations (Goldring, Tsuchimochi, & Ijiri, 2006; Karamboulas, Dranse, & Underhill, 2010; Green et al., 2015). Chondrogenesis and osteogenesis are known to be majorly regulated by TGF-ß1 and TGF-ß2 (Sandberg, Autio-Harmainen, & Vuorio, 1998; Carrington, Roberts, & Flanders, 1988). TGF-ß also signals the transition from proliferation to the onset of chondrocyte differentiation (Cleary, van Osch, & Brama, 2015). This progression has been widely studied both *in vivo*, during embryonic cartilage development, and *in vitro*, using MSCs (Augustyniak, Trzeciak, & Richter, 2015). TGF-ß has been demonstrated to mediate the differentiation of chondroprogenitor cells into chondrocytes in addition to inhibiting proliferation (Rosen, Stempien, & Thompson, 1986). TGF- ß has long served as the primary growth factor model for inducing chondrogenesis in MSCs *in vitro* (Johnstone, Hering, & Caplan, 1998). TGF-ß, especially TGF-ß1 and TGF-ß3, activates Smad-dependent pathways (e.g., Smad2/3 and Smad1/5/8) to initiate and regulate chondrogenic differentiation (Cleary, van Osch, & Brama, 2015; Green et al., 2015; Chen et al., 2021). In MSCs, TGF-ß activates MAPK proteins, including p38, ERK, and JNK, which subsequently downregulate N-cadherin expression by inhibiting Wnt-mediated ß-catenin nuclear translocation (Tuli, Tuli, & Nandi, 2003; Rodríguez-García et al., 2017; Luo, 2017; Loh et

al., 2019). This reduction in N-cadherin expression is a critical step required for the transition from cell condensation to differentiation (Oh, Chang, & Yoon, 2000; Tufan, &Tuan, 2001; Tufan et al., 2002; Loh et al., 2019).

BMPs have been identified as key positive regulators in ectopic chondrogenesis and endochondral ossification (Guo et al., 2014). Inhibiting BMP signaling has been shown to reduce cartilage formation. BMP family members are essential for chondro-progenitor cell condensation and chondrocyte differentiation *in vivo* and *in vitro* (Yoon, Ovchinnikov, & Yoshii, 2005; Hidaka, & Goldring, 2008; Long, & Ornitz, 2013). Joint development, which also involves chondrogenesis, depends on BMPs such as BMP-2, BMP-4, and GDF5 (Coleman & Tuan, 2003; Hidaka, & Goldring, 2008). Application of BMP-2, -4, -6, -7, -9, -13, and -15 enhance type II collagen synthesis in articular chondrocytes *in vitro* (Chubinskaya, & Kuettner, 2003; Gründer, Gaissmaier, & Fritz, 2004; Hidaka, & Goldring, 2008; Cleary, van Osch, & Brama, 2015). The implications of BMP signaling are probably concentration-dependent and time-dependent, although BMPs participate in the condensation and differentiation stages, which are displayed to be mutually exclusive (Hidaka, & Goldring, 2008).

2.4.2 Transcriptional regulation

2.4.2.1 Sox9

Sox9 is a prochondrogenic transcription factor that is arguably the single most important regulator of chondrogenesis. During chondrogenesis, multiple signaling pathways regulate the expression and activity of Sox9 (Fig 2.5), (Augello, & De Bari, 2010; Kozhemyakina, Lassar, & Zelzer, 2015). Full expression of the chondrocyte phenotype requires high levels of Sox9 (Lefebvre, & Dvir-Ginzberg, 2016). Sox9 expression is induced by compressive force and is influenced by a variety of prochondrogenic factors, including RelA, Pref-1, and Arib5b, as well as two other Sox transcription factors, Sox5 and Sox6 (Ushita, Saito, & Ikeda, 2009; Wang, & Sul, 2009; Yamashita, Miyaki, & Kato, 2012; Hata, Takashima, & Amano, 2013; Juhász, Matta, & Somogyi, 2014). Sox9, as a transcription factor, directly regulates the genes it interacts with through its mechanism of action. It activates elements in the promoters of Col2a1, Col9a1, Col11a2, Bbf2h7-sec23a, and ACAN by interacting with two binding sites for HMG-domain proteins (Akiyama, Chaboissier, & Martin, 2002; Akiyama, 2008; Leung et al., 2011; Hino, Saito, & Kido, 2014). Sox9 primarily regulates chondrocyte proliferation

and differentiation by directly controlling the expression of various chondrocyte-specific genes. Furthermore, many studies have highlighted Sox9's role in the earlier phases of chondrogenesis, particularly during condensation. Research indicates that Sox9 expression levels are significantly elevated in condensing mesenchymal progenitors, both *in vitro* and *in vivo*, whereas Sox9 knockout mice are unable to undergo mesenchymal cell condensation (Mori-Akiyama et al., 2003; Akiyama, Lyons, & Mori-Akiyama, 2004; Quintana, Zur Nieden, & Semino, 2009). Sox9 induces MSCs differentiate into chondrocytes and promotes proliferation (Chen et al., 2021) FGF2 induces chondrocyte proliferation by upregulating the expression levels of Sox9 (Pan, Yu, & Chen, 2008; Shi, Wang, & Acton, 2015). Sox9 enhances the prochondrogenic properties of BMP2 while also inhibiting BMP2-induced osteogenic differentiation and endochondral ossification (Liao, Hu, & Zhou, 2014). Sox9 blocks the activation of the transcription factor Runx2, which is the key factor in reducing chondrocyte maturation, thereby repressing chondrocyte hypertrophy (Zhou et al., 2006). Sox9 inhibits Wnt signaling by interacting with β-catenin, which is known to promote chondrocyte hypertrophy (Topol et al., 2009). Sox9 may inhibit the expression of genes associated with hypertrophic chondrocytes, including Col10a1 and VEGFA (Hattori et al., 2010; Leung et al., 2011).

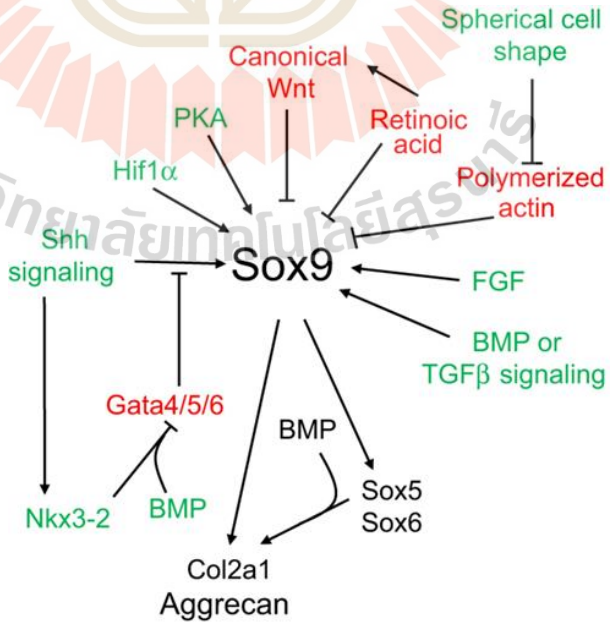


Figure 2.5 Multiple signaling pathways regulate the expression and activity of Sox9 during chondrogenesis (Kozhemyakina, Lassar, & Zelzer, 2015).

2.4.2.2 Runt-related transcription factors (Runx)

Runx proteins, which include Runx2 and Runx3, are a family of transcription factors that play crucial roles in chondrogenesis and are essential for the maturation of chondrocytes (Yoshida et al., 2004; Komori, 2018; Rashid, Chen, & Javed, 2021; Nakata et al., 2022, Komori, 2024). Runx2 regulates osteogenesis and chondrocyte proliferation through its interaction with PI3K-Akt signaling (Fujita, Azuma, & Fukuyama, 2004). Sox9 plays a dominant role in controlling Runx2, particularly in MSCs committed to the chondrogenic lineage (Akiyama et al., 2005; Zhou, Zheng, & Engin, 2006; Chan et al., 2021). This regulatory relationship becomes more complex with the involvement of CypA, which promotes the expression of both Runx2 and Sox9 in chondro-progenitor cells. As expected, knockdown of CypA results in reduced chondrogenesis and impaired endochondral ossification (Guo, Shen, & Kwak, 2015). Another regulatory mechanism for controlling Runx2 involves Nkx3.2. This factor represses Runx2 activity by directly interacting with its promoter, and such repression is essential for the progression of BMP-induced chondrogenesis (Lengner, Hassan, & Serra, 2005; Kawato et al., 2011; Rainbow, Won, & Zeng; 2014). The regulation of Runx2 expression plays a crucial role in chondrogenesis, as it influences multiple processes in MSCs. Runx2 is expressed at low levels in proliferating chondrocytes but increases as these cells exit the cell cycle. Notably, Runx2 expression in proliferating chondrocytes has been found to accelerate their transition to hypertrophy (Stricker et al., 2002; Hinoi et al., 2006; Chen et al., 2021; Rashid, Chen, & Javed, 2021; Komoti, 2022; Nagata et al., 2022; Rashid et al., 2024), the removal of RUNX2 inhibits normal mineralization of hypertrophic cartilage. Conversely, several studies show that ectopic expression of Runx2 in immature chondrocytes induces the expression of hypertrophic markers, including Col10a1, MMP13, and VEGF (Takeda et al., 2001; Stricker et al., 2002; Zheng, Zhou, & Morello, 2003; Li et al., 2011; Wang et al., 2014; Kozhemyakina, Lassar, & Zelzer, 2015; Rashid, Chen, & Javed, 2021; Chen et al., 2023).

2.5 Animal model for chondrocytes transplantation

cartilage regeneration. Mice, guinea pig, rabbits, dogs, goats, pigs, and horses models are frequently employed in cartilage repair studies due to their relevance to human joint mechanics and cartilage structure (Chu, Szczodry, & Bruno, 2010; Cook

et al., 2014; Kuyinu et al., 2016). Animal models provide valuable insights into developing and testing tissue-engineering techniques, helping researchers refine methods to repair damaged articular cartilage (Reinholz et al., 2004). A systematic review covering literature from 2000 to 2022 highlights advances and trends in using animal models for articular cartilage research, emphasizing their importance for improving transplantation techniques (Vinod et al., 2023).

Animal studies have provided valuable insights into the progression and treatment of OA in different models. Unilateral focal degeneration was noted in the medial tibial plateau of 2 out of 5 three-month-old guinea pigs, with age-related severity increasing to moderate-to-severe degeneration in all animals by 12-18 months, while the lateral knee joint remained unaffected (Bendele & Hulman, 1988). In animal study involving adult merino sheep, intraarticular hyaluronic acid (HA) injections post-unilateral medial meniscectomy were shown to mitigate changes in articular cartilage and subchondral bone associated with early OA (Armstrong, Read, & Ghosh, 1994). In 2003, Tessier et al. used MRI to assess longitudinal changes in knee joint integrity in guinea pigs with spontaneous OA, revealing cartilage swelling and fragmentation over time, culminating in a 36% loss of tibial cartilage volume between 9 and 12 months. A reliable histological scoring system was developed for OA assessment in guinea pigs (Kraus et al., 2010). Research by Ebihara et al., 2012 demonstrated that layered chondrocyte sheets can promote cartilage repair in a minipig model, though some defects remained. Sato et al., 2012 investigated the transplantation of MSCs in HA into guinea pig knees, finding partial cartilage repair in the HA-MSC group. Yan et al., 2014 documented age-related degeneration of cartilage and subchondral bone in female Dunkin Hartley guinea pigs, starting from 3 months, but found no significant correlations with estradiol levels or bone mineral density.

The use of Dunkin Hartley guinea pigs as an animal model for studying spontaneous cartilage degeneration in the knee joint, which is similar to OA in humans, has been well established (Tessier et al., 2003; Yan et al., 2014; Wang et al., 2021). Researchers reported that the knee joint of guinea pigs closely resembles that of humans affected by OA (Fernandez et al., 1997; Kraus et al., 2010). Moreover, spontaneous cartilage degeneration in the Dunkin-Hartley guinea pigs are used for study (Bendele & Hulman, 1988). Previous studies demonstrated that injecting MSCs

with hyaluronic acid (HA) into the articular cartilage of guinea pigs with OA led to recovery (Sato et al., 2012). HA-based formulations are currently delivered into the joint to relieve pain and improve joint mobility of OA patients by partial restoration of the rheological properties of the synovial fluid (La Gatta et al., 2021). These findings highlight how animal models, especially guinea pigs and sheep, are instrumental in studying OA mechanisms and developing new therapeutic strategies.

2.6 References

Aigner, T., Söder, S., Gebhard, P. M., McAlinden, A., & Hagg, J. (2007). Mechanisms of Disease: role of chondrocytes in the pathogenesis of osteoarthritis-structure, chaos and senescence. **Nat. Rev. Rheumatol.** 3: 391–399.

Akiyama, H. (2008). Control of chondrogenesis by the transcription factor Sox9. **Mod. Rheumatol.** 18: 213-219.

Akiyama, H., Chaboissier, M. C., & Martin, J. F. (2002). The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. **Genes. Dev.** 16: 2813-2828.

Akiyama, H., Kim, J. E., Nakashima, K., Balmes, G., Iwai, N., Deng, J. M., Zhang, Z., Martin, J. F., Behringer, R. R., & Nakamura, T. (2005). Osteo-chondroprogenitor cells are derived from Sox9 expressing precursors. **Proc. Natl. Acad. Sci. USA.** 102: 14665-14670.

Akiyama, H., Lyons, J. P., & Mori-Akiyama, Y. (2004). Interactions between Sox9 and beta-catenin control chondrocyte differentiation. **Genes. Dev.** 18: 1072-1087.

Ambrosi, T. H., Scialdone, A., Graja, A., Gohlke, S., Jank, A.-M., Bocian, C., Woelk, L., Fan, H., Logan, D. W., Schürmann, A., Saraiva, L. S., & Schulz, T. J. (2017). Adipocyte Accumulation in the Bone Marrow during Obesity and Aging Impairs Stem Cell-Based Hematopoietic and Bone Regeneration. **Cell Stem Cell.** 20: 771-784.

Ariosa-Morejon, Y., Santos, A., Fischer, R., Davis, S., Charles, P., Thakker, R., Wann, A. K., & Vincent T. (2021). Age-dependent changes in protein incorporation into collagen-rich tissues of mice by *in vivo* pulsed SILAC labelling. **Elife.** 10: e66635.

Armstrong, S., Read, R., & Ghosh, P. (1994). The effects of intraarticular hyaluronan on cartilage and subchondral bone changes in an ovine model of early osteoarthritis. **J. Rheumatol.** 21: 680-688.

Asakura, A., Komaki, M. & Rudnicki, M. (2001). Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. **Differentiation.** 68(4-5): 245-253.

Ashton, B. A., Allen, T. D., Howlett, C. R., Eaglesom, C. C., Hattori, A., & Owen, M. (1980). Formation of bone and cartilage by marrow stromal cells in diffusion chambers *in vivo*. **Clin. Orthop. Relat. Res.** 151: 294-307.

Augello, A., & De Bari, C. (2010). The regulation of differentiation in mesenchymal stem cells. **Hum. Gene. Ther.** 21(10): 1226-1238.

Augustyniak, E., Trzeciak, T., & Richter, M. (2015). The role of growth factors in stem cell-directed chondrogenesis: a real hope for damaged cartilage regeneration. **Int. Orthop.** 39: 995-1003.

Azadniv, M., Myers, J. R., McMurray, H. R., Guo, N., Rock, P., Coppage, M. L., Ashton, J., Becker, M. W., Calvi, L. M., & Liesveld, J. L. (2020). Bone Marrow Mesenchymal Stromal Cells from Acute Myelogenous Leukemia Patients Demonstrate Adipogenic Differentiation Propensity with Implications for Leukemia Cell Support. **Leukemia.** 34: 391-403.

Bab, I., Ashton, B. A., Gazit, D., Marx, G., Williamson, M. C. & Owen, M. E. (1986). Kinetics and differentiation of marrow stromal cells in diffusion chambers *in vivo*. **J. Cell Sci.** 84: 139-151.

Bacenkova, D., Trebunova, M., Demeterova, J., & Zivcak, J. (2023). Human Chondrocytes, Metabolism of Articular Cartilage, and Strategies for Application to Tissue Engineering. I**nt. J. Mol. Sci.** 24(23): 17096.

Baddoo, M., Hill, K., Wilkinson, R., Gaupp, D., Hughes, C., Kopen, G. C., & Phinney, D. G. (2003). Characterization of Mesenchymal Stem Cells Isolated from Murine Bone Marrow by Negative Selection. **J. Cell. Biochem.** 89: 1235-1249.

Bagno, L., Hatzistergos, K. E., Balkan, W., & Hare, J. M. (2018). Mesenchymal Stem Cell-Based Therapy for Cardiovascular Disease: Progress and Challenges. **Mol. Ther.** 26(7): 1610-1623.

Barlian, A., Judawisastra, H., Ridwan, A., Wahyuni, A. R., & Lingga, M. E. (2020). Chondrogenic differentiation of Wharton's Jelly mesenchymal stem cells on silk spidroin-fibroin mix scaffold supplemented with L-ascorbic acid and platelet rich plasma. **Sci. Rep.** 10(1): 19449.

Barna, M., & Niswander L. (2007). Visualization of cartilage formation: insight into cellular properties of skeletal progenitors and chondrodysplasia syndromes. **Dev. Cell.** 12: 931-941.

Baumann, C. A., Hinckel, B. B., Bozynski, C. C., & Farr, J. (2019). Articular Cartilage: Structure and Restoration. In Yanke, A., & Cole, B. (eds). *Joint Preservation of the Knee* (pp. 3-24). Cham: Springer.

Bendele, A. M., & Hulman, J. F. (1988). Spontaneous cartilage degeneration in guinea pigs. **Arthritis Rheum.** 31(4): 561-565.

Benayahu, D. (2022). Mesenchymal stem cell differentiation and usage for biotechnology applications: tissue engineering and food manufacturing. **Biomater. Transl.** 3(1): 17-23.

Bharti, D., Shivakumar, S. B., Park, J. K., Ullah, I., Subbarao, R. B., Park, J. S., Lee, S. L., Park, B. W., & Rho, G. J. (2018). Comparative analysis of human Wharton's jelly mesenchymal stem cells derived from different parts of the same umbilical cord. **Cell Tissue Res.** 372(1): 51-65.

Bradley, E. W., & Drissi, M. H. (2011). Wnt5b regulates mesenchymal cell aggregation and chondrocyte differentiation through the planar cell polarity pathway. **J. Cell. Physiol.** 226: 1683-1693.

Campagnoli, C., Roberts, I. A., Kumar, S., Bennett, P. R., Bellantuono, I. & Fisk, N. M. (2001). Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. **Blood.** 98(8): 2396-2402.

Carrington, J. L., Roberts, A. B., & Flanders, K. C. (1988). Accumulation, localization, and compartmentation of transforming growth factor beta during endochondral bone development. **J. Cell Biol.** 107: 1969-1975.

Castro-Malaspina, H., Gay, R. E., Resnick, G., Kapoor, N., Meyers, P., Chiarieri, D., McKenzie, S., Broxmeyer, H. E., & Moore, M. A. (1980). Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. **Blood.** 56(2): 289-301.

Chan, W. C. W., Tan, Z., To, M. K. T., & Chan, D. (2021). Regulation and Role of Transcription Factors in Osteogenesis. **Int. J. Mol. Sci.** 22(11): 5445.

Chen, H., Tan, X-N., Hu, S., Liu, R-Q., Peng, L-H., Li, Y-M., & Wu, P. (2021). Molecular Mechanisms of Chondrocyte Proliferation and Differentiation. **Cell Dev. Biol.** 9: 664168.

Chen, N., Wu, R. W. H., Lam, Y., Chan, W. C. W., & Chan, D. (2023). Hypertrophic chondrocytes at the junction of musculoskeletal structures. **Bone Rep.** 19: 101698.

Chen, Q., Shou, P., Zheng, C., Jiang, M., Cao, G., Yang, Q., Cao, J., Xie, N., Velletri, T., Zhang, X., Xu, C., Zhang, L., Yang, H., Hou, J., Wang, Y., & Shi, Y. (2016). Fate decision of mesenchymal stem cells: Adipocytes or osteoblasts. **Cell Death Differ.** 23(7): 1-12.

Chen, W-H., Lai, M-T., Wu, A., Wu, C-C., Gelovani, J. G., Lin, C-T., Hung, S-C., Chiu, W-T., & Deng, W-P. (2009). *In vitro* stage-specific chondrogenesis of mesenchymal stem cells committed to chondrocytes. **Arthritis Rheumatol.** 60(2): 450-459.

Chen, Y., Zhang, C., Zhang, S., Qi, H., Zhang, D., Li, Y., & Fang, J. (2022). Novel advances in strategies and applications of artificial articular cartilage. **Front. Bioeng. Biotechnol.** 10: 987999.

Cheng, B., Feng, F., Shi, F., Huang, J., Zhang, S., Quan, Y., Tu, T., Liu, Y., Wang, J., Zhao, Y., & Zhang, M. (2022). Distinctive Roles of Wnt Signaling in Chondrogenic Differentiation of BMSCs under Coupling of Pressure and Platelet-Rich Fibrin. **Tissue Eng. Regen. Med.** 19(4): 823-837.

Chong, P. P., Selvaratnam, L., Abbas, A. A. & Kamarul, T. (2012). Human peripheral blood derived mesenchymal stem cells demonstrate similar characteristics and chondrogenic differentiation potential to bone marrow derived mesenchymal stem cells. **J. Orthop. Res.** 30(4): 634-642.

Chu, C. R., Szczodry, M., & Bruno, S. (2010). Animal models for cartilage regeneration and repair. **Tissue Eng. Part B Rev.** 16(1): 105-115.

Cleary, M. A., van Osch, G. J., & Brama, P. A. (2015). FGF, TGF-β and Wnt crosstalk: embryonic to *in vitro* cartilage development from mesenchymal stem cells. **J. Tissue Eng. Regen.** 9: 332-342.

Coleman, C. M. & Tuan, R. S. (2003). Growth/differentiation factor 5 enhances chondrocyte maturation. **Dev. Dyn.** 228: 208-216.

Cong, B., Sun, T., Zhao, Y., & Chen, M. (2023). Current and Novel Therapeutics for Articular Cartilage Repair and Regeneration. **Ther. Clin. Risk Manag.** 19: 485-502.

Cook, J. L., Hung, C. T., Kuroki, K., Stoker, A. M., Cook, C. R., Pfeiffer, F. M., Sherman, S. L., & Stannard, J. P. (2014). Animal models of cartilage repair. **Bone Joint Res.** 3(4): 89-94.

Copp, G., Robb, K. P. & Viswanathan, S. (2023). Culture-expanded mesenchymal stromal cell therapy: does it work in knee osteoarthritis? A pathway to clinical success. **Cell Mol. Immunol.** 20: 626-650.

Correa, D., Somoza, R. A., & Lin, P. (2015). Sequential exposure to fibroblast growth factors (FGF) 2, 9 and 18 enhances hMSC chondrogenic differentiation. **Osteoarthritis Cartilage.** 23: 443-453.

Correia, C. D., Ferreira, A., Fernandes, M. T., Silva, B. M., Esteves, F., Leitão, H. S., Bragança, J., & Calado, S. M. (2023). Human Stem Cells for Cardiac Disease Modeling and Preclinical and Clinical Applications-Are We on the Road to Success? **Cells.** 12(13): 1727.

Cortes-Medina, M., Bushman, A. R., Beshay, P. E., Adorno, J. J., Menyhert, M. M., Hildebrand, R. M., Agarwal, S. S., Avendano, A., & Song, J. W. (2023). Chondroitin sulfate, dermatan sulfate, and hyaluronic acid differentially modify the biophysical properties of collagen-based hydrogels. **bioRxiv.** 541626.

Chubinskaya, S., &Kuettner, K. E. (2003). Regulation of osteogenic proteins by chondrocytes. **Int. J. Biochem. Cell Biol.** 35: 1323-1340.

Davies, J. E., Walker, J. T., & Keating, A. (2017). Concise Review: Wharton's Jelly: The Rich, but Enigmatic, Source of Mesenchymal Stromal Cells. **Stem Cells Transl. Med.** 6(7): 1620-1630.

de Crombrugghe, B., Lefebvre, V., Behringer, R., Bi, W., Murakami, S., & Huang, W. (2000). Transcriptional mechanisms of chondrocyte differentiation. **Matrix Biol.** 19: 389-394.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., & Horwitz, E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. **Cytotherapy.** 8: 315-317.

Drobiova, H., Sindhu, S., Ahmad, R., Haddad, D., Al-Mulla, F., & Al Madhoun, A. (2023). Wharton's jelly mesenchymal stem cells: a concise review of their secretome and prospective clinical applications. **Cell Dev. Biol.** 11: 1211217.

Ebihara, G., Sato, M., Yamato, M., Mitani, G., Kutsuna, T., Nagai, T., Ito, S., Ukai, T., Kobayashi, M., & Kokubo, M. (2012). Cartilage repair in transplanted scaffold-free chondrocyte sheets using a minipig model. **Biomaterials.** 33: 3846–3851.

Fernandez, M. L., Vergara-Jimenez, M., Conde, K., Behr, T., & Abdel-Fattah, G. (1997). Regulation of apolipoprotein B-containing lipoproteins by dietary soluble fiber in guinea pigs. **Am. J. Clin. Nutr.** 65(3): 814-822.

Frech, T. M., & Clegg, D. O. (2007). The utility of nutraceuticals in the treatment of osteoarthritis. **Curr. Rheumatol. Rep.** 9(1): 25-30.

Friedenstein, A. J., Chailakhjan, R. K., & Lalykina, K. S. (1970). The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. **Cell Tissue Kinet.** 3: 393–403.

Friedenstein, A. J., Gorskaja, J. F., & Kulagina, N. N. (1976). Fibroblast precursors in normal and irradiated mouse hematopoietic organs. **Exp. Hematol.** 4(5): 267-274.

Fujii, Y., Liu, L., Yagasaki, L., Inotsume, M., Chiba, T., & Asahara, H. (2022). Cartilage Homeostasis and Osteoarthritis. **Int. J. Mol. Sci.** 23(11): 6316.

Fujita, T., Azuma, Y., & Fukuyama, R. (2004). Runx2 induces osteoblast and chondrocyte differentiation and enhances their migration by coupling with PI3K-Akt signaling. **J. Cell Biol.** 166: 85-95.

Gentili, C., & Cancedda, R. (2009). Cartilage and bone extracellular matrix. **Curr. Pharm. Des.** 15 (12): 1334-1348.

Goldring, M., Tsuchimochi, K., & Ijiri, K. (2006). The control of chondrogenesis. **J. Cell Biochem.** 97: 33-44.

Goldring, M. B. (2007). Update on the Chondrocyte Lineage and Implications for Cell Therapy in Osteoarthritis. In Sharma, L., & Berenbaum, F. (eds). *Osteoarthritis:*

A companion to rheumatology. (1st Ed, pp. 53-76) Philadelphia: Mosby, Inc., an affiliate of Elsevier, Inc.

Goldring, M. B., & Goldring, S. R. (2007). Osteoarthritis. **J. Cell Physiol.** 213: 626-634.

Goldring, M. B., & Marcu, K. B. (2009). Cartilage homeostasis in health and rheumatic diseases. **Arthritis Res. Ther.** 11:224.

Goldring, M. B. (2012). Chondrogenesis, chondrocyte differentiation, and articular cartilage metabolism in health and osteoarthritis. **Ther. Adv. Musculoskelet. Dis.** 4(4): 269-285.

Green, J. D., Tollemar, V., Dougherty, M., Yan, Z., Yin, L., Ye, J., Collier, Z., Mohammed, M. K., Haydon, R. C., Luu, H. H., Kang, R., Lee, M. J., Ho, S. H., He, T. C., Shi, L. L., & Athiviraham, A. (2015). Multifaceted signaling regulators of chondrogenesis: Implications in cartilage regeneration and tissue engineering. **Genes Dis.** 2(4): 307-327.

Green, K. A., & Lund, L. R. (2005). ECM degrading proteases and tissue remodelling in the mammary gland. **Bioessays.** 27: 894–903.

Gruber, H. E., Deepe, R., Hoelscher, G. L., Ingram, J. A., Norton, H. J., Scannell, B., Loeffler, B. J., Zinchenko, N., Hanley, E. N., & Tapp, H. (2010). Human adipose-derived mesenchymal stem cells: direction to a phenotype sharing similarities with the disc, gene expression profiling, and coculture with human annulus cells. **Tissue Eng. Part A.** 16: 2843-2860.

Gründer, T., Gaissmaier, C., & Fritz, J. (2004). Bone morphogenetic protein (BMP)-2 enhances the expression of type II collagen and aggrecan in chondrocytes embedded in alginate beads. **Osteoarthritis Cartilage.** 12: 559-567.

Guadix, J. A., Zugaza, J. L., & Gálvez-Martin, P. (2017). Characteristics, applications and prospects of mesenchymal stem cells in cell therapy. **Med. Clin. (English).** 148(9): 408-414.

Guillot, P.V., Gotherstrom, C., Chan, J., Kurata, H. & Fisk, N. M. (2007). Human first-trimester fetal MSC express pluripotency markers and grow faster and have longer telomeres than adult MSC. **Stem Cells.** 25(3): 646-654.

Guo, P., Shi, Z-L., Liu, A., Lin, T., Bi, F., Shi, M., & Yan, S. G. (2014). Effects of cartilage oligomeric matrix protein on bone morphogenetic protein-2-induced differentiation of mesenchymal stem cells. **Orthop. Surg.** 6(4): 280-287.

Guo, M., Shen, J., & Kwak, J. H. (2015). A novel role of Cyclophilin A in regulation of chondrogenic commitment and endochondral ossification. **Mol. Cell Biol.**

Handorf, A., & Li, W-J. (2011). Fibroblast Growth Factor-2 Primes Human Mesenchymal Stem Cells for Enhanced Chondrogenesis. **PloS one.** 6(7): e22887.

Hartmann, C., & Tabin, C. J. (2000). Dual roles of Wnt signaling during chondrogenesis in the chicken limb. **Development.** 127: 3141-3159.

Hartmann, C. A. (2006). Wnt canon orchestrating osteoblastogenesis. **Trends Cell Biol**. 16: 151-158.

Hartmann, C. (2007). Skeletal Development - Wnts Are in Control. **Mol. Cells.** 24(2): 177-184.

Hass, R., Kasper, C., Bohm, S. & Jacobs, R. (2011). Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC. **Cell Commun. Signal.** 9: 8-12.

Hata, K., Takashima, R., & Amano, K. (2013). Arid5b facilitates chondrogenesis by recruiting the histone demethylase Phf2 to Sox9-regulated genes. **Nat. Commun.** 4: 2850.

Hattori, T., Müller, C., Gebhard, S., Bauer, E., Pausch, F., Schlund, B., Bösl, M. R., Hess, A., Surmann-Schmitt, C., von der Mark, H., de Crombrugghe, B., & von der Mark, K. (2010). SOX9 is a major negative regulator of cartilage vascularization, bone marrow formation and endochondral ossification. **Development.** 137(6): 901-911.

He, Y., Li, Z., Alexander, P. G., Ocasio-Nieves, B. D., Yocum, L., Lin, H., & Tuan, R. S. (2020). Pathogenesis of Osteoarthritis: Risk Factors, Regulatory Pathways in Chondrocytes, and Experimental Models. **Biology.** 9: 194.

Heijink, A., Gomoll, A. H., & Madry, H. (2012). Biomechanical considerations in the pathogenesis of osteoarthritis of the knee, Knee Surgery, Sports Traumatology. **Arthroscopy.** 20: 423-435.

Heinegard, D., & Saxne, T. (2011). The Role of the Cartilage Matrix in Osteoarthritis. **Nat. Rev. Rheumatol.** 7 (1): 50–56.

Hellingman, C. A., Koevoet, W., & Kops, N. (2010). Fibroblast growth factor receptors in *in vitro* and *in vivo* chondrogenesis: relating tissue engineering using adult

mesenchymal stem cells to embryonic development. **Tissue Eng. Part A.** 16: 545–556.

Hidaka, C., & Goldring, M. B. (2008). Regulatory mechanisms of chondrogenesis and implications for understanding articular cartilage homeostasis. **Curr. Rheumatol. Rev.** 4: 136-147.

Hino, K., Saito, A., & Kido, M. (2014). Master regulator for chondrogenesis, Sox9, regulates transcriptional activation of the endoplasmic reticulum stress transducer BBF2H7/CREB3L2 in chondrocytes. **J. Biol. Chem.** 289: 13810-13820.

Hinoi, E., Bialek, P., Chen, Y. T., Rached, M. T., Groner, Y., Behringer, R. R., Ornitz, D. M., & Karsenty, G. (2006). Runx2 inhibits chondrocyte proliferation and hypertrophy through its expression in the perichondrium. **Genes Dev.** 20(21): 2937-42.

Hu, L., Chen, W., Qian, A., & Li, Y-P. (2024). Wnt/ß-catenin signaling components and mechanisms in bone formation, homeostasis, and disease. **Bone Res.** 12: 39.

Hunter, D. J., & Felson, D. T. (2006). Osteoarthritis. **BMJ.** 332(7542): 639-642.

In't Anker, P. S., Scherjon, S. A., Kleijburg-van der Keur, C., de Groot-Swings, G. M., Claas, F. H., Fibbe, W. E. & Kanhai, H. H. (2004). Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. **Stem Cells.** 22(7): 1338-1345.

Ishige, I., Nagamura-Inoue, T., Honda, M. J., Harnprasopwat, R., Kido, M., Sugimoto, M., Nakauchi, H., & Tojo, A. (2009). Comparison of mesenchymal stem cells derived from arterial, venous, and Wharton's jelly explants of human umbilical cord. **Int. J. Hematol.** 90: 261-269.

Ito, T., Sawada, R., Fujiwara, Y., & Tsuchiya, T. (2008). FGF-2 increases osteogenic and chondrogenic differentiation potentials of human mesenchymal stem cells by inactivation of TGF-beta signaling. **Cytotechnology.** 56: 1-7.

Johnstone, B., Hering, T. M., & Caplan, A. I. (1998). *In vitro* chondrogenesis of bone marrow-derived mesenchymal progenitor cells. **Exp. Cell Res.** 238: 265-272.

Juhász, T., Matta, C., & Somogyi, C. (2014). Mechanical loading stimulates chondrogenesis via the PKA/CREB-Sox9 and PP2A pathways in chicken micromass cultures. **Cell. Signal**. 26: 468-482**.**

Kamal, M. M., & Kassem, D. H. (2020). Therapeutic Potential of Wharton's Jelly Mesenchymal Stem Cells for Diabetes: Achievements and Challenges. **Front. Cell Dev. Biol.** 8: 16.

Kamel, G., Hoyos, T., & Rochard, L. (2013). Requirements for frzb and fzd7a in cranial neural crest convergence and extension mechanisms during zebrafish palate and jaw morphogenesis. **Dev. Biol.** 381: 423-433.

Karamboulas, K., Dranse, H. J., & Underhill, T. M. (2010). Regulation of BMP-dependent chondrogenesis in early limb mesenchyme by TGF-ß signals. **J. Cell Sci.** 123: 2068-2076.

Kawato, Y., Hirao, M., Ebina, K., Tamai, N., Shi, K., Hashimoto, J., Yoshikawa, H., & Myoui, A. (2011). Nkx3.2-induced suppression of Runx2 is a crucial mediator of hypoxia-dependent maintenance of chondrocyte phenotypes. **Res. Commun.** 416(1-2): 205-210.

Kern, S., Eichler, H., Stoeve, J., Kluter, H. & Bieback, K. (2006). Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. **Stem Cells.** 24(5): 1294-1301.

Kolasinski, S. L., Neogi, T., Hochberg, M. C., Oatis, C., Guyatt, G., Block, J., Callahan, L., Copenhaver, C., Dodge, C., & Felson, D. (2020). American college of rheumatology/arthritis foundation guideline for the management of osteoarthritis of the hand, hip, and knee. **Arthritis Rheumatol.** 72: 220–233.

Komori, T. (2018). Runx2, an inducer of osteoblast and chondrocyte differentiation. **Histochem. Cell Biol.** 49: 313-323.

Komori, T. (2022). Whole Aspect of Runx2 Functions in Skeletal Development. **Int. J. Mol. Sci.** 23(10): 5776.

Komori, T. (2024). Regulation of Skeletal Development and Maintenance by Runx2 and Sp7. **Int. J. Mol. Sci.** 25(18): 10102.

Kozhemyakina, E., Lassar, A. B., & Zelzer, E. (2015). A pathway to bone: signaling molecules and transcription factors involved in chondrocyte development and maturation, **Development**. 142(5): 817-831.

Kraus, V. B., Huebner, J. L., DeGroot, J., & Bendele, A. (2010). The OARSI histopathology initiative - recommendations for histological assessments of

osteoarthritis in the guinea pig. **Osteoarthritis Cartilage.** 18 Suppl 3(Suppl 3): S35-52.

Kuyinu, E. L., Narayanan, G., Nair, L. S., & Laurencin, C. T. (2016). Animal models of osteoarthritis: classification, update, and measurement of outcomes. **J. Orthop. Surg. Res.** 11: 19.

La Gatta, A., Stellavato, A., Vassallo, V., Di Meo, C., Toro, G., Iolascon, G., & Schiraldi, C. (2021). Hyaluronan and derivatives: an *in vitro* multilevel assessment of their potential in viscosupplementation. **Polymers.** 13: 3208.

Lee, O. K., Kuo, T. K., Chen, W. M., Lee, K. D., Hsieh, S. L., & Chen, T. H. (2004). Isolation of multipotent mesenchymal stem cells from umbilical cord blood. **Blood.** 103: 1669-1675.

Lee, J. M., Kim, J. Y., & Cho, K. W. (2008). Wnt11/Fgfr1b cross-talk modulates the fate of cells in palate development. **Dev. Biol.** 314: 341-350.

Lefebvre, V., & Dvir-Ginzberg, M. (2016). SOX9 and the many facets of its regulation in the chondrocyte lineage. **Connect. Tissue Res.**

Leijten, J. C., Emons, J., & Sticht, C. (2012), Gremlin 1, frizzled-related protein, and Dkk-1 are key regulators of human articular cartilage homeostasis. **Arthritis Rheum.** 64: 3302-3312.

Lengner, C. J., Hassan, M. Q., & Serra, R. W. (2005). Nkx3.2-mediated repression of Runx2 promotes chondrogenic differentiation. **J. Biol. Chem.** 280: 15872-15879.

Leung, V., Gao, B., Leung, K., Melhado, I., Wynn, S., Au, T., Dung, N., Lau, J., Mak, A., Chan, D., & Cheah, K. S. E. (2011). SOX9 governs differentiation stage-specific gene expression in growth plate chondrocytes via direct concomitant transactivation and repression. **PLoS Genet.** 7: e1002356.

Li, F., Lu, Y., Ding, M., Napierala, D., Abbassi, S., Chen, Y., Duan, X., Wang, S., Lee, B., & Zheng, Q. (2011). Runx2 contributes to murine Col10a1 gene regulation through direct interaction with its cis-enhancer. **J. Bone Miner. Res.** 26: 2899-2910.

Liao, J., Hu, N., & Zhou, N. (2014). Sox9 potentiates BMP2-induced chondrogenic differentiation and inhibits BMP2-induced osteogenic differentiation. **PLoS One.** 9: e89025.

Liau, L. L., Ruszymah, B. H. I., Ng, M. H., & Law, J. X. (2019). Characteristics and clinical applications of Wharton's jelly-derived mesenchymal stromal cells. **Curr. Res. Transl. Med.** 68(1): 5-16.

Lim, S. K., & Khoo, B. Y. (2021). An overview of mesenchymal stem cells and their potential therapeutic benefits in cancer therapy. **Oncol. Lett.** 22(5): 785.

Lin, W., Huang, L., Li, Y., Fang, B., Li, G., Chen, L., & Xu, L. (2019). Mesenchymal Stem Cells and Cancer: Clinical Challenges and Opportunities. **Biomed Res. Int.** 2019: 2820853.

Liu, C. F., Samsa, W. E., Zhou, G., & Lefebvre, V. (2017). Transcriptional control of chondrocyte specification and differentiation. **Semin. Cell Dev. Biol.** 62: 34-49.

Liu, J., Xiao, Q., Xiao, J. Niu, C., Li, Y., Zhang, X., Zhou, Z., Shu, G., & Yin, G. (2022). Wnt/ β-catenin signalling: function, biological mechanisms, and therapeutic opportunities. **Signal Transduct. Target Ther.** 7: 3.

Loh, C-Y., Chai, J. Y., Tang, T. F., Wong, W. F., Sethi, G., Shanmugam, M. K., Chong, P. P., & Looi, C. Y. (2019). The E-Cadherin and N-Cadherin Switch in Epithelial-to-Mesenchymal Transition: Signaling, Therapeutic Implications, and Challenges. **Cells**. 8(10): 1118.

Long, F., & Ornitz, D. M. (2013). Development of the endochondral skeleton. **Cold Spring Harb. Perspect Biol.** 5: a008334.

Lu, P., Takai, K., Weaver, V. M., & Werb, Z. (2011). Extracellular matrix degradation and remodeling in development and disease. **Cold Spring Harb. Perspect Biol.** 3(12): a005058.

Luo, K. (2017). Signaling Cross Talk between TGF-ß/Smad and Other Signaling Pathways. **Cold Spring Harb. Perspect Biol.** 9(1): a022137.

Lv, Z., Cai, X., Bian, Y., Wei, Z., Zhu, W., Zhao, X., & Weng, X. (2023). Advances in Mesenchymal Stem Cell Therapy for Osteoarthritis: From Preclinical and Clinical Perspectives. **Bioengineering (Basel).** 10(2): 195.

Maiese, K., Li, F., Chong, Z. Z., & Shang, Y. C. (2008). The Wnt signaling pathway: Aging gracefully as a protectionist? **Pharmacol. Ther.** 118(1): 58-81.

Maldonado, V. V., Patel, N. H., Smith, E. E., Barners, C. L., Gustafson, M. P., Rao, R. R., & Samsonraj, R. M. (2023). Clinical utility of mesenchymal stem/stromal cells in regenerative medicine and cellular therapy. **J. Biol. Eng.** 17: 44.

Man, G. S., & Mologhianu, G. (2014). Osteoarthritis pathogenesis – a complex process that involves the entire joint. **J. Med. Life.** 7(1): 37-41.

Margiana, R., Markov, A., Zekiy, A.O., Hamza, M-U., Al-Dabbagh, K. A., Al-Zubaidi, S. H., Hameed, N. M., Ahmad, I., Sivaraman, R., Kzar, H. H., Al-Gazally, M. E., Mustafa, Y-F., & Siahmansouri, H. (2022). Clinical application of mesenchymal stem cell in regenerative medicine: a narrative review. **Stem Cell Res. Ther.** 13: 366.

Mariani, E., Pulsatelli, L., & Facchini, A. (2014). Signaling Pathways in Cartilage Repair. **Int. J. Mol. Sci.** 15(5): 8667-8698.

Marino, L., Castaldi, M. A., Rosamilio, R., Ragni, E., Vitolo, R., Fulgione, C., Castaldi, S. G., Serio, B., Bianco, R., Guida, M., & Selleri. C. (2019). Mesenchymal Stem Cells from the Wharton's Jelly of the Human Umbilical Cord: Biological Properties and Therapeutic Potential. **Int. J. Stem Cells.** 12(2): 218-226.

Mattei, V., & Delle Monache, S. (2024). Mesenchymal Stem Cells and Their Role in Neurodegenerative Diseases. **Cells.** 13(9): 779.

McNulty, A. L., & Guilak, F. (2015). Mechanobiology of the Meniscus. **J. Biomech.** 48 (8).

Mebarki, M., Abadie, C., Larghero, J., & Cras, A. (2021). Human umbilical cord-derived mesenchymal stem/stromal cells: a promising candidate for the development of advanced therapy medicinal products. **Stem Cell Res. Ther.** 12: 152.

Michelacci, Y. M., Baccarin, R. Y. A., & Rodrigues, N. N. P. (2023). Chondrocyte Homeostasis and Differentiation: Transcriptional Control and Signaling in Healthy and Osteoarthritic Conditions. **Life (Basel).** 13(7): 1460.

Mishra, s., Sevak, J. K., Das, A., Arimbasseri, G. A., Bhatnagar, S., & Gopinath, S. D. (2020). Umbilical cord tissue is a robust source for mesenchymal stem cells with enhanced myogenic differentiation potential compared to cord blood. **Sci. Rep.** 10: 18978.

Mori-Akiyama, Y., Akiyama, H., Rowitch, D. H., de & Crombrugghe, B. (2003). Sox9 is required for determination of the chondrogenic cell lineage in the cranial neural crest. **Proc. Natl. Acad. Sci. U. S. A.** 100: 9360-9365.

Morrison, S. J., Shah, N. M., & Anderson, D. J. (1997). Regulatory mechanisms in stem cell biology. **Cell.** 88: 287-298.

Mow, V. C., Yong GU, W., & Hui Chen, F. (2005). Structure and function of articular carilage and meniscus. In Mow, V.C., & Huiskers, R. (eds). *Basic Orthopaedic Biomechanics and Mechano-Biology*. (3rd Ed, pp. 181-258). Philadelphia: Lippincot Williams & Wilkins.

Musial-Wysocka, A., Kot, M., & Majka, M. (2019). The Pros and Cons of Mesenchymal Stem Cell-Based Therapies. **Cell Transplant.** 28(7): 801-812.

Naba, A., Clauser, K. R., Ding, H., Whittaker, C. A., Carr, S. A., & Hynes, R. O. (2016). The extracellular matrix: Tools and insights for the “omics” era. **Matrix Biol.** 49:10-24.

Nagata, K., Hojo, H., Chang, S. H. Okada, H., Yano, F., Chijimatsu, R., Omata, Y., Mori, D., Makii, Y., Kawata, M., Kaneko, T., Iwanaga, Y., Nakamoto, H., Maenohara, Y., Tachibana, N., Ishikura, H., Higuchi, J., Taniguchi, Y., Ohba, S., Chung, U-I., Tanaka, S., & Saito, T. (2022). Runx2 and Runx3 differentially regulate articular chondrocytes during surgically induced osteoarthritis development. **Nat. Commun.** 13: 6187.

Nahian, A., & Sapra, A. (2023). Histology, Chondrocytes. (NBK557576) Island: StatPearls Publishing LLC.

Nimeskern, L., Pleumeekers, M. M., Pawson, D. J., Koevoet, W. L. M., Lehtoviita, I., Soyka, M. B., Roosli, C., Holzmann, D., van Osch, G. J. V. M., Muller, R., & Stok, K. S. (2015). Mechanical and Biochemical Mapping of Human Auricular Cartilage for Reliable Assessment of Tissue-Engineered Constructs. **J. Biomech.** 48 (10), 1721–1729.

Oh, C. D., Chang, S. H., & Yoon, Y. M. (2000). Opposing role of mitogen-activated protein kinase subtypes, erk-1/2 and p38, in the regulation of chondrogenesis of mesenchymes. **J. Biol. Chem.** 275: 5613-5619.

Ohba, S., He, X., Hojo, H., & McMahon, A. P. (2015). Distinct Transcriptional Programs Underlie Sox9 Regulation of the Mammalian Chondrocyte. **Cell Rep.** 12(2): 229-243.

Ornitz, D. M., & Marie, P. J. (2015). Fibroblast growth factor signaling in skeletal development and disease. **Genes Dev.** 29(14): 1463-86.

Oseni, A. O., Crowley, C., Boland, M. Z., Bulter, P. E., & Seifalian, A. M. (2011). Cartilage tissue engineering: the application of nanomaterials and stem cell technology. **Tissue Eng. Regen. Med.** 233-266.

O'Shea, D. G., Curtin, C. M., & O'Brien, F. J. (2022). Articulation inspired by nature: A review of biomimetic and biologically active 3D printed scaffolds for cartilage tissue engineering. **Biomater. Sci.** 10: 2462-2483.

Pan, Q., Yu, Y., & Chen, Q. (2008). Sox9, a key transcription factor of bone morphogenetic protein-2-induced chondrogenesis, is activated through BMP pathway and a CCAAT box in the proximal promoter. **J. Cell Physiol.** 217: 228-241.

Pelusi, L., Schiavone, V., Bologna, G., Cerritelli, P., Lezza, D., Pantalone, A., & Mandatori, D. (2021) Chondrogenic Differentiation of Human Wharton's Jelly and Bone Marrow-Derived Mesenchymal Stem Cells: Focus on the Role of an Acrylamide-Free Cross-Linked Hyaluronic Acid Hydrogel. **J. Orthop. Res. Ther.** 6: 1209.

Perry, B. C., Zhou, D., Wu, X., Yang, F. C., Byers, M. A., Chu, T. M., Hockema, J. J., Woods, E. J. & Goebel, W. S. (2008). Collection, cryopreservation, and characterization of human dental pulp-derived mesenchymal stem cells for banking and clinical use. **Tissue Eng. Part C: Methods.** 14(2): 149-156.

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S., & Marshak, D. R. (1999). Multilineage potential of adult human mesenchymal stem cells. **Science.** 284: 143-147.

Pittenger, M. F., Discher, D. E., Péault, B. M., Phinney, D. G., Hare, J. M., & Caplan, A. I. (2019). Mesenchymal stem cell perspective: cell biology to clinical progress. **Regen. Med.** 4: 22.

Ponnaiyan, D., Bhat, K. M., & Bhat, G. S. (2012). Comparison of immuno-phenotypes of stem cells from human dental pulp and periodontal ligament. **Int. J. Immunopathol. Pharmacol.** 25: 127-134.

Qin, K., Yu, M., Fan, J., Wang, H., Zhao, P., Zhao, G., Zeng, W., Chen, C., Wang, Y., Wang, A., Schwartz, Z., Hong, J., Song, L., Wagstaff, W., Haydon, R. C., Luu, H. H., Ho, S. H., Strelzow, J., Reid, R. R., He, T. C., & Shi, L. L. (2023). Canonical

and noncanonical Wnt signaling: Multilayered mediators, signaling mechanisms and major signaling crosstalk. **Genes Dis.** 11(1): 103-134.

Quintana, L., Zur Nieden, N. I., & Semino, C. E. (2009). Morphogenetic and regulatory mechanisms during developmental chondrogenesis: new paradigms for cartilage tissue engineering. **Tissue Eng. B.** 15 :29-41.

Rainbow, R. S., Won, H. K., & Zeng, L. (2014). The role of Nkx3.2 in chondrogenesis. **Front. Biol. (Beijing).** 9(5): 376-381.

Rashid, H., Chen, H., & Javed, A. (2021). Runx2 is required for hypertrophic chondrocyte mediated degradation of cartilage matrix during endochondral ossification. **Matrix Biol. Plus.** 12: 100088.

Rashid, H., Smith, C. M., Convers, V., Clark, K., & Javed, A. (2024). Runx2 deletion in hypertrophic chondrocytes impairs osteoclast mediated bone resorption. **Bone**. 181: 117014.

Rebelatto, C. K., Aguiar, A. M., Moretao, M. P., Senegaglia, A. C., Hansen, P., Barchiki, F., Oliveira, J., Martins, J., Kuligouski, C., Mansur, F., Christofis, A., Amaral, V. F., Brofman, P. S., Goldenberg, S., Nakao, L. S. & Correa, A. (2008). Dissimilar differentiation of mesenchymal stem cells from bone marrow, umbilical cord blood, and adipose tissue. **Exp. Biol. Med.** 233(7): 901-913.

Reinholz, G. G., Lu, L., Saris, D. B. F., Yaszemski, M. J., & O'Driscoll, S. W. (2004). Animal models for cartilage reconstruction. **Biomaterials.** 25(9): 1511-1521.

Richter, W., Bock, R., Hennig, T., & Weiss, S. (2009). Influence of FGF-2 and PTHrP on chondrogenic differentiation of human mesenchymal stem cells. **J. Bone Joint Surg. Br.** 91-B (suppl III): 444.

Rodríguez-García, A., Samsó, P., Fontova, P., Simon-Molas, H., Manzano, A., Castaño, E., Rosa, J. L., Martinez-Outshoorn, U., Ventura, F., Navarro-Sabaté, À., & Bartrons, R. (2017). TGF- β1 targets Smad, p38 MAPK, and PI3K/Akt signaling pathways to induce PFKFB3 gene expression and glycolysis in glioblastoma cells. **FEBS J.** 284(20): 3437-3454.

Romanov, Y. A., Darevskaya, A. N., Merzlikina, N. V., & Buravkova, B. (2005). Mesenchymal stem cells from human bone marrow and adipose tissue: isolation, characterization and differentiation potentialities. Bulletin **Exp. Biol. Med.** 140: 138-143.

Rosen, D. M., Stempien, S. A., & Thompson, A. Y. (1986). Differentiation of rat mesenchymal cells by cartilage-inducing factor. Enhanced phenotypic expression by dihydrocytochalasin B. **Exp. Cell Res.** 165: 127-138.

Rozemuller, H., Prins, H. J., Naaijkens, B., Staal, J., Buhring, H. J. & Martens, A. C. (2010). Prospective isolation of mesenchymal stem cells from multiple mammalian species using cross-reacting anti-human monoclonal antibodies. **Stem Cells Dev.** 19(12): 1911-1921.

Saeedi, P., Halabian, R., Imani Fooladi. A. A. (2019). A revealing review of mesenchymal stem cells therapy, clinical perspectives and Modification strategies. **Stem Cell Investig.** 6: 34.

Sandberg, M., Autio-Harmainen, H., & Vuorio, E. (1998). Localization of the expression of types I, III, and IV collagen, TGF-beta 1 and c-fos genes in developing human calvarial bones. **Dev. Biol.** 130: 324-334.

Sato, M., Uchida, K., Nakajima, H., Miyazaki, T., Guerrero, A. R., Watanabe, S., Roberts, S., & Baba, H. (2012). Direct transplantation of mesenchymal stem cells into the knee joints of Hartley strain guinea pigs with spontaneous osteoarthritis. **Arthritis Res. Ther.** 14(1): R31.

Shen, Q., Guo, Y., Wang, K., Zhang, C., & Ma, Y. (2023). A Review of Chondroitin Sulfate's Preparation, Properties, Functions, and Applications. **Molecules.** 28(20): 7093.

Shi, S., Wang, C., & Acton, A. J. (2015). Role of Sox9 in growth factor regulation of articular chondrocytes. **J. Cell. Biochem.** 116: 1391-1400.

Shum, L., & Nuckolls, G. (2002). The life cycle of chondrocytes in the developing skeleton. **Arthritis Res.** 4(2): 94-106.

Solchaga, L. A., Penick, K., Porter, J. D., Goldberg, V. M., Caplan, A. I., & Welter, J. F. (2005). FGF-2 enhances the mitotic and chondrogenic potentials of human adult bone marrow-derived mesenchymal stem cells. **J. Cell Physiol.** 203(2): 398-409.

Sophia Fox, A. J., Bedi, A., & Rodeo, S. A. (2009). The basic science of articular cartilage: structure, composition, and function. **Sports Health.** 1 (6): 461-468.

Staff, N. P., Jones, D. T., & Singer, W. (2019). Mesenchymal Stromal Cell Therapies for Neurodegenerative Diseases. **Mayo Clin. Proc.** 94(5): 892-905.

Stevens, M. M., Marini, R. P., & Martin, I. (2004). FGF-2 enhances TGF- β1-induced periosteal chondrogenesis. **J. Orthop. Res.** 25: 1114-1119.

Stricker, S., Fundele, R., Vortkamp, A., & Mundlos, S. (2002). Role of Runx genes in chondrocyte differentiation. **Dev. Biol.** 245(1): 95-108.

Suthon, S., Perkins, R. S., Bryja, V., Miranda-Carboni, G. A., & Krum, S. A. (2021). WNT5B in Physiology and Disease. **Front. Cell Dev. Biol.** 9: 667581.

Taghizadeh, R. R., Cetrulo, K. J., & Cetrulo, C. L. (2011). Wharton's Jelly stem cells: Future clinical applications. **Placenta.** 32(4): S311-S315.

Takeda, S., Bonnamy, J-P., Owen, M.J., Ducy, P., & Karsenty, G. (2001). Continuous expression of Cbfa1 in nonhypertrophic chondrocytes uncovers its ability to induce hypertrophic chondrocyte differentiation and partially rescues Cbfa1-deficient mice. **Genes Dev.** 15(4): 467-481.

Talens-Visconti, R., Bonora, A., Jover, R., Mirabet, V., Carbonell, F., Castell, J. V. & Go'mez-Lecho'n, M. J. (2006). Hepatogenic differentiation of human mesenchymal stem cells from adipose tissue in comparison with bone marrow mesenchymal stem cells. **World J. Gastroenterol.** 12(36): 5834-5845.

Teng, Y., Li, X., Chen, Y., Cai, H., Cao, W., Chen, X., Sun, Y., Liang, J., Fan, Y., & Zhang, X. (2017). Extracellular matrix powder from cultured cartilage-like tissue as cell carrier for cartilage repair. **J. Mater. Chem. B.** 5(18): 3283-3292.

Tessier, J. J., Bowyer, J., Brownrigg, N. J., Peers, I. S., Westwood, F. R., Waterton, J. C., & Maciewicz, R. A. (2003). Characterisation of the guinea pig model of osteoarthritis by *in vivo* three-dimensional magnetic resonance imaging. **Osteoarthritis Cartilage.** 11(12): 845-853.

Timothy, F. D., Xizhi, G., Lisa, G-B., & Yingzi, Y. (2005). Wnt/β-Catenin Signaling in Mesenchymal Progenitors Controls Osteoblast and Chondrocyte Differentiation during Vertebrate Skeletogenesis. **Dev. Cell.** 8(5): 739-750.

Tivig, I., Vallet, L., Moisescu, M. G., Fernandes, R., Andre, F. M., Mir, L. M., & Savapol, T. (2024). Early differentiation of mesenchymal stem cells is reflected in their dielectrophoretic behavior. **Sci. Rep.** 14: 4330.

Tondreau, T., Meuleman, N., Delforge, A., Dejeneffe, M., Leroy, R., Massy, M., Mortier, C., Bron, D., & Lagneaux, L. (2005). Mesenchymal stem cells derived from

CD133-positive cells in mobilized peripheral blood and cord blood: proliferation, Oct4 expression, and plasticity. **Stem Cells.** 23: 1105-1112.

Topol, L., Chen, W., Song, H., Day, T. F., & Yang, Y. (2009). Sox9 inhibits Wnt signaling by promoting β-catenin phosphorylation in the nucleus. **J. Biol. Chem.** 284(5): 3323-3333.

Troeberg, L., & Nagase, H. (2012). Proteases involved in cartilage matrix degradation in osteoarthritis. **Biochim. Biophys. Acta.** 1824(1): 133-45.

Troyer, D. L., & Weiss, M. L. (2008). Wharton's jelly-derived cells are a primitive stromal cell population. **Stem Cells.** 26(3): 591-599.

Tsai, M. S., Lee, J. L., Chang, Y. J. & Hwang, S. M. (2004). Isolation of human multipotent mesenchymal stem cells from second-trimester amniotic fluid using a novel two-stage culture protocol. **Hum. Reprod.** 19(6): 1450-1456.

Tufan, A. C., & Tuan, R. S. (2001). Wnt regulation of limb mesenchymal chondrogenesis is accompanied by altered N-cadherin-related functions. **FASEB. J.** 15: 1436-1438.

Tufan, A. C., Daumer, K. M., DeLise, A. M., & Tuan, R. S. (2002). AP-1 transcription factor complex is a target of signals from both WnT-7a and N-cadherin-dependent cell-cell adhesion complex during the regulation of limb mesenchymal chondrogenesis. **Exp. Cell Res.** 273: 197-203.

Tuli, R., Tuli, S., & Nandi, S. (2003). Transforming growth factor-beta-mediated chondrogenesis of human mesenchymal progenitor cells involves N-cadherin and mitogen-activated protein kinase and Wnt signaling cross-talk. **J. Biol. Chem.** 278: 41227-41236.

Urbani, S., Caporale, R., Lombardini, L., Bosi, A., & Saccardi, R. (2006). Use of CFDA-SE for evaluating the *in vitro* proliferation pattern of human mesenchymal stem cells. **Cytotherapy.** 8: 243 253.

Ushita, M., Saito, T., & Ikeda, T. (2009). Transcriptional induction of SOX9 by NF-kappaB family member RelA in chondrogenic cells. **Osteoarthritis Cartilage.** 17: 1065-1075.

Vasanthan, J., Gurusamy, N., Rajasingh, S., Sigamani, V., Kirankumar, S., Thomas, E. L., & Rajasingh. J. (2020). Role of Human Mesenchymal Stem Cells in Regenerative Therapy. **Cells.** 10(1): 54.

Veeman, M. T., Axelrod, J. D., & Moon, R. T. (2003). A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. **Dev. Cell.** 5: 367-377.

Vicinanza, C., Lombardi, E., Da Ros, F., Marangon, M., Durante, C., Mazzucato, M., & Agostini, F. (2022). Modified mesenchymal stem cells in cancer therapy: A smart weapon requiring upgrades for wider clinical applications. **World J. Stem Cells.** 14(1): 54-75.

Vinod, E., Padmaja, K., Ramasamy, B., & Sathishkumar, S. (2023). Systematic review of articular cartilage derived chondroprogenitors for cartilage repair in animal models. **J. Orthop.** 35: 43-53.

Vlashi, R., Zhang, X., Wu, M., & Chen, G. (2023). Wnt signaling: Essential roles in osteoblast differentiation, bone metabolism and therapeutic implications for bone and skeletal disorders. **Genes & Diseases.** 10 (4):1291-1317.

Voisin, C., Cauchois, G., Reppel, L., Laroye, C., Louarn, L., Schenowitz, C., Sonon, P., Poras, I., Wang, V., Carosella. E., Benkirane-Jessel, N., Moreau, P., Rouas-Freiss, N., Bensoussan, D., & Huselstein, C. (2020). Are the Immune Properties of Mesenchymal Stem Cells from Wharton's Jelly Maintained during Chondrogenic Differentiation? **J. Clin. Med.** 9(2): 423.

Wang, W-J., Sun, C., Liu, Z., Sun, X., Zhu, F., Zhu, Z. Z., & Qiu, Y. (2014). Transcription factor Runx2 in the low bone mineral density of girls with adolescent idiopathic scoliosis. **Orthop. Surg.** 6(1): 8-14.

Wieland, H. A., Michaelis, M., Kirschbaum, B. J. & Rudolphi, K. A. (2005). Osteoarthritis-an untreatable disease? **Nat. Rev. Drug Discov.** 4: 331-344.

Wise, C. (2010). Osteoarthritis. In Dale, D. C., & Federman, D. D. (eds). *ACP Medicine* (section 15, chap. 10, pp. 1-12.) New York: WebMD.

Witkowska-Zimny, M., & Wrobel, E. (2011). Perinatal sources of mesenchymal stem cells: Wharton's Jelly, amnion and chorion. **Cell Mol. Biol. Lett.** 16(3): 493-514.

Wolock, S. L., Krishnan, I., Tenen, D. E., Matkins, V., Camacho, V., Patel, S., Agarwal, P., Bhatia, R., Tenen, D. G., Klein, A. M., & Welner, R. S. (2019). Mapping Distinct Bone Marrow Niche Populations and Their Differentiation Paths. **Cell Rep.** 28: 302-311.

Wang, M., Wu, Y., Li, G., Lin, Q., Zhang, W., Liu, H., & Su, J. (2024). Articular cartilage repair biomaterials: strategies and applications. **Mater. Today Bio.** 24: 100948.

Wang, R. N., Green, J., Wang, Z., Deng, Y., Qiao, M., Peabody, M., Zhang, Q., Ye, J., Yan, Z., Denduluri, S., Idowu, O., Li, M., Shen, C., Hu, A., Haydon, R. C., Kang, R., Mok, J., Lee, M. J., Luu, H. L., & Shi, L. L. (2014). Bone Morphogenetic Protein (BMP) signaling in development and human diseases. **Genes Dis.** 1(1): 87-105.

Wang, X., Wei, L., Xue, Y., & Li, R-S. (2021). Experimental observation of the sequence of tibial plateau chondrocyte and matrix degeneration in spontaneous osteoarthritis in Guinea pigs. **BMC Musculoskelet. Disord.** 22: 395.

Wang, Y. & Sul, H. S. (2009). Pref-1 regulates mesenchymal cell commitment and differentiation through Sox9. **Cell Metab.** 9: 287-302.

Wong, C-C., Ou, K-L., Lin, Y-H., Lin, M-F., Yang, T-L., Chen, C-H., & Chan, W. P. (2020). Platelet-rich fibrin facilitates one-stage cartilage repair by promoting chondrocytes viability, migration, and matrix synthesis. **Int. J. Mol. Sci.** 21: 577.

Wu, M., Wu, S., Chen, W., & Li, Y-P. (2024). The roles and regulatory mechanisms of TGF-β and BMP signaling in bone and cartilage development, homeostasis and disease. **Cell Res.** 34: 101-123.

Yamashita, S., Miyaki, S., & Kato, Y. (2012). L-Sox5 and Sox6 proteins enhance chondrogenic miR-140 microRNA expression by strengthening dimeric Sox9 activity. **J. Biol. Chem.** 287: 22206-22215.

Yan, J-Y., Tian, F-M., Wang, W-Y., Cheng, Y., Xu, H-F., Song, H-P., Zhang, Y-Z., & Zhang, L. (2014). Age dependent changes in cartilage matrix, subchondral bone mass, and estradiol levels in blood serum, in naturally occurring osteoarthritis in Guinea pigs. **Int. J. Mol. Sci.** 15(8): 13578-13595.

Yang, Y. H. K., Ogando, C. R., Wang See, C., Chang, T-Y., & Barabino, G. A. (2018). Changes in phenotype and differentiation potential of human mesenchymal stem cells aging *in vitro*. **Stem Cell Res. Ther.** 9: 131.

Yang, X., Tian, S., Fan, L. Niu, R, Yan, M., Chen, S., Zheng, M., & Zhang, S. (2022). Integrated regulation of chondrogenic differentiation in mesenchymal stem cells and differentiation of cancer cells. **Cancer Cell Int.** 22: 169.

Yano, F., Kugimiya, F., & Ohba, S. (2005). The canonical Wnt signalling pathway promotes chondrocyte differentiation in a Sox9- dependent manner. **Biochem. Biophys. Res. Commun.** 333: 1300-1308.

Yea, J. H, Kim, Y., & Jo, C. H. (2023). Comparison of mesenchymal stem cells from bone marrow, umbilical cord blood, and umbilical cord tissue in regeneration of a full-thickness tendon defect *in vitro* and *in vivo*. **Biochem. Biophys. Rep.** 34: 101486.

Yoshida, C. A., Yamamoto, H., Fujita, T., Furuichi, T., Ito, K., Inoue, K., Yamana, K., Zanma, A., Takada, K., Ito, Y., & Komori, T. (2004). Runx2 and Runx3 are essential for chondrocyte maturation, and Runx2 regulates limb growth through induction of Indian hedgehog. **Genes Dev.** 18(8): 952-63.

Yoon, B. S., Ovchinnikov, D. A., & Yoshii, I. (2005). Bmpr1a and Bmpr1b have overlapping functions and are essential for chondrogenesis *in vivo*. **Proc. Natl. Acad. Sci. U. S. A.** 102: 5062-5067.

Zhang, L., Hu, J., & Athanasiou, K. A. (2009). The role of tissue engineering in articular cartilage repair and regeneration. **Crit. Rev. Biomed. Eng.** 37: 1-57.

Zhang, Q., Pan, Y., Ji, J., Xu, Y., Zhang, Q., & Qin, L. (2021). Roles and action mechanisms of WNT4 in cell differentiation and human diseases: a review. **Cell Death Discov.** 7: 287.

Zheng, Q., Zhou, G., & Morello, R. (2003). Type X collagen gene regulation by Runx2 contributes directly to its hypertrophic chondrocyte-specific expression *in vivo*. **J. Cell. Biol.** 162: 833-842.

Zhou, G., Zheng, Q., Engin, F., Munivez, E., Chen, Y., Sebald, E., Krakow, D., & Lee, B. (2006). Dominance of SOX9 function over RUNX2 during skeletogenesis. **Proc. Natl. Acad. Sci. U. S. A.** 103(50): 19004-19009.

Zhou, S., Eid, K., & Glowacki, J. (2004). Cooperation between TGF-ß and Wnt pathways during chondrocyte and adipocyte differentiation of human marrow stromal cells. **J. Bone Min. Res.** 19: 463-470.

Zhuang, W. Z., Lin, Y. H., Su, L. J., Su, L-J., Wu, M-S., Jeng, H-Y., Chang, H-C., Huang, Y-H., & Ling, T-Y. (2021). Mesenchymal stem/stromal cell-based therapy: mechanism, systemic safety and biodistribution for precision clinical applications. **J. Biomed. Sci.** 28: 28.

Zuk, P. A., Zhu, M., Mizuno, H., Huang, J., Futrell, J. W., Katz, A. J., Benhaim, P., Lorenz, H. P. & Hedrick, M. H. (2001). Multilineage cells from human adipose tissue: implications for cell-based therapies. **Tissue Eng.** 7(2): 211- 228.

Zuscik, M. J., Hilton, M. J., Zhang, X., Chen, D., & O'Keefe, R. J. (2008). Regulation of chondrogenesis and chondrocyte differentiation by stress. **J. Clin. Invest.** 118(2): 429-38.

CHAPTER III

ESTABLISHMENT OF HUMAN WHARTON'S JELLY MESENCHYMAL STEM CELLS AND INDUCTION TO BE CHONDROCYTES

3.1 Abstract

Human wharton's jelly mesenchymal stem cells (hWJ-MSCs) were successfully isolated from umbilical cords of two donors. Two cell lines of the hWJ-MSCs were obtained and characterized. The analyzed characteristics included cell surface markers, colony forming unit, population doubling time, surface protein expression, and differentiation potencies into three cell lineages (adipogenic, chondrogenic and osteogenic). The results revealed that only one cell line exhibited appropriate mesenchymal stem cells (MSCs) characteristics and it was recruited to do in the next experiments. In the determination of cell surface markers, flow cytometry was used instead of cell staining on culture dish because it can provide quantitative values of the positive cell proportion. hWJ-MSCs were induced to be chondrocytes via the induction of chondrogenic differentiation for 28 days. The obtained chondrocytes were characterized by immunofluorescent staining, qPCR, and immunobloting. In this study, only type II collagen was proposed to be determined in the immunofluorescent staining and immunoblotting. However, Sox9, Aggrecan, and type X collagen were also detected in the immunofluorescent staining, and type X collagen was further determined in the immunoblotting to strongly confirm the results. Moreover, a human cartilage was included in the research for chondrocyte isolation and protein extraction. The human cartilage-derived chondrocytes were used to compare the gene expressions with the hWJ-MSC-derived chondrocytes.

3.2 Introduction

Osteoarthritis (OA) is prevalent among elderly individuals, obese patients, and physically active people who exert significant stress on their knee joints. The disease can be triggered by genetic and non-genetic factors (Jones et al., 2019; Kong et al.,

2017). Initial symptoms involve deterioration of articular cartilage leading to pain, bone dysfunction, and difficulties in performing daily activities. Current OA treatments are commonly determined based on disease severity, and the physician's recommendations including medication, physical therapy, and surgery. However, there is a possibility of recurrence and complications after treatment in many patients. Consequently, a novel and more effective procedure for osteoarthritis is indispensable, like application of cartilage cells. However, extraction of these cells from a human requires invasive surgery, which is complicated and expensive (Ebihara et al., 2012; Wong et al., 2020). Research is currently underway to explore the potential use of MSCs in treating osteoarthritis. MSCs have demonstrated a number of desirable characteristics, including tri-mesodermal differentiation into osteoblasts, chondrocytes, and adipocytes, plastic attachment, self-renewal, and positive expression of unrestricted MSCs markers (Baddoo et al., 2003; Dominici et al., 2006; Ambrosi et al., 2017; Wolock et al., 2019; Azadniv et al., 2020). For numerous of these reasons, MSCs are currently an excellent candidate for therapeutic purpose in clinical applications. MSCs possess the unique ability to stimulate the growth of cartilage cells and other types of cells. MSCs are utilized in treating various disorders and can be sourced from several different locations, including bone marrow, adipose tissue (Romanov et al., 2005; Gruber et al., 2010), blood (Tondreau et al., 2005), dental pulp (Ponnaiyan et al., 2012), umbilical cord blood and umbilical cord tissue. They can be isolated and cultured with a high level of proliferation activity. Previous studies have identified Wharton's jelly, found in the umbilical cord of humans, as a common source of MSCs. This tissue can be collected from pregnant women following childbirth, with no complex collection process required (Troyer & Weiss, 2008; Ishige et al., 2009; Witkowska-Zimny & Wrobel, 2011; Mishra et al., 2020; Mebarki et al., 2021; Yea et al., 2023). As a result, MSCs isolated from the Wharton's jelly of human umbilical cords are a promising area of interest for future clinical trials. In this study, we isolated MSCs from human Wharton's jelly of the umbilical cord and induced them into cartilage cells.

3.3 Materials and Methods

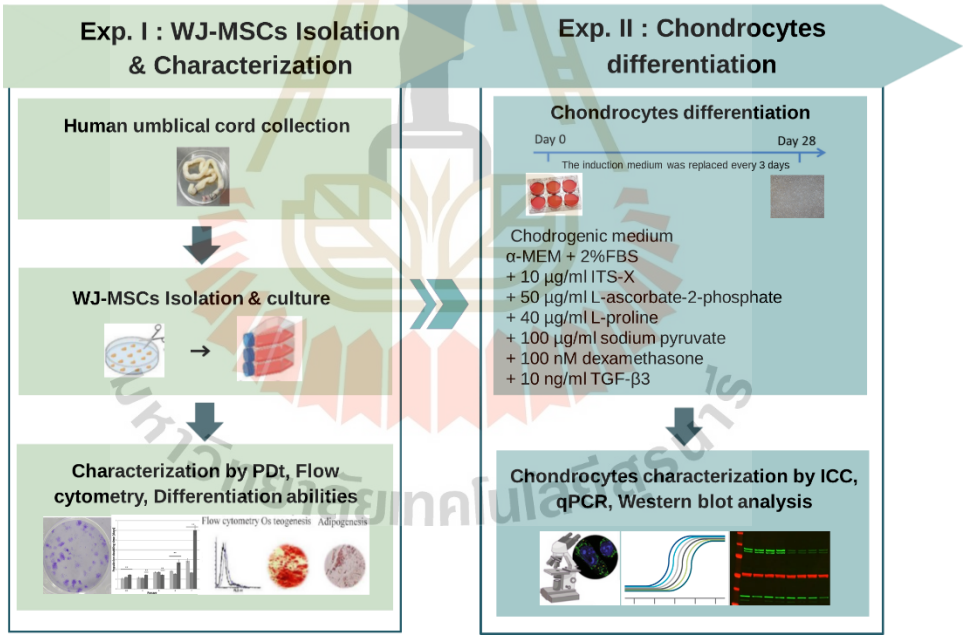
3.3.1 Ethics Statement

In this study, ethical approval was granted from the Ethics Committee for Researches Involving Human Subjects, Suranaree University of Technology (EC-61-56), Nakhon Ratchasima, Thailand.

3.3.2 Reagents

All chemical compounds were purchased from Sigma-Aldrich Corporation (St. Louis, Missouri, USA), antibodies were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The cell culture media and cell culture ware was obtained from Gibco (Paisley, UK) and SPL Life Science (Gyeonggi-do, South Korea), respectively. Unless stated otherwise.

3.3.3 Experimental design



3.3.4 hWJ-MSCs isolation and culture

The human umbilical cords (n=2) were obtained from Maharat Nakhon Ratchasima Hospital (Nakhon Ratchasima, Thailand) with mother's informed consent. hWJ-MSCs were isolated from the umbilical cord and cultured as previously described (Petsa et al., 2009; Tanthaisong et al., 2017). The cords, which were approximately 7-10 cm in length, were washed using phosphate buffered saline (PBS(-)).The

hWJ-MSCs were isolated from the umbilical cord and cultured as previously described by Tanthaisong et al. (2017). Briefly, the gelatinous Wharton's Jelly tissues were collected and sliced into small pieces (2-5 mm^2). These pieces were placed in 90x15 mm culture dishes and grown in alpha modification of Eagle's medium (α-MEM) enriched with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS). The hWJ-MSCs were then expanded until passage 3, cryopreserved with 10% dimethyl sulfoxide (DMSO) in culture media, and stored in liquid nitrogen.

3.3.5 hWJ-MSCs characterization

3.3.5.1 Colony forming unit

To evaluate the colony forming ability, the colony forming unit assay was conducted by seeding 200 MSCs in a 6-well dish and culturing them for two weeks, with the medium being replaced every two days. Following this, the MSCs were fixed using 4% paraformaldehyde (PFA) for 20 minutes and then subjected to staining with 0.5% crystal violet to permit visual assessment of the colony. The assessment was carried out for passages 4, 5, 6, 7, and 10 of MSCs. Subsequently, the stained cells were scrutinized using an inverted microscope (Eclipse Ti-S, Nikon Imaging Japan Inc.) through the NIS-Elements D program (Nikon Imaging Japan Inc., Tokyo, Japan). An aggregate of no less than 50 cells was regarded as a colony. The numbers of colonies were then calculated following equation below with each condition being tested thrice.

$$\% \text{ CFU} = (\text{Total number of colony} \times 100) / \text{Initial cells seeded (\%)}$$

3.3.5.2 Population doubling time (PDt)

Triplicates of cells ranging from passages 4 to 10 were seeded onto a 35 mm culture dish at a density of 4,000 cells/cm^2 and then cultured in α-MEM supplemented with 10% FBS. Following 72 hours of culture, the number of viable cells was determined using 0.4% trypan Blue staining. PDt was calculated using the formula below (Redaelli et al., 2012).

$$PDt = (t \times \log 2 / (\log NF - \log NI)$$

Where NI = Initial cells seeded, NF = Final numbers of cells, t = Time (hours)

3.3.5.3 Flow cytometric analysis

To verify the surface markers of MSCs, a flow cytometric analysis was carried out. In this analysis, MSCs at passage 5 were mixed with PBS(-) and incubated with various antibodies, including anti-CD73-APC, anti-CD90-APC/A750, anti-CD105-PE (dilution 1:100, Biolegend, San Diego, California, USA), anti-CD34-PE (dilution 1:10, Beckman Coulter, Brea, California, USA) and anti-CD45-FITC (dilution 1:20, Bio-legend). As negative controls, isotype control antibodies were used. The incubation was carried out in the dark for 20 minutes, after which the samples were washed with PBS(-) and analyzed using an Attune™ NxT Flow Cytometer (Attune™ NXT, Thermo Fisher Scientific, Cleveland, OH, USA).

3.3.5.4 Differentiation ability

To induce osteogenic differentiation in hWJ-MSCs, cells at passage 5 were cultured in 4-well culture plates coated with 0.1% gelatin until they reached 70% confluence. The induction medium contained α-MEM medium supplemented with 100 nM dexamethasone, 0.2 mM L-ascorbate-2-phosphate, 10 mM β-glycero-phosphate, 100 U/ml penicillin, and 100 µg/ml streptomycin. The induction medium was replaced every 3 days and the cells were cultured for 21 days. Following this, calcium deposits from the cells were stained with Alizarin red and visualized under an inverted microscope.

To induce adipogenic differentiation in hWJ-MSCs at passage 5, they were cultured in 4-well culture plates coated with 0.1% gelatin until they reached 70% confluence. The induction medium was α-MEM medium supplemented with 10 µM insulin, 100 µM indomethacin, 1 µM dexamethasone, and 0.5 mM isobutyl-methylxanthine (IBMX). After 7 days of induction, IBMX was removed from the medium. The medium was replaced every 3 days, and the cells were cultured for a total of 21 days. Following this, the cells were stained with Oil Red O to observe oil droplets, which were visualized under an inverted microscope.

3.3.5.5 Chondrocyte differentiation

hWJ-MSCs at passage 5, they were cultured in 4-well culture plates coated with 0.1% gelatin until they reached 70% confluence. The cells were then treated with an induction medium consisting of α-MEM medium supplemented

with 10 µg/ml ITS-X, 50 µg/ml L-ascorbate-2-phosphate, 40 µg/ml L-proline, 100 µg/ml sodium pyruvate, 100 nM dexamethasone, 10 ng/ml TGF-β3, and 2% FBS. The induction medium was replaced every 3 days, and the cells were cultured for 28 days. The glycosaminoglycan extracellular matrix was evaluated using Alcian blue 8X staining and examined under an inverted microscope.

3.3.5.6 Chondrocyte characterization by Immunocytochemistry staining (ICC)

Following 28 days of chondrocyte induction from MSCs, the cells were fixed in 4% PFA for 20 minutes, permeabilized, and blocked with a solution consisting of 5% BSA, 5% normal goat serum, and 0.1% Triton-X-100 at 37°C for 1 hour. Subsequently, the cells were incubated with specific primary antibodies, including anti-collagen type II antibody (dilution 1:100), anti-collagen type X antibody (dilution 1:100), anti-Sox9 antibody (dilution 1:100, obtained from Abcam, Cambridge, UK), and anti-Aggrecan antibody (dilution 1:100, obtained from Abcam), at 4°C overnight. On the following day,

the cells were incubated with secondary antibodies, including Alexa fluor® 594 goat anti-rat IgG (dilution 1:250, obtained from Invitrogen, Carlsbad, CA, USA) and Alexa fluor® 488 goat anti-mouse IgG (dilution 1:1000, obtained from Invitrogen), for 2 hours at room temperature. Finally, the cells were stained with 6-diamino-2-phenylindole (DAPI) at a dilution of 1:1000 and mounted with Vectashield antifade mounting medium (Vector Laboratories, Burlingame, CA, USA). Samples were observed using a fluorescence inverted microscope (Eclipse Ti-S, Nikon Imaging Japan Inc.) by the NIS-Elements D program (Nikon Imaging Japan Inc., Tokyo, Japan).

3.3.4 Chondrocyte characterization by gene expression analysis

After hWJ-MSCs were induced to undergo chondrogenic differentiation for 28 days, total RNA was prepared by a kit from RBC Real Genomics, RBC Bioscience based in Taipei, Taiwan. Then, cDNA synthesis was performed using oligo-dT primers and the iScript™ Reverse Transcription Supermix for RT-qPCR from BioRad, Hercules, CA, USA. To examine gene expression, KAPA SYBR-Green PCR Master Mix from Applied Biosystems, Carls-bad, CA, USA was used with the QuantStudio 5 real-time PCR system also from Applied Biosystems, Carlsbad, CA, USA. The specificity of the specific primers (listed in Table 3.1) was confirmed by conducting a melting curve analysis

(Tanthaisong et al., 2017). The reference gene used to standardize the target genes was *GAPDH*, and the expression fold change was determined in relation to the control cells. To ensure accuracy, qPCR was performed three times, and relative changes in gene expression analysis were carried out using the $2^{-\Delta\Delta CT}$ method.

Chondrocytes obtained from human cartilage were also isolated and utilized as a positive control. It is worth noting that the use of chondrocytes derived from human cartilage was authorized by the Ethics Committee for Research Involving Human Subjects at Suranaree University of Technology, with the reference number EC-61-56.

Table 3.1 Primers used for gene expression analysis.

Genes	Accession number	Primer sequence (5'-3')	Product size (bp)	References
ACAN	001113455.1	F: ACTTCCGCTGGTCAGATGGA R: TCTCGTGCCAGATCATCACC	111	Bwalya et al., 2018
Sox9	000346.4	F: ACACACAGCTCACTCGACCTTG R: GGGAATTCTGGTTGGTCCTCT	103	Tao et al., 2016
Col2a1	001844.4	F: GAGACAGCATGACGCCGAG R: GCGGATGCTCTCAATCTGGT	67	Tanthaisong et al., 2017
Col10a1	000493.4	F: CCCTCTTGTTAGTGCCAACC R: AGATTCCAGTCCTTGGGTCA	155	Tanthaisong et al., 2017
Runx2	001015051.4	F: ATACCGAGTGACTTTAGGGATGC R: AGTGAGGGTGGAGGGAAGAAG	131	Tanthaisong et al., 2017
β-catenin	001330729.2	F: AATGCTTGGTTCACCAGTG R: GGCAGTCTGTCGTAATAGCC	176	Tanthaisong et al., 2017
GAPDH[*]	002046.7	F: TGCACCACCACCTGCTTAGC R: GGCATGGACTGTGGTCATGAG	87	Kee et al., 2018

[*]Reference gene

3.3.5 Chondrocyte characterization by Western blot analysis

After 28 days of chondrogenic differentiation, the total proteins were extracted using a lysis buffer consisting of 10% sodium dodecyl sulfate (SDS), 0.1 M dithio-threitol (DTT), 1% glycerol, 1.2% urea, 1M Tris- HCl pH 7.4 along with a complete protease inhibitor. The total protein concentration was determined using

the Bradford assay. Subsequently, 20 µg of total protein was separated using SDS-PAGE (10% resolving gel) and the separated protein was transferred onto PVDF membranes (Immun-Blot® PVDF Membrane, Bio-Rad Laboratories). Next, the membranes were blocked in TBST (Tris-buffered saline with 0.1% Tween 20) containing 5% skim milk at RT for 1 hour. For detection of collagen type II and type X proteins, membranes were incubated overnight at 4°C with primary antibody solutions (1% BSA in TBS, Tris-buffered saline). The membranes were then washed with TBST and incubated at RT for 1 hour with secondary antibodies conjugated with horseradish peroxidase (HRP; Abcam) that were diluted 1:2000 in 5% skim milk in TBST. The chemiluminescent substrate was added using an ECL substrate kit (Ultra-high sensitivity, Abcam) following the manufacturer's instructions. Protein bands were visualized using ImageQuant™ LAS 500 (GE Healthcare Life Sciences, Massachusetts, USA). For collagen type II and type X protein normalization, β-actin was used as a housekeeping control. Data were compared to negative control cells, and bands with saturated pixels were excluded.

3.3.6 Statistical Analysis

Statistical analysis was conducted on three to five samples, and the data were presented as the mean ± standard deviation (S.D.). To compare differences between two groups, a one-way analysis of variance (ANOVA) was employed, followed by Tukey-Kramer Honest Significant Difference (HSD) Post hoc test. Results with a *p*-value less than 0.05 were regarded as significant, whereas those with a *p*-value less than 0.01 were deemed highly significant.

3.4 Results

3.4.1 Isolation and characterization of hWJ-MSCs

hWJ-MSCs were obtained from two freshly collected umbilical cords (named as WJ01 and WJ07) at Maharat Nakhon Ratchasima Hospital, Thailand. Characteristics of hWJ-MSCs, including cell surface protein expression, colony forming unit, population doubling time, and differentiation ability, were determined.

Colony forming unit results from WJ01 and WJ07 cell line at passage 4, 5, 6, 7 and 10 are showed in Figure 3.1 A. Colony forming unit results of WJ01 at

passage 4, 5, 6, 7 and 10 are between 18.17 ± 2.08 to 24.67 ± 5.48. Colony forming unit results of WJ07 at passage 4, 5, 6, 7 and 10 are between 35.17 ± 2.08 to 38.17 ± 1.76. Thus, the results of the colony forming unit of the WJ01 cell line were significantly lower than WJ07 cell line in all passages.

PDts were comparable between two cell lines (Figure 3.1 B). PDt of both cell lines at P4-P7 and P10 was varied from 38.47 ± 4.16 h to 51.30 ± 1.72 h. There was no significant difference between two cell lines. Both hWJ-MSCs were positive for CD73, CD90, and CD105 and negative for CD34 and CD45 (Figure 3.1 C and Table 3.2). However, the proportion of WJ07 cells positive for CD105 was very low (32.01%). Both cell lines had adipogenic and osteogenic induction abilities (Figure 3.1 D). Lipid droplets were much more detectable in WJ07 cells than in WJ01 but osteogenic differentiation potentials of both cell lines were similar.

Table 3.2 Percentage of cell surface protein expression.

Cell line	Percentage of cell surface protein expression				
	CD73	CD90	CD105	CD34	CD45
WJ01	99.641	97.820	97.599	0.010	0.523
WJ07	99.310	99.990	32.430	0.030	0.450

There is no obvious difference among the adipogenic and osteogenic differentiation ability of the two cell lines but only WJ01 cell line qualifies the standard of MSCs surface markers. WJ07 had higher CFU-F but low expression of CD105 than WJ01 while no difference of PDt between two groups. Therefore, the WJ01 cell line was used to be transplanted into guinea pigs with osteoarthritis.

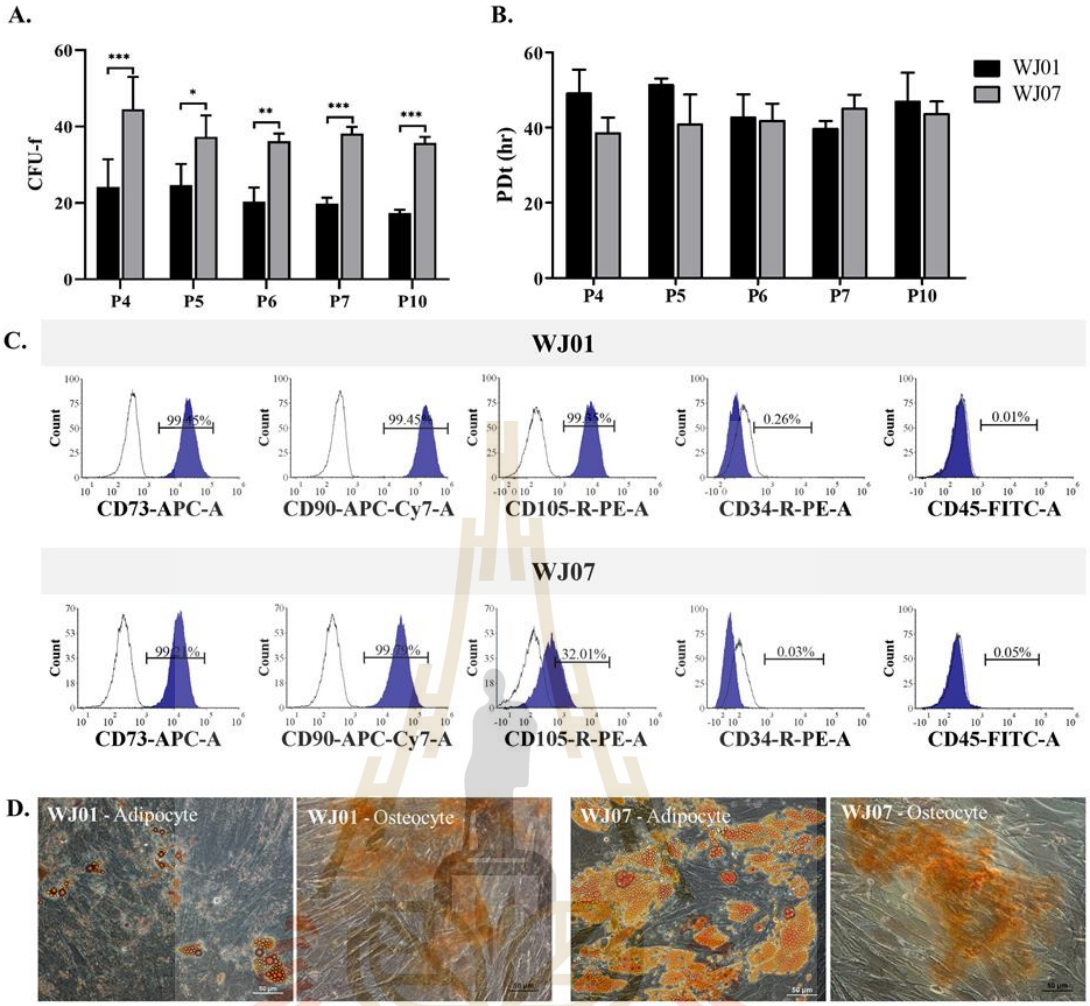


Figure 3.1 Characterization of hWJ-MSCs. (A) Colony forming unit results from WJ01 and WJ07 cell line at passage 4, 5, 6, 7 and 10 (* $P< 0.05$, ** $P< 0.01$, *** $P< 0.001$). (B) Population doubling time results of WJ01 and WJ07 cell line. (C) Cell surface marker expression of WJ01 and WJ07 cell lines. (D) Osteogenic and adipogenic differentiation of WJ01 and WJ07 cell lines. Scale bar = 50 μm.

3.4.2 Characterization of chondrocytes derived from hWJ-MSCs

After 28 days of chondrogenic induction, Sox9 (early chondrocyte stage) and type II collagen (mature chondrocyte stage) were highly expressed in chondrocytes derived from MSCs (Figure 3.2 A, C) cells. However, Aggrecan (mature chondrocyte stage) and type X collagen (hypertrophic chondrocyte stage), were low expressed in these differentiated cells (Figure 3.2 B, D).

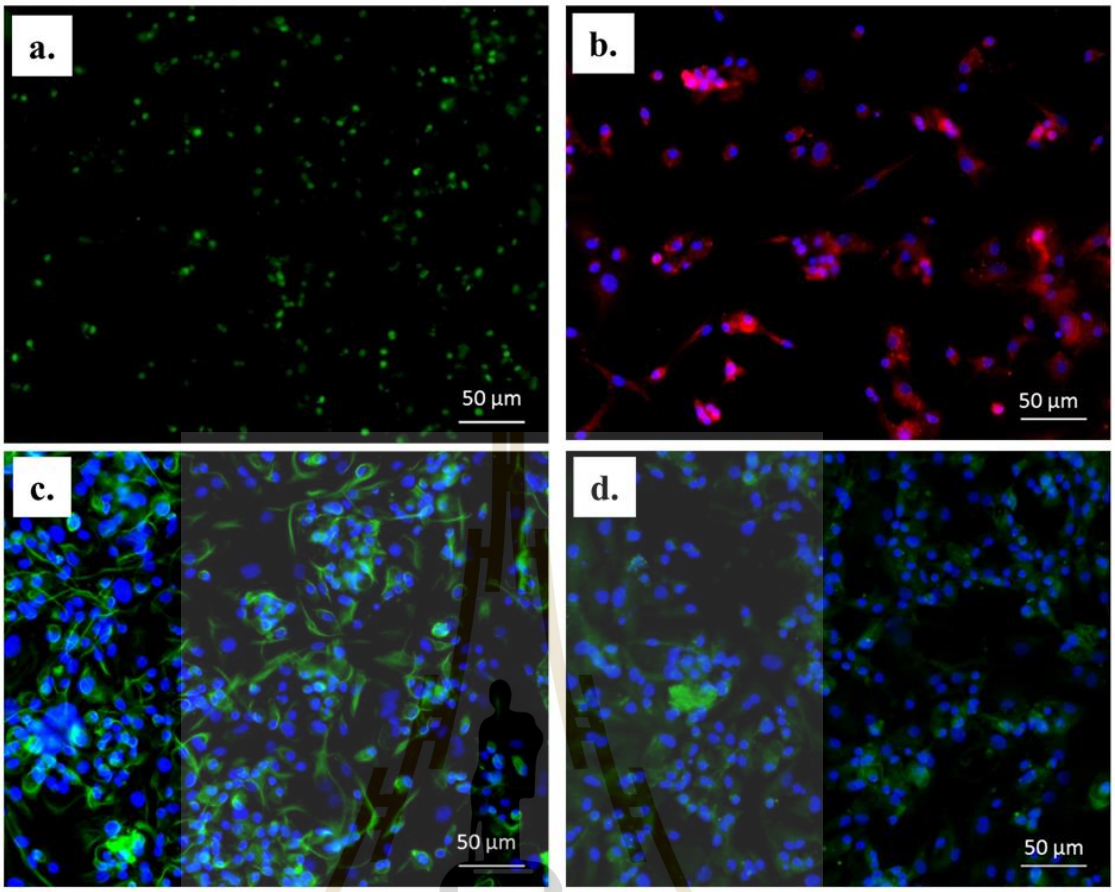


Figure 3.2 Chondrocyte characterization by ICC. (A) Sox9 (green; nuclear marker). (B) Aggrecan (red). (C) type II collagen (green). (D) type X collagen (green). Scale bar = 50 µm.

Gene expression was examined by qPCR at days 0, 3, 7, 14 and 28 of chondrogenic differentiation and compared with cartilage cells isolated from dissected human knee cartilages (Figure 3.3). *β-catenin* gene expression was significantly higher in the chondrogenic differentiation groups (day 3 and 28) than the control group ($P<0.01$), undifferentiated MSCs. But there was no significant difference of *β-catenin* gene expression between differentiated cells at day 3, 7 and 14. However, level of *β-catenin* gene was significantly higher in cells at day 28 than them at day 14 and the positive control (chondrocyte) but not different between group day 28 with chondrocyte group. ($P<0.01$). Expression of *Sox9* gene was significantly higher in chondrogenic induction cells on day 14 and 28 than the control group ($P< 0.01$). After days 3, 7 and 14 of induction, *Runx2*, *Col2a1*, *Col10a1* and *ACAN* gene expression showed no significant

difference between groups. Until day 28, the expression of *Runx2* and *ACAN* was significantly higher than the control group ($P< 0.01$). The results of *Col2a1* and *Col10a1* expression were significantly higher than the control group ($P< 0.05$). After the MSCs induction, *Sox9*, *Runx2*, *Col2a1*, *Col10a1* and *ACAN* gene expression were highest on day 28, but still lower than the positive control.

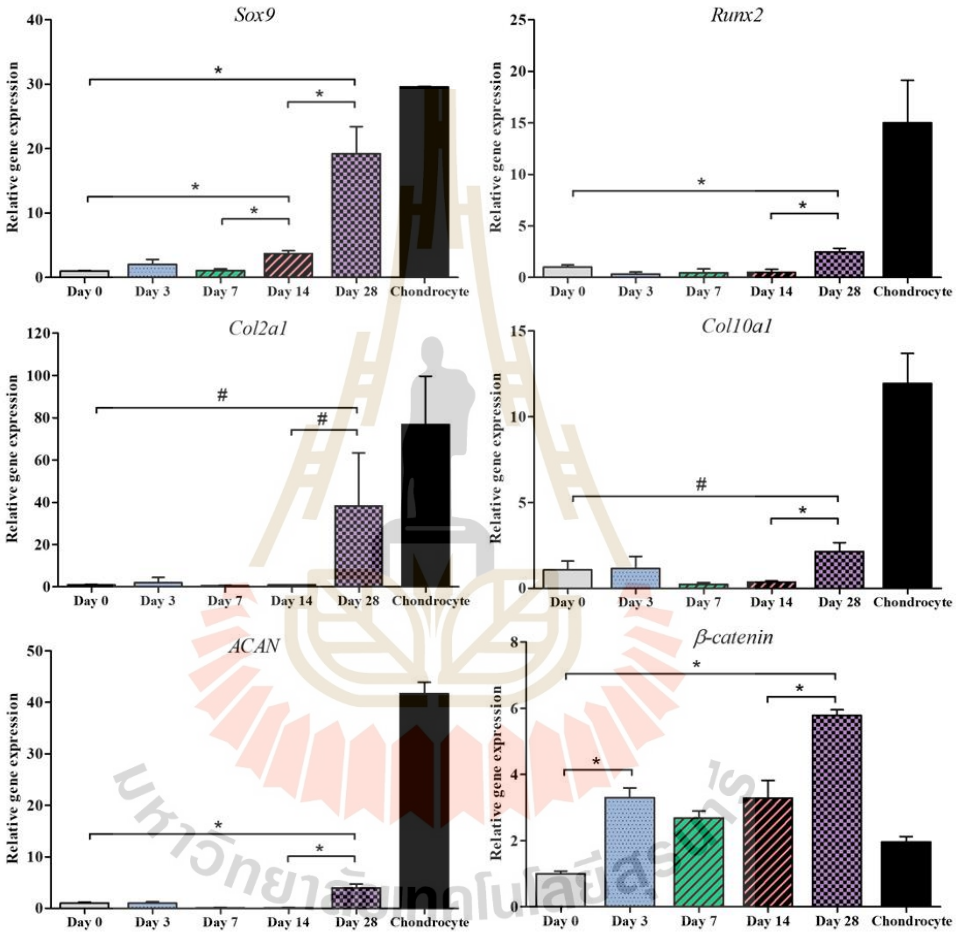


Figure 3.3 Gene expression analysis of chondrocyte by qPCR, *Sox9*, *Runx2*, *Col2a1*, *Col10a1*, *ACAN* and *β-Catenin* genes. The targeted gene was normalized to *GAPDH* as a reference gene and calculated the relative expression compared to each group. (*$P< 0.01$, #$P< 0.05$).

Type II collagen proteins of chondrogenic differentiated cells on day 28 were compared to MSCs by immunoblot. MSCs before induction expressed very low levels of type II collagen, but chondrogenic differentiated cells expressed very high

level of type II collagen. At the same time, low levels of type X collagen expression were also found (Figure 3.4).

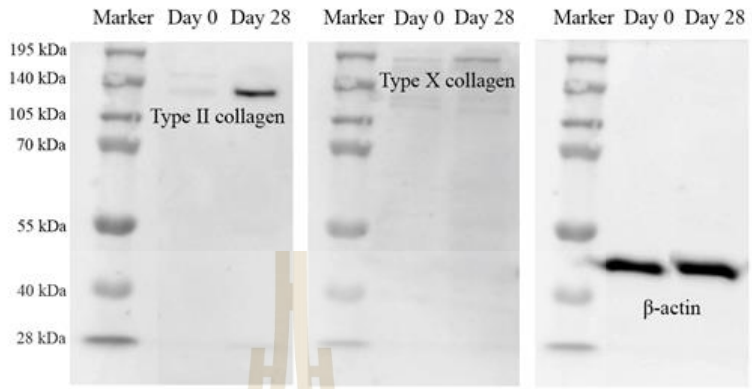


Figure 3.4 Type X collagen protein expression analysis of chondrocyte differentiated cells on day 28 by immunoblot and β-actin protein was used as an internal control.

3.5 Discussion

hWJ-MSCs were obtained and expanded from two types of cell lines. The characteristics of both types of cell lines were determined by various methods such as CFU assay, PDT, MSCs surface markers, and their capability to differentiate into osteocytes, adipocytes, and chondrocytes. In particular, cell line WJ01 had more than 95% of MSCs surface markers, including CD73, CD90, and CD105, while negative markers CD34 and CD45 were less than 2%. Moreover, cell line WJ01 demonstrated the ability to differentiate into osteocytes, adipocytes, and chondrocytes, in accordance with the standards set by the International Society for Cell and Gene Therapy (Dominici et al., 2006; Fong et al., 2012; Wang et al., 2004) . The doubling times for cell line WJ01 were around 40-50 hrs for passages 4, 5, 6, 7, and 10, which were similar to the results obtained for MSCs from the Wharton's jelly tissue of human umbilical cords (Yoon et al., 2013; Bharti et al., 2018). A specific MSCs cell line was selected and utilized to generate cartilage cells over a period of 28 days. As per a previously established method (Tanthaisong et al., 2017), MSCs can be stimulated to differentiate into cartilage cells. The outcomes demonstrated the production of high levels of proteoglycan and strong Alcian blue staining in the

generated cartilage cells. Additionally, immunofluorescence staining was performed to examine the expression of various proteins such as Sox9, Aggrecan, Type II collagen, and Type X collagen (Grassel et al., 2007; Stromps et al., 2014). The mature cartilage cells exhibited significant expression of the Sox9 and Type II collagen proteins, while moderate expression of the aggrecan protein was observed. However, the findings indicated a low expression of type X collagen in the aged cartilage cells, with only minimal staining observed in the induced cells. Consistent with this observation, the results of protein quantification via immunoblot indicated high expression of type II collagen and low expression of type X collagen. The expression of several genes was analyzed at different time points during the study. The *ß-Catenin* gene expression demonstrated a rapid increase on day 3, indicating the involvement of the Wnt/ß-Catenin signaling pathway in chondrogenic differentiation (Day et al., 2005; Yang et al., 2012). Similarly, the *Sox9* gene expression also displayed a quick increase, with the highest levels observed on day 28. However, the *ACAN*, *Col10a1*, and *Runx2* genes only exhibited a slight increase in expression on day 28.

3.6 Conclusions

In summary, only one cell line was chosen to participate in the following experiments. This cell line WJ01 exhibited the proper mesenchymal stem cells (MSCs) characteristics, such as cell surface markers, colony forming unit, population doubling time, surface protein expression, and differentiation potencies into three cell lineages. Following a 28-day stimulation of chondrogenic differentiation, hWJ-MSCs were transformed into chondrocytes and assessed using immunofluorescent staining, qPCR, and immunoblotting. It was suggested that only type II collagen would be measured in this investigation using immunofluorescent staining and immunoblotting. To further firmly corroborate the findings, type X collagen was further determined by immunoblotting, and Sox9, Aggrecan, and type X collagen were also determined by immunofluorescent labeling. hWJ-MSCs derived-chondrocyte can be utilized for research studies on OA treatment *in vivo* model.

3.7 References

Ambrosi, T. H., Scialdone, A., Graja, A., Gohlke, S., Jank, A.-M., Bocian, C., Woelk, L., Fan, H., Logan, D. W., Schürmann, A., Saraiva, L. S., & Schulz, T. J. (2017). Adipocyte Accumulation in the Bone Marrow during Obesity and Aging Impairs Stem Cell-Based Hematopoietic and Bone Regeneration. **Cell Stem Cell.** 20: 771-784.

Azadniv, M., Myers, J. R., McMurray, H. R., Guo, N., Rock, P., Coppage, M. L., Ashton, J., Becker, M. W., Calvi, L. M., & Liesveld, J. L. (2020). Bone Marrow Mesen-chymal Stromal Cells from Acute Myelogenous Leukemia Patients Demonstrate Adipogenic Differentiation Propensity with Implications for Leukemia Cell Support. **Leukemia**. 34: 391-403.

Baddoo, M., Hill, K., Wilkinson, R., Gaupp, D., Hughes, C., Kopen, G. C., & Phinney, D.G. (2003). Characterization of Mesenchymal Stem Cells Isolated from Murine Bone Marrow by Negative Selection. **J. Cel. Biochem.** 89: 1235-1249.

Bharti, D., Shivakumar, S.B., Park, J. K., Ullah, I., Subbarao, R. B., Park, J. S., Lee, S. L., Park, B. W., & Rho, G. J. (2018). Comparative analysis of human Wharton's jelly mesenchymal stem cells derived from different parts of the same umbilical cord. **Cell Tissue Res.** 372(1): 51-65.

Bwalya, E. C., Wijekoon, H. S., Fang, J., Kim, S., Hosoya, K., & Okumura, M. (2018). Independent chondrogenic potential of canine bone marrow-derived mesenchymal stem cells in monolayer expansion cultures decreases in a passage-dependent pattern. **J. Vet. Med. Sci.** 80(11): 1681-7.

Day, T. F., Guo, X., Garrett-Beal, L., & Yang, Y. (2005). Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. **Dev. Cell.** 8(5): 739-50.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, D., Keating, A., Prockop, D., & Horwitz, E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. **Cytotherapy.** 8(4): 315-7.

Ebihara, G., Sato, M., Yamato, M., Mitani, G., Kutsuna, T., Nagai, T., Ito, S., Ukai, T., Kobayashi, M., Kokubo, M., Okano, T., & Mochidaet, J. (2012). Cartilage repair in transplanted scaffold-free chondrocyte sheets using a minipig model. **Biomaterials**. 33(15): 3846-51.

Grassel, S., & Ahmed, N. (2007). Influence of cellular microenvironment and paracrine signals on chondrogenic differentiation. **Front. Biosci.** 12: 4946-56.

Gruber, H. E., Deepe, R., Hoelscher, G. L., Ingram, J. A., Norton, H. J., Scannell, B., Loeffler, B. J., Zinchenko, N., Hanley, E. N., & Tapp, H. (2010). Human adipose-derived mesenchymal stem cells: direction to a phenotype sharing similarities with the disc, gene expression profiling, and coculture with human annulus cells. **Tissue Eng. Part A.** 16: 2843-2860.

Fong, C. Y., Subramanian, A., Gauthaman, K., Venugopal, J., Biswas, A., Ramakrishna, S., & Bongso, A. (2012). Human umbilical cord Wharton's jelly stem cells undergo enhanced chondrogenic differentiation when grown on nanofibrous scaffolds and in a sequential two-stage culture medium environment. **Stem Cell Rev. Rep.** 8(1): 195-209.

Ishige, I., Nagamura-Inoue, T., Honda, M. J., Harnprasopwat, R., Kido, M., Sugimoto, M., Nakauchi, H., & Tojo, A. (2009). Comparison of mesenchymal stem cells derived from arterial, venous, and Wharton's jelly explants of human umbilical cord. **Int. J. Hematol.** 90: 261-269.

Jones, I. A., Togashi, R., Wilson, M. L., Heckmann, N., & Vangsness, C. T. (2019). Intra-articular treatment options for knee osteoarthritis. **Nat. Rev. Rheumatol**. 15(2): 77-90.

Kee, J. Y., Han, Y. H., Mun, J. G., Park, S. H., Jeon, H. D., & Hong, S. H. (2018). Gomisin A suppresses colorectal lung metastasis by inducing AMPK/p38-mediated apoptosis and decreasing metastatic abilities of colorectal cancer cells. **Front. Pharmacol.** 986(9).

Kong, L., Zheng, L. Z., Qin, L., & Ho, K .K. W. (2017). Role of mesenchymal stem cells in osteoarthritis treatment. **J. Orthop. Translat**. 9: 89-103.

Mebarki, M., Abadie, C., Larghero, J., & Cras, A. (2021). Human umbilical cord-derived mesenchymal stem/stromal cells: a promising candidate for the development of advanced therapy medicinal products. **Stem Cell Res & Ther.** 12: 152.

Mishra, s., Sevak, J. K., Das, A., Arimbasseri, G. A., Bhatnagar, S., & Gopinath, S. D. (2020). Umbilical cord tissue is a robust source for mesenchymal stem cells

with enhanced myogenic differentiation potential compared to cord blood. **Sci. Rep.** 10: 18978.

Petsa, A., Gargani, S., Felesakis, A., Grigoriadis, N., & Grigoriadis, I. (2009). Effectiveness of protocol for the isolation of Wharton's Jelly stem cells in large-scale applications. **In Vitro Cell Dev. Biol. Anim**. 45(10): 573.

Ponnaiyan, D., Bhat, K. M., & Bhat, G. S. (2012). Comparison of immuno-phenotypes of stem cells from human dental pulp and periodontal ligament. **Int. J. Immunopathol. Pharmacol.** 25: 127-134.

Redaelli, S., Bentivegna, A., Foudah, D., Miloso, M., Redondo, J., Riva, G., Baronchelli, S., Dalprà, L., & Tredici, G. (2012). From cytogenomic to epigenomic profiles: monitoring the biologic behavior of *in vitro* cultured human bone marrow mesenchymal stem cells. **Stem Cell Res. Ther.** 3(6): 47-47.

Romanov, Y. A., Darevskaya, A. N., Merzlikina, N. V., & Buravkova, B. (2005). Mesenchymal stem cells from human bone marrow and adipose tissue: isolation, characterization and differentiation potentialities. Bulletin **Exp. Biol. Med.** 140: 138-143.

Stromps, J. P., Paul, N. E., Rath, B., Nourbakhsh, M., Bernhagen, J., & Pallua, N. (2014). Chondrogenic differentiation of human adipose-derived stem cells: a new path in articular cartilage defect management? **Biomed. Res. Int.** 740926.

Tanthaisong, P., Imsoonthornruksa, S., Ngernsoungnern, A., Ngernsoungnern, P., Ketudat-Cairns, M., & Parnpai, R. (2017). Enhanced chondrogenic differentiation of human umbilical cord Wharton's jelly derived mesenchymal stem cells by GSK-3 inhibitors. **PloS one.** 12(1): e0168059.

Tao, K., Frisch, J., Rey-Rico, A., Venkatesan, J. K., Schmitt, G., Madry, H., Lin. J., & Cucchiarini, M. (2016). Co-overexpression of TGF-ß and SOX9 via rAAV gene transfer modulates the metabolic and chondrogenic activities of human bone marrow-derived mesenchymal stem cells. **Stem Cell Res. Ther.** 7(1): 20.

Tondreau, T., Meuleman, N., Delforge, A., Dejeneffe, M., Leroy, R., Massy, M., Mortier, C., Bron, D., & Lagneaux, L. (2005). Mesenchymal stem cells derived from CD133-positive cells in mobilized peripheral blood and cord blood: proliferation, Oct4 expression, and plasticity. **Stem Cells.** 23: 1105-1112.

Troyer, D. L., & Weiss, M. L. (2008). Wharton's jelly-derived cells are a primitive stromal cell population. **Stem Cells**. 26(3): 591-9.

Wang, H. S., Hung, S. C., Peng, S. T., Huang, C. C., Wei, H. M. Guo, Y. J., Fu, Y. S., Lai, M. C., & Chen, C. C. (2004). Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. **Stem Cells.** 22(7): 1330-7.

Witkowska-Zimny, M., & Wrobel, E. (2011). Perinatal sources of mesenchymal stem cells: Wharton's Jelly, amnion and chorion. **Cell Mol. Biol. Lett.** 16(3): 493-514.

Wolock, S. L., Krishnan, I., Tenen, D. E., Matkins, V., Camacho, V., Patel, S., Agarwal, P., Bhatia, R., Tenen, D. G., Klein, A. M., & Welner, R. S. (2019). Mapping Distinct Bone Marrow Niche Populations and Their Differentiation Paths. **Cell Rep.** 28: 302-311.

Wong, C. C., Ou, K. L., Lin, Y. H., Lin, M. F., Yang, T. L., Chen, C. H., & Chan, W. P. (2020). Platelet-rich fibrin facilitates one-stage cartilage repair by promoting chondrocytes viability, migration, and matrix synthesis. **Int. J. Mol. Sci**. 21(2): 577.

Yang, Z., Zou, Y., Guo, X. M., Tan, H. S., Denslin, V., Yeow, C. H., Ren, X. F., Liu, T. M., Hui, J. H. P., & Lee, E. H. (2012). Temporal activation of β-catenin signaling in the chondrogenic process of mesenchymal stem cells affects the phenotype of the cartilage generated. **Stem Cells Dev.** 21(11): 1966-76.

Yea, J. H, Kim, Y., & Jo, C. H. (2023). Comparison of mesenchymal stem cells from bone marrow, umbilical cord blood, and umbilical cord tissue in regeneration of a full-thickness tendon defect *in vitro* and *in vivo*. **Biochem. Biophys. Rep.** 34: 101486.

Yoon, J. H., Roh, E. Y., Shin, S., Jung, N. H., Song, E. Y., Chang, J. Y., Kim, B. J., & Jeon, H. W. (2013). Comparison of explant-derived and enzymatic digestion-derived MSCs and the growth factors from Wharton's jelly. **Biomed. Res. Int.** 2013: 428726.

CHAPTER IV

TRANSPLANTATION OF HUMAN WHARTON'S JELLY MESENCHYMAL STEM CELLS DERIVED-CHONDROCYTES IN GUINEA PIG MODEL WITH SPONTANEOUS OSTEOARTHRITIS

4.1 Plants and Phenotypic Similarities

Osteoarthritis (OA) is a degenerative joint disease commonly found in elderly people, and obese patients. Currently, OA treatments are determined based on their condition severity and medical professional's advice. The aim of this study was to differentiate human Wharton's jelly-derived mesenchymal stem cells (hWJ-MSCs) into chondrocytes for transplantation in OA-suffered guinea pigs. Early stage chondrocytes can be obtained by chondrogenic differentiation induction of MSCs for 14 days. For transplantation, Then, early OA-suffered guinea pigs were injected with hyaluronic acid (HA) containing either MSCs or 14-days old hWJ-MSCs-derived chondrocytes. Results showed that hWJ-MSCs-derived chondrocytes expressed specific markers of chondrocytes including Aggrecan, type II collagen, and type X collagen proteins, and *ß-catenin*, *Sox9*, *Runx2*, *Col2a1*, *Col10a1* and *ACAN* gene expression markers. Administration of HA plus hWJ-MSCs-derived chondrocytes (HA-CHON) produced better recovery rate of degenerative cartilages than HA plus MSCs or only HA. Histological assessments demonstrated no significant difference in Mankin's scores of recovered cartilages between HA-CHON-treated guinea pigs and normal articular cartilage guinea pigs. Transplantation of hWJ-MSCs-derived chondrocytes was more effective than undifferentiated hWJ-MSCs or hyaluronic acid for OA treatment in guinea pigs. This study provides a promising treatment used in early OA patients to promote the recovery and prevent the disease progression to severe osteoarthritis.

4.2 Introduction

Articular cartilage is a specialized type of connective tissue composed of cartilage cells and typically found in synovial joints. These cells produce extracellular matrix (ECM) and preserve the function of the tissue. Articular cartilage does not possess self-healing ability due to the absence of blood vessels, lymphatic vessels, and the nervous system (Sophia Fox et al., 2009). Arthritic cartilage degeneration can cause various symptoms, including growth abnormalities in children, injuries caused by stress from trauma, and age-related osteoarthritis (Song et al., 2004).

Current OA treatments are commonly determined based on disease severity, and the physician's recommendations including pharmacological and non-pharmacological therapies. The first-line pharmacologic treatment is acetaminophen to cure mild and intermittent symptoms, and then followed by non-steroidal anti-inflammatory drugs (NSAIDs) when acetaminophen is ineffective to alleviate pain. However, NSAIDs prescription should be considered due to their gastric ulcer complication, and cardiovascular risk (Yusuf, 2016). Combination of pharmacological and non-pharmacologic treatment, namely diet and weight loss, physical therapy and exercise, and nutritional supplements (glucosamine and chondroitin sulfate) is a common advice for OA treatment to reduce symptoms and improve functional performance of the joint. Surgery is an invasive procedure that should be conducted when the combined therapy is unsuccessful to produce desired outcomes (Yusuf, 2016). Additionally, there is a possibility of recurrence and complications after surgery in many patients. Consequently, a novel and more effective procedure for osteoarthritis is indispensable, like application of cartilage cells. However, extraction of these cells from a human requires invasive surgery, which is complicated and expensive (Ebihara et al., 2012; Wong et al., 2020). Research is currently underway to explore the potential use of mesenchymal stem cells (MSCs) in treating osteoarthritis. The stem cells possess the unique ability to stimulate the growth of cartilage cells and other types of cells. MSCs are utilized in treating various disorders and can be sourced from several different locations, including bone marrow, blood, adipose tissue, and dental pulp. They can be isolated and cultured with a high level of proliferation activity. Previous studies have identified Wharton's jelly, found in the

umbilical cord of humans, as a common source of MSCs. This tissue can be collected from pregnant women following childbirth, without complex collection process required (Troyer et al., 2008). As a result, MSCs isolated from the Wharton's jelly of human umbilical cords are a promising area of interest for future clinical trials.

The use of Dunkin Hartley guinea pigs as an animal model for studying spontaneous cartilage degeneration in the knee joint, which is similar to osteoarthritis in humans, has been well established (Tessier et al., 2003; Yan et al., 2014). Researchers reported that the knee joint of guinea pigs closely resembles that of humans affected by osteoarthritis (Fernandez et al., 1997; Kraus et al., 2010). Moreover, spontaneous cartilage degeneration in the Dunkin-Hartley guinea pigs are used for study (Bendele & Hulman, 1988). Previous studies demonstrated that injecting mesenchymal stem cells (MSCs) with hyaluronic acid (HA) into the articular cartilage of guinea pigs with osteoarthritis led to recovery (Sato et al., 2012). HA-based formulations are currently delivered into the joint to relieve pain and improve joint mobility of OA patients by partial restoration of the rheological properties of the synovial fluid (La Gatta et al., 2021).

In this study, we isolated MSCs from human Wharton's jelly of the umbilical cord and induced them into cartilage cells. We then transplanted the early chondrogenic differentiated MSCs into the guinea pigs which have osteoarthritis and monitored their progress to evaluate the effectiveness of the treatment. The results demonstrated promising outcomes in the experimental animals, suggesting that this treatment approach using early chondrogenic differentiated MSCs could be developed into a viable treatment option for patients with osteoarthritis. Furthermore, this method is simpler and less invasive than surgical treatments, making it a potentially safer option for patients. Therefore, the purposes of this study were to differentiate human Wharton’s jelly-derived mesenchymal stem cells (hWJ-MSCs) isolated from human umbilical cord tissues into chondrocytes, and characterize hWJ-MSCs-derived chondrocytes prior to transplantation in OA-suffered guinea pigs.

4.3 Materials and Methods

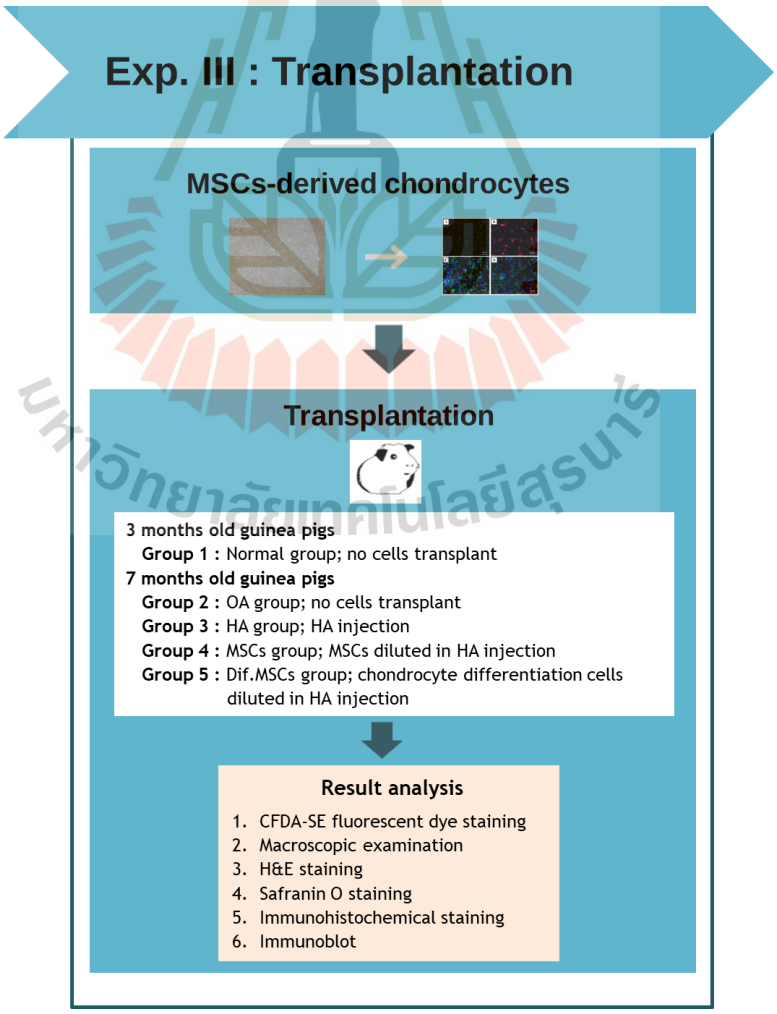
4.3.1 Ethics Statement

Ethical approval for this study was obtained from the Animal Ethics Committee of Suranaree University of Technology, Thailand (Approval Number: U1-03131-2559).

4.3.2 Reagents

All chemical compounds were purchased from Sigma-Aldrich Corporation (St. Louis, Missouri, USA), antibodies were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The cell culture media and cell culture ware was obtained from Gibco (Paisley, UK) and SPL Life Science (Gyeonggi-do, South Korea), respectively. Unless stated otherwise.

4.3.3 Experimental design



4.3.4 Experimental animals

Guinea pigs of 3 months age were identified as normal articular cartilage guinea pigs, while guinea pigs of 7 months were identified as having minor osteoarthritis (OA). The study randomly assigned guinea pigs to five groups (N= 10/group) using a Completely Randomized Design (CRD) depicted in Figure 4.1: (1) Normal group consisting of guinea pigs of 3 months with no cells transplant (2) OA group consisting of OA guinea pigs of 7 months with no cells transplant, (3) HA group consisting of OA guinea pigs of 7 months given Hyaluronic acid (HA) injection (4) HA+MSCs group consisting of OA guinea pigs of 7 months given Hyaluronic acid (HA) injection with hWJ-MSCs, and (5) HA+dif.MSCs group consisting of OA guinea pigs of 7 months given Hyaluronic acid (HA) injection with chondrogenic differentiated cells. The guinea pigs were marked by notching on the pinna of the ear for identification among treatments. Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Thermo Fischer Scientific) was used to label cells before transplantation (Yoon et al., 2013).

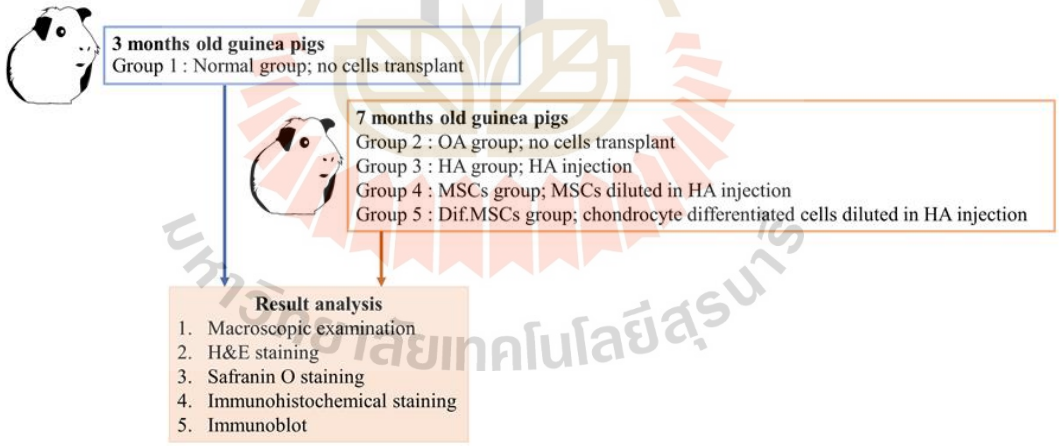


Figure 4.1 Experimental design of cell transplantation.

4.3.5 Preparation of chondrocytes derived from hWJ-MSCs

At day 14th of chondrogenic induction, chondrocytes derived from hWJ-MSCs at passage 5 were collected for transplantation. The hWJ-MSCs-derived chondrocytes were detached and separated by a 30 minute digestion process using

0.2% collagenase type II followed by 0.25% Trypsin. Before injection, these cells were labeled with CFDA-SE.

4.3.6 Cell transplantation

In group 4 and 5, each guinea pigs received a 100 µl injection containing a total of 1×10^6 cells (hWJ-MSCs in group 4 and chondrocytes derived from hWJ-MSCs in group 5) that were labeled with CFDA-SE and suspended in HA (Hyruan®III; LG Chem, Seoul, South Korea). The injection was administered into the medial compartment of the knees of the guinea pigs (Sato et al., 2012).

4.3.7 Macroscopic examination

Five weeks following cell transplantation, the guinea pigs were euthanized using carbon dioxide fumigation. The proximal tibiae of each guinea pigs were then opened, and their cartilage repair in the degenerative knee was examined (Sato et al., 2012). This study used five proximal tibia samples to perform the macroscopic examination. The tibiae's distal heads were first stained with India ink for a minute. The cartilage surfaces were then washed with PBS(-) before being examined and scored following a criteria in Table 4.1.

Table 4.1 Scoring criteria for osteoarthritis symptoms according to cartilage damage examined by India ink staining.

Score	cartilage surface
0	normal, perfectly smooth surface, no black areas
1	small area of rough surface, only stick black on small area < 10%
2	medium area of rough surface, stick black on small area 10 - 30%
3	large area of rough surface, stick black on wide and dark area >30%
4	Cartilage loss areas are deep but not damaged to the bone
5	Cartilage loss areas are deep and damaged to the bone

4.3.8 Histology and Immunohistochemistry

Five proximal tibia samples were fixed in 10% buffered formaldehyde at 4°C for 72 hours. To decalcify the tissues, they were soaked in 5% nitric acid for seven days, and then embedded in optimal cutting temperature (OCT) compound. Tissue slices with a thickness of 15 mm were sectioned (Kawamoto and Shimizu,

2000). The study conducted histology, immunohistochemistry, and fluoroscopic analysis on guinea pigs tissue series. The tissue slides were stained using hematoxylin and eosin (H&E) and safranin O before being examined and scored following the Mankin et al.'s (Mankin et al., 1971) and Armstrong et al.'s (Armstrong et al., 1994) methodology. In the case of immunohistochemistry, the tissue slides were incubated using primary polyclonal antibodies (rabbits against type II collagen) with a 1:200 dilution (COSMO BIO, Tokyo, Japan) for an hour at room temperature. After washing with PBS, the tissue slides were incubated in HRP anti-mouse IgG secondary antibody for 30 minutes at room temperature. The tissue slides were visualized using VECTA STAIN ABC Reagent (Vectastain Elite Kit; Vector Laboratories). To conduct fluoroscopic analysis, CFDA-SE labeled cells were studied in a tibial frontal section using a fluorescence microscope with 492 nm and 517 nm settings (Sato et al., 2012).

4.3.9 Immunoblot analysis

Proteins were extracted from tissues and separated via SDS-PAGE using a 15% resolving gel. After electrotransfer to PVDF membranes (Immun-Blot® PVDF Mem-brane), the membranes were blocked using a solution of 5% skim milk in TBST (Tris-buffered saline with 0.1% Tween 20) for 1 hour at room temperature. To detect type I collagen, type II collagen, and matrix metalloproteinase-13 (MMP13 dilution 1:20)), the membranes were incubated with primary antibody solutions (1% BSA in TBS Tris-buffered saline) overnight at 4°C. After being washed with TBST, secondary antibodies (goat anti-rabbit or goat anti-mouse) conjugated with horseradish peroxidase (HRP; Abcam, Cambridge, U.K) were applied for 1 hour at room temperature at a dilution of 1:2000 in 5% skim milk in TBST. The chemiluminescent substrate was applied using an ECL substrate kit (Ultra-high sensitivity, Abcam, Cambridge, UK) following the manufacturer’s suggestions. The protein bands were then imaged using Image Quant™ LAS 500 (GE Healthcare Life Sciences, Massachusetts, USA), with β-actin used as a were not quantified.

4.3.10 Statistical Analysis

Statistical analysis was conducted on three to five samples, and the data were presented as the mean ± standard deviation (S.D.). To compare differences between the control and treated groups, a one-way analysis of variance (ANOVA) was employed, followed by Tukey-Kramer Honest Significant Difference (HSD) Post hoc

test. Results with a *p*-value less than 0.05 were regarded as significant, whereas those with a *p*-value less than 0.01 were deemed highly significant.

4.4 Results

4.4.1 Chondrocyte transplantation results

Dunkin Hartley guinea pigs were used as animal models. Guinea pigs were divided into five groups: 1) 3-month-old guinea pigs with normal knee joints (normal), 2) 7-month-old guinea pigs with no treatment (spontaneous osteoarthritis; OA), 3) 7-month-old guinea pigs with HA injections, 4) 7-month-old guinea pigs with HA+MSCs injections, 5) 7-month-old guinea pigs with HA+ differentiated chondrogenic MSCs. The differentiated chondrogenic MSCs were stained with CFDA-SE fluorescent dye before transplantation. After staining with CFDA-SE, the stained cells survived and grew normally when cultured (Figure 4.2). Intra-articular injection was performed at the knee joints of the guinea pigs. After transplantation, all the guinea pigs were healthy, and there were no observable any abnormalities at the knee joints after the injection.

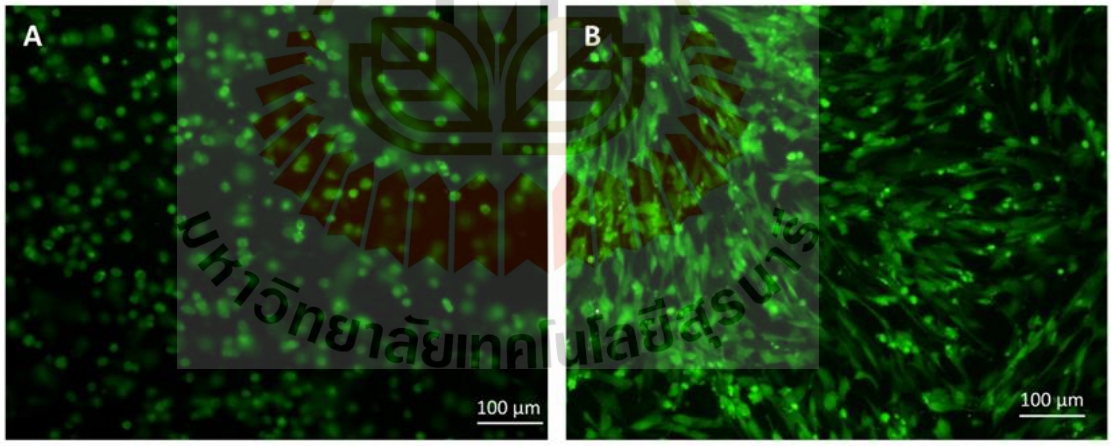


Figure 4.2 The cells stained with CFDA-SE fluorescent dye: (A) cells suspended in HA, (B) cells cultured for 7 days. Scale bar = 100 µM.

4.4.2 Macroscopic examination results

Five samples of the proximal tibia were obtained from each guinea pig group and dyed with India ink as shown in Figure 4.3. The rough cartilage surface clearly showed black color of India ink particles. In group 1, there were no blacked

out areas on the cartilage surfaces. Only the medial sides of the tibial cartilages in groups 2-5 of 7-month-old guinea pigs were blacked out. The cartilage of the group 2 (not- transplanted), the blacked areas were wider than group 3, 4 and 5. Degenerative symptom scores of each group are displayed in Figure 4.3 F. The degenerative scores of the 7-month old guinea pigs in groups 2 – 5 were significantly higher than group 1 (the 3-month old guinea pigs; $P< 0.01$).

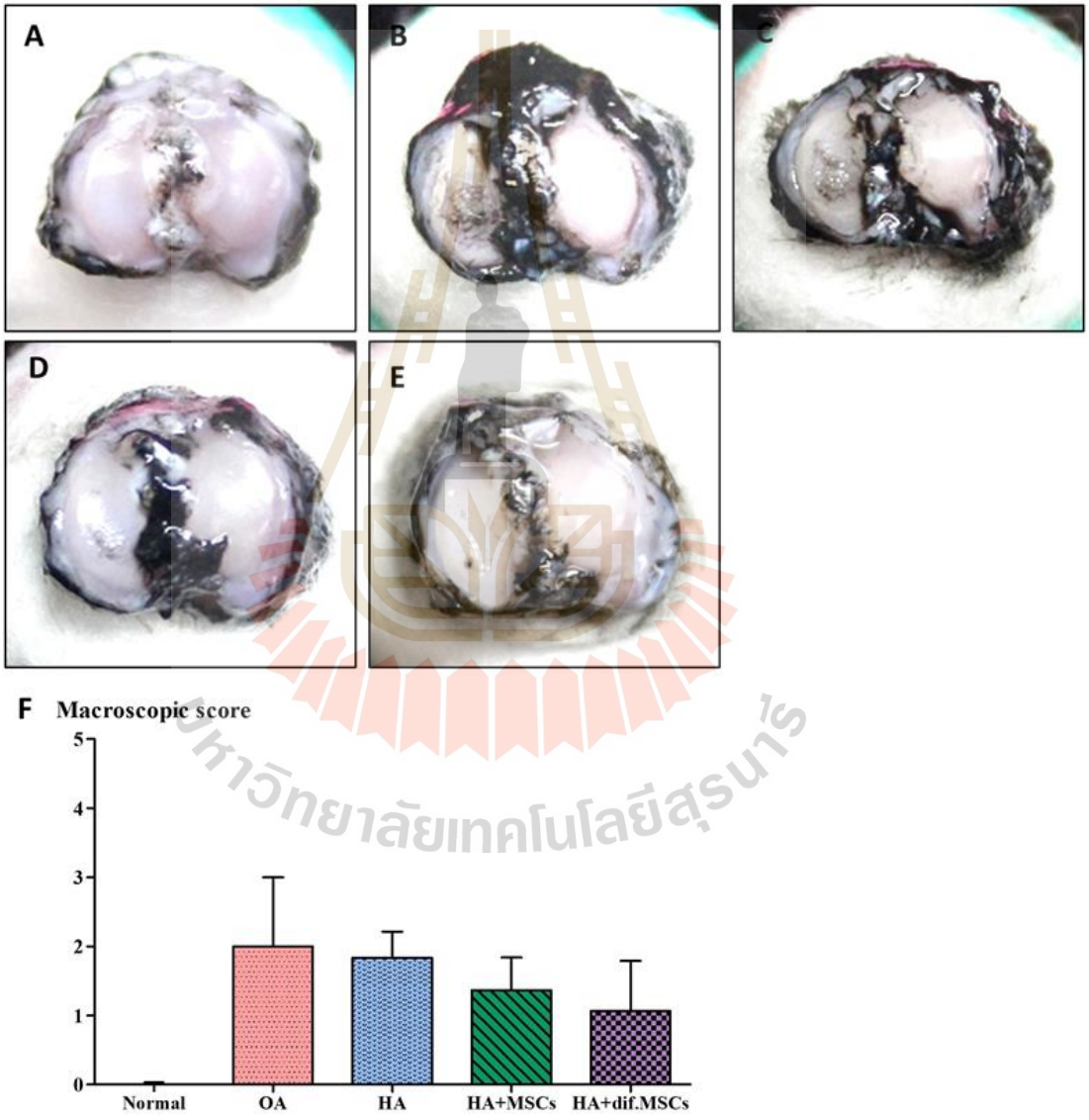


Figure 4.3 The osteoarthritis scores of each group were examined by (A-E) India ink staining and (F) macroscopic score.

4.4.3 Histology results

CFDA-SE fluorescence-stained cells in the knee sample of guinea pigs were examined using cryosection method and observed under a fluorescent microscope. Human nuclei were also stained with red fluorescent antibody. Green fluorescent cells of CFDA-SE with red staining were observed at the human nuclei. This confirmed that the cells attached to the cartilage surface were human cells that were transplanted into the guinea pig's knee joint (Figure 4.4). The results also pointed out that hWJ-MSCs-derived chondrocytes could adhere to the cartilage surfaces together with recovery of the damaged cartilage.

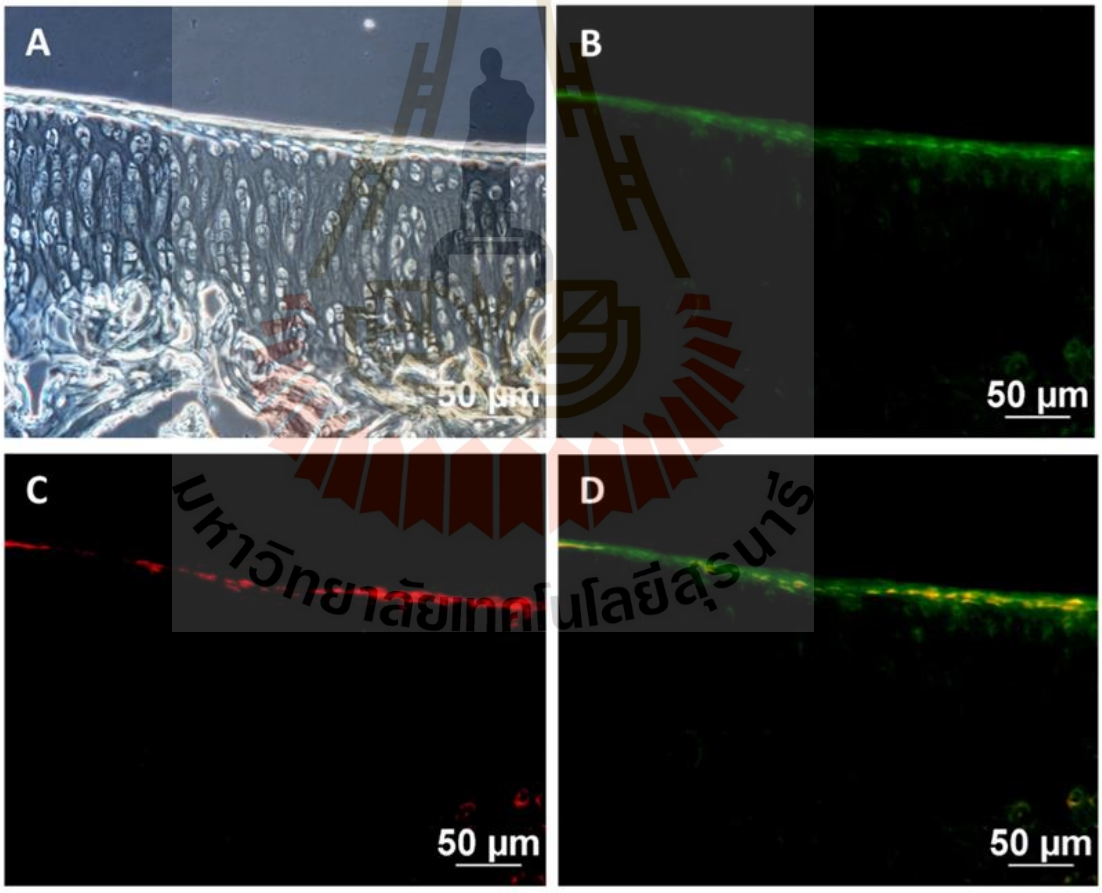


Figure 4.4 Cell tracking after transplantation: (A) bright field images, (B) CFDA-SE-stained transplanted cells (green), (C) human nuclei (red), (D) merged images. Scale bar = 50 um.

The knee joints of guinea pig were cryosectioned and examined histologically using H&E and Safranin O staining techniques (Figure 4.5-4.6), respectively. A smooth cartilage surface, a uniform cartilage surface layer, and the extracellular matrix were present in group 1. However, the cartilage of 7-month-old guinea pigs in groups 2-5 showed the cartilage imperfection at the medial side of the tibia indicated by un-smooth thickness of the cartilage surface layer, the rupture of the cartilage surface (the arrows in Figure 4.5-4.6, extracellular matrix loss, and the formation of a cavity within the cartilage tissue. Group 2 had the most severe cartilage damage among all groups. Cartilage damage was reduced in group 3 (HA injection) and group 4 (HA injection with MSCs). On average, MSCs caused the least cartilage damage across all samples. In group 5 (HA injection with chondrogenic differentiated cells), the lowest cartilage damage was noticeable, compared to other groups. Cartilage damage scores based on the Mankin criterion procedure are illustrated in Figure 4.7. The knee joints of guinea pig in groups 1, 2, 3, 4 and 5 showed cartilage damage scores as 1.6 ± 0.5, 6.6 ± 1.8, 6.4 ± 2.1, 4.8 ± 1.5, and 3.0 ± 1.9, respectively. The values in groups 2, 3, and 4 were significantly higher than group 1 ($P< 0.01$), but there was no difference between group 5 and group 1.

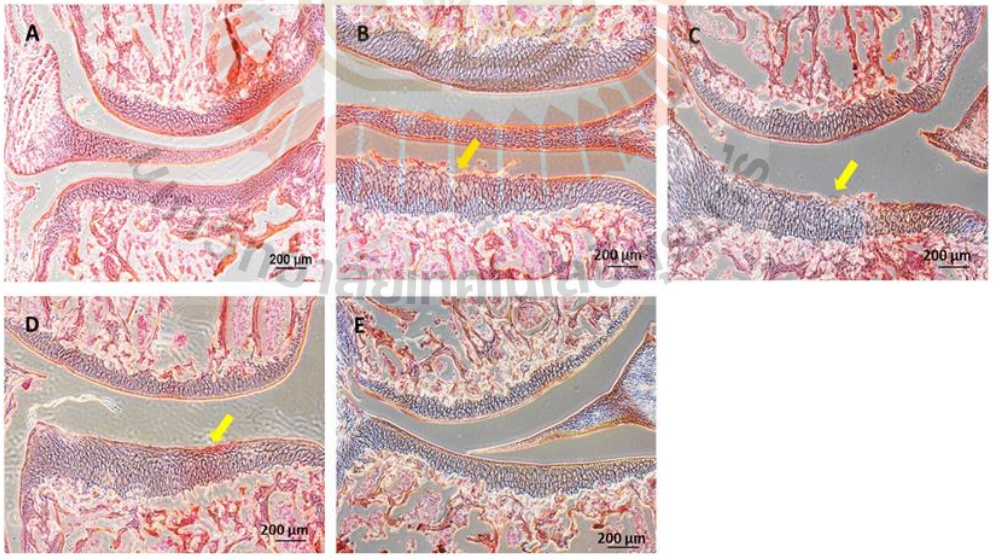


Figure 4.5 Histological examination by H&E staining: (A) group 1; 3 months old, (B) group 2; 7 months old, (C) group 3; 7 months old with HA injection, (D) group 4; 7 months old with HA + MSCs injection, (E) group 5, 7 months old with HA + chondrocyte differentiated cells injection. Scale bar = 200 um.

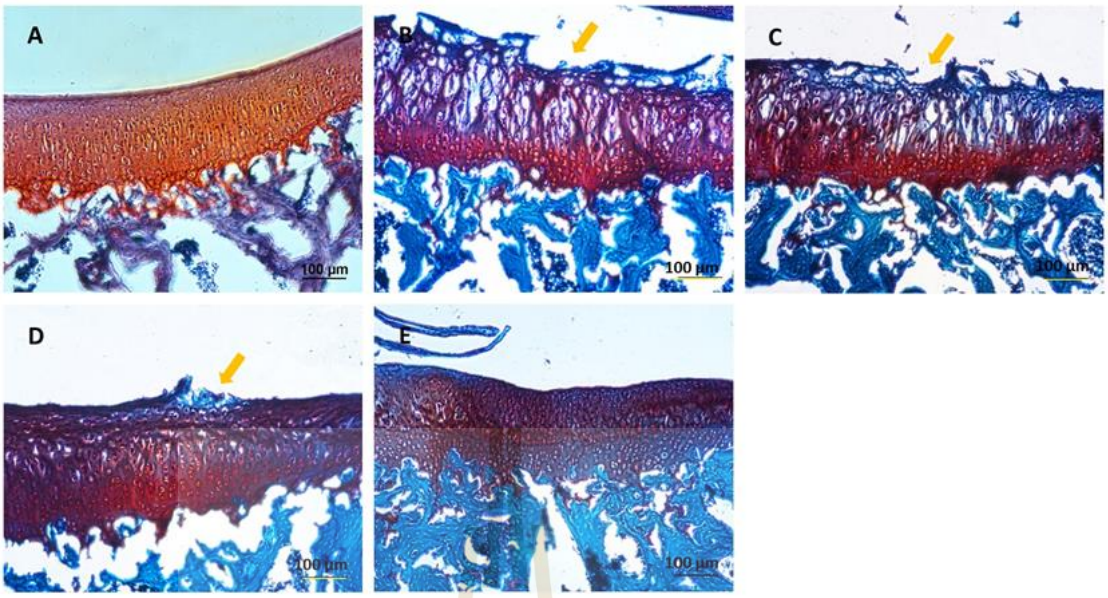


Figure 4.6 Histological examination by Safranin O staining: (A) group 1; 3 months old, (B) group 2; 7 months old, (C) group 3; 7 months old with HA injection, (D) group 4; 7 months old with HA + MSCs injection, (E) group 5, 7 months old with HA + chondrocyte differentiated cells injection. Scale bar = 100 μm.

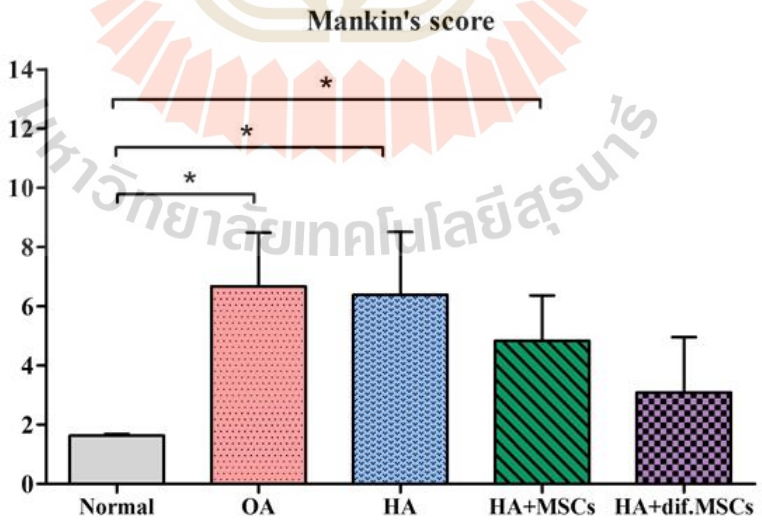


Figure 4.7 Cartilage damage scores based on the Mankin criteria (* *P*< 0.01).

4.4.4 Immunohistochemistry results

collagen (Figure 4.8). The area with a high type II collagen expression will be darkened. The cartilage of the group 1 showed high type II collagen expression, dark colored covering over the cartilage. Even though the cartilage of group 2-5 showed unequal expression, there was no difference between among groups.

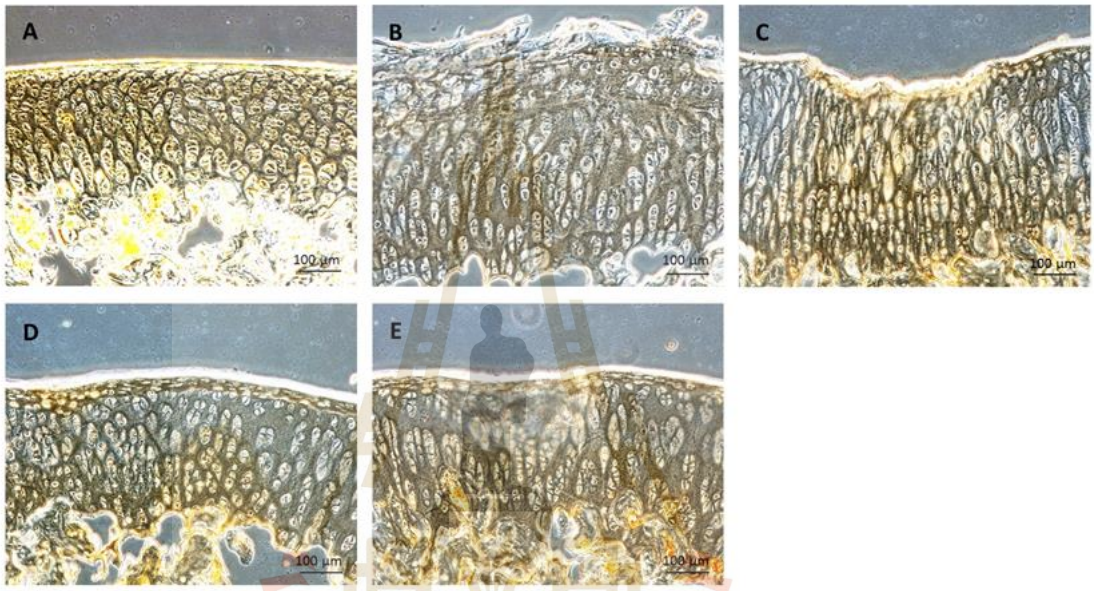


Figure 4.8 Immunohistochemistry for type II collagen: (A) group 1; 3 months old, (B) group 2; 7 months old, (C) group 3; 7 months old with HA injection, (D) group 4; 7 months old with HA + MSCs injection, (E) group 5, 7 months old with HA + chondrocyte differentiated cells injection. Scale bar = 100 um.

4.4.5 Immunoblot results

Total proteins from guinea pig tibia cartilages were extracted and analyzed for type II collagen, type I collagen, MMP13 and β-actin proteins. The results were compared with human knee cartilage. Protein bands isolated by gel electrophoresis and immunoblot are shown in Figure 4.9. Intensity changes of type II collagen, type I collagen and MMP13 versus β-actin protein, acts as an internal control are present in Figure 4.10.

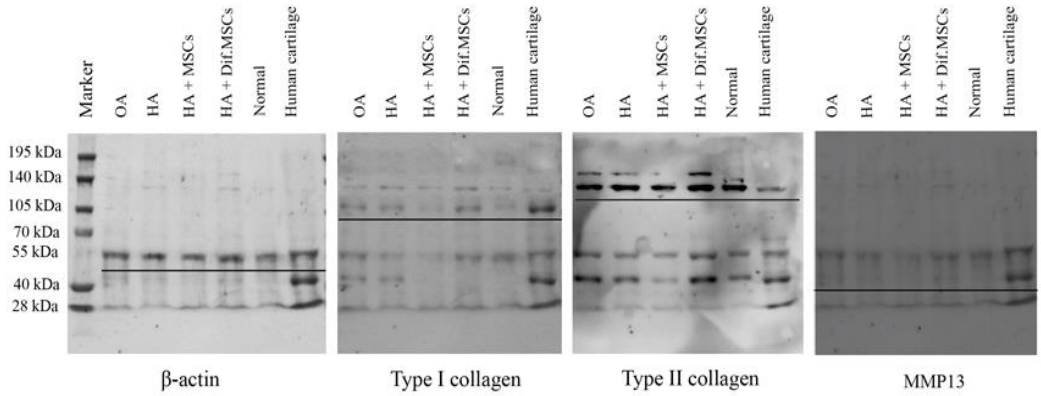


Figure 4.9 Immunoblot analysis after protein bands were isolated by gel electrophoresis.

Intensity changes of Col-II/ β-actin in the cartilage of group 1 were significantly higher than groups 2-3 ($P< 0.05$), but there was no difference among group 1 and group 4 and group 5. The results of the intensity changes of Col-I/ β-actin, on the other hand, revealed no difference in cartilage in all groups. For the results of human cartilage with severe osteoarthritis, the intensity changes of Col-II/ β-actin in cartilage were lower than all groups of guinea pigs. However, the intensity changes of Col-I/ β-actin in human cartilage were greater than in all other groups. MMP13/ β-actin intensity changes were similarly low in all guinea pig groups and lower than in human osteoarthritis cartilage. The highest Col-II/Col-I protein expression was seen in group 1. Col-II/Col-I protein expression in human osteoarthritis cartilage was very low and lower than those in guinea pigs in all experimental groups.

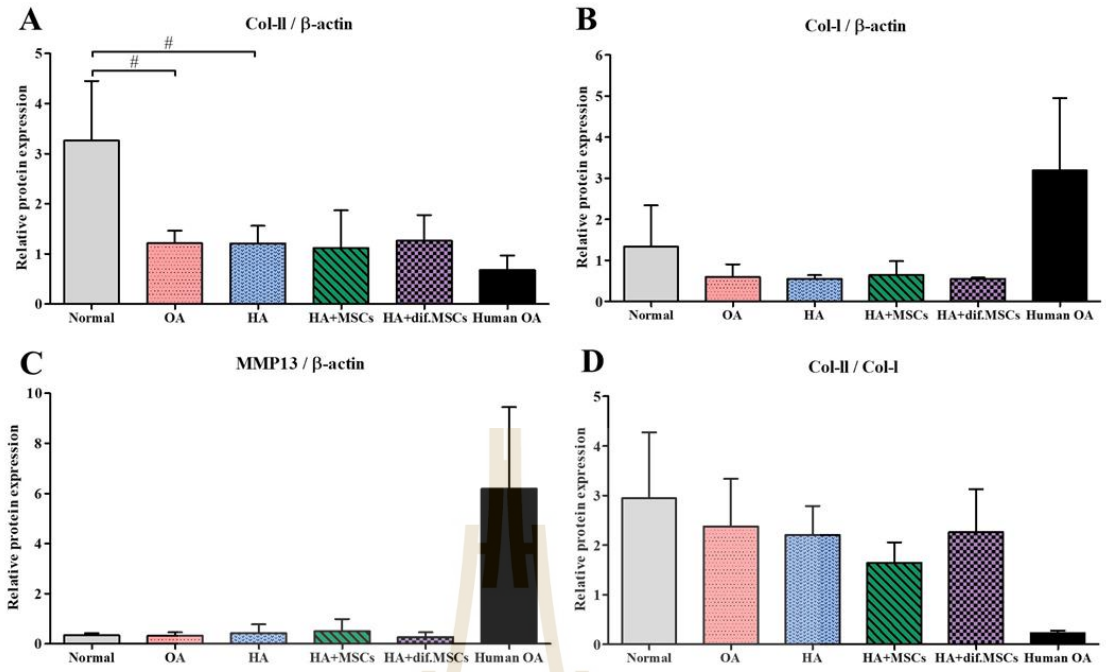


Figure 4.10 (A) Intensity changes of type II collagen, (B) type I collagen and (C) MMP13 proteins in guinea pig cartilage and human cartilage with osteoarthritis, compared with β-actin protein used as an internal control (#$P< 0.05$), and (D) intensity changes of type II collagen/type I collagen in guinea pig cartilage and human cartilage with osteoarthritis.

4.5 Discussion

The current study compared the effects of cell transplantation for knee osteoarthritis between MSCs and MSCs-derived chondrocytes. It was observed that induction of cartilage cells led to the production of hypertrophic chondrocytes, a common type of cartilage cell found in osteoarthritis patients in the long term (Armiento et al., 2019; Ding et al., 2012). In this work, MSCs were induced into cartilage cells in the early stage of chondrogenic differentiation for 14 days. At day 14, the gene expression of Sox9 and β-Catenin increased, while Col10a1 and Runx2, both of which are associated with hypertrophic chondrocyte aging in cartilage, did not express (Armiento et al., 2019; Ding et al., 2012). In this study, Dunkin Hartley guinea pigs were used, as they are prone to develop osteoarthritis with age. The normal articular cartilage of three-month-old guinea pigs was compared to that of seven-month-old guinea pigs with early-stage osteoarthritis (Kraus et al., 2010). The

knees of the guinea pigs were injected with either cell-free HA (Hyruan®III), or with either MSCs or chondrogenic differentiated cells. In the study, it was observed that seven-month-old guinea pigs that received only one injection of cell-free HA did not show any significant difference compared to the not-injected group. HA-based formulations, viscosupplements, are intended to recover the rheological properties of the synovial fluid, resulting in improvement of pain and joint mobility (Salgado et al., 2021). Chemically modified HA improved mechanical performance during high-frequency solicitation and showed a prolonged viscosupplementation effect, compared to the unmodified, linear HA-based product (La Gatta et al., 2021). However, the guinea pigs that received injections of HA containing either MSCs or early cartilage-differentiated cells showed a reduction in osteoarthritis. The injected cells adhered to the cartilage surface, thereby repairing the damaged cartilage, and making it smoother, similar to normal cartilage. Histological analysis showed that the cartilage tissue in the injected cells had a smoother surface compared to the non-injected group. It was concluded that using HA containing early chondrogenic differentiated cells from MSCs could be an effective method for restoring articular cartilage and could be more effective than using MSCs alone for treating osteoarthritis. Inflammatory responses made of xenogeneic/allogenic materials in case of cells or organ transplantation are notable problems. Allogeneic chondrocytes seeded on xenogeneic scaffolds did not suppress graft inflammation, but induced variable inflammatory responses involving mast cells and macrophages (Klabukov et al., 2023). Although allogenic MSCs were shown to support cartilage regeneration and decrease the symptoms of OA (de Windt et al., 2017), the inflammatory responses from xenogeneic MSCs and MSCs-derived chondrocytes remained poor studied and should be further investigated.

4.6 Conclusions

For transplantation, MSCs-derived early stage chondrocytes were intra-articular injected into the knee joints of 7-month old guinea pigs which has symptom of early spontaneous osteoarthritis. After transplantation for 5 weeks, the joints were collected. The results from the injection of MSCs-derived early stage chondrocytes were compared with those from the injection of undifferentiated MSCs and the

injection of Hyaluronic acid. The results revealed that the transplanted cells were integrated into the guinea pig cartilage surfaces and restored the degenerated cartilages. The injection of MSCs-derived early stage chondrocytes recovered the degenerated cartilages better than the injection of undifferentiated MSCs and the injection of Hyaluronic acid. The tissues of the recovered cartilages after the injection of MSCs-derived early stage chondrocytes were resembled to the cartilages of 3-month old guinea pigs, which has no symptom of osteoarthritis, with no significant difference in the Mankin's scores from the histological assessment.

4.7 References

Armstrong, S., Read, R., & Ghosh, P. (1994). The effects of intraarticular hyaluronan on cartilage and subchondral bone changes in an ovine model of early osteoarthritis. **J. Rheumatol.** 21(4): 680-8.

Armiento, A. R., Alini, M., & Stoddart, M. J. (2019). Articular fibrocartilage - Why does hyaline cartilage fail to repair? **Adv. Drug. Deliv. Rev.** 146: 289-305.

Bendele, A. M., & Hulman, J. F. (1988). Spontaneous cartilage degeneration in guinea pigs. **Arthritis Rheum.** 31(4): 561-5.

de Windt, T. S., Vonk, L. A., Slaper-Cortenbach, I. C. M., Nizak, R., van Rijen, M. H. P., & Saris, D. B. F. (2017). Allogeneic MSCs and recycled autologous chondrons mixed in a one-stage cartilage cell transplantation: a first-in-man trial in 35 patients. **Stem Cells.** 35: 1984-93.

Ding, M., Lu, Y., Abbassi, S., Li, F., Li, X., Song, Y., Geoffroy, V., Im, H. J., & Zheng, Q. (2012). Targeting Runx2 expression in hypertrophic chondrocytes impairs endochondral ossification during early skeletal development. **J. Cell. Physiol.** 227(10): 3446-56.

Ebihara, G., Sato, M., Yamato, M., Mitani, G., Kutsuna, T., Nagai, T., Ito, S., Ukai, T., Kobayashi, M., Kokubo, M., Okano, T., & Mochidaet, J. (2012). Cartilage repair in transplanted scaffold-free chondrocyte sheets using a minipig model. **Biomaterials.** 33(15): 3846-51.

Fernandez, M. L., Vergara-Jimenez, M., Conde, K., Behr, T., & Abdel-Fattah, G. (1997). Regulation of apolipoprotein B-containing lipoproteins by dietary soluble fiber in guinea pigs. **Am. J. Clin. Nutr.** 65(3): 814-22.

Kawamoto ,T., & Shimizu, M. A. (2000). method for preparing 2- to 50-µm-thick fresh-frozen sections of large samples and undecalci-fied hard tissues. **Histochem. Cell Biol.** 113: 331-9.

Klabukov, I., Atiakshin, D., Kogan, E., Ignatyuk, M., Krasheninnikov, M., Zharkov, N., Yakimova, A., Grinevich, V., Pryani-kov, P., Parshin, V., Sosin, D., Kostin, A. A, Shegay, P., Kaprin, A. D., & Baranovskii, D. (2023). Post-implantation inflammatory responses to xenogeneic tissue-engineered cartilage implanted in rabbit trachea: the role of cultured chondrocytes in the modification of inflammation. **Int. J. Mol. Sci.** 24: 16783.

Kraus, V. B., Huebner, J. L., DeGroot, J., & Bendele, A. (2010). The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the guinea pig. **Osteoarthritis Cartilage.** 18 (3): 35-52.

La Gatta, A., Stellavato, A., Vassallo, V., Di Meo, C., Toro, G., Iolascon, G., & Schiraldi, C. (2021). Hyaluronan and derivatives: an *in vitro* multilevel assessment of their potential in viscosupplementation. **Polymers.** 13: 3208.

Mankin, H. J., Dorfman, H., Lippiello, L., & Zarins, A. (1971). Biochemical and metabolic abnormalities in articular cartilage from os-teo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data. **J. Bone Joint Surg. Am.** 53(3): 523-37.

Salgado, C., Jordan, O., & Allémann, E. (2021). Osteoarthritis *in vitro* models: Applications and implications in development of in-tra-articular drug delivery systems. **Pharmaceutics.** 13: 60.

Sato, M., Uchida, K., Nakajima, H., Miyazaki, T., Guerrero, A. R., Watanabe, S., Roberts, S., & Baba, H. (2012). Direct transplantation of mesenchymal stem cells into the knee joints of Hartley strain guinea pigs with spontaneous osteoarthritis. **Arthritis Res. Ther.** 14(1): R31.

Tessier, J. J., Bowyer, J., Brownrigg, N. J., Peers, I. S., Westwood, F. R., Waterton, J. C., & Maciewicz, R. A. (2003). Characterisation of the guinea pig model of osteoarthritis by *in vivo* three-dimensional magnetic resonance imaging. **Osteoarthritis and Cartilage.** 11(12): 845-853.

Troyer, D. L., & Weiss, M. L. (2008). Wharton's jelly-derived cells are a primitive stromal cell population. **Stem Cells.** 26(3): 591-599.

Wong, C-C., Ou, K-L., Lin, Y-H., Lin, M-F., Yang, T-L., Chen, C-H., & Chan, W. P. (2020). Characterization Platelet-Rich Fibrin Facilitates One-Stage Cartilage Repair by Promoting Chondrocytes Viability, Migration, and Matrix Synthesis. **Int. J. Mol. Sci.** 21(2): 577.

Yoon, J. H., Roh, E. Y., Shin, S., Jung, N. H., Song, E. Y., Chang, J. Y., Kim, B. J., & Jeon, H. W. (2013). Comparison of explant-derived and enzymatic diges-tion-derived MSCs and the growth factors from Wharton's jelly. **Biomed. Res. Int.** 2013:428726.

CHAPTER V
OVERALL CONCLUSION

At present, the treatments available for osteoarthritis are not sufficiently effective, and many patients with early-stage osteoarthritis show severe symptoms. Knee replacement surgery is an expensive treatment, and many patients are unable to afford it. As a result, there is a need for more effective treatment options for this condition. This study was to differentiate human Wharton's jelly-derived mesenchymal stem cells (hWJ-MSCs) into chondrocytes for transplantation in OA-suffered guinea pigs. hWJ-MSCs were isolated using explant culture method and then their proliferation, phenotypes, and differentiation ability were evaluated. Subsequently, hWJ-MSCs-derived chondrocytes were induced and characterized based on immunofluorescent staining, qPCR, and immunoblotting techniques. Then, early OA-suffered guinea pigs were injected with hyaluronic acid (HA) containing either MSCs or 14-days old hWJ-MSCs-derived chondrocytes. This study demonstrates that early intervention with intra-articular injection of MSCs is effective in preventing the progression of early-stage osteoarthritis to severe osteoarthritis. These results are superior to those of HA injections, which are commonly used to treat early-stage osteoarthritis. In addition, the injection of early-stage chondrogenic differentiated cells from MSCs, rather than MSCs alone or HA, was found to be more effective in treating knee osteoarthritis. Novel method of injecting early-stage cartilage differentiated cells from MSCs was developed in this study and offers a superior treatment option for osteoarthritis compared to undifferentiated MSCs or HA alone. Thus, this approach could serve as a guide for the treatment of early osteoarthritis patients, where injection of chondrogenic differentiated cells into the knee joint could lead to recovery of the cartilage and prevent disease progression to severe osteoarthritis. This study demonstrated that injection of 1×10^6 chondrogenic differentiated cells into the knee joints of guinea pigs was effective for early

osteoarthritis treatment. However, further research is needed to optimize the treatment method for improved efficacy, including cell numbers, solvents, and numbers of injection. Additionally, future studies could investigate the treatment effects on moderate and severe osteoarthritis, ultimately leading to improved treatment options for human osteoarthritis patients.

APPENDIX

APPENDIX

Additionally

Gor, I., **Gulrez, N**., Bataev, H., and Dorofeev, A. (2021). Prevalence and Structure of Periodontal Disease and Oral Cavity Condition in Patients with Coronary Heart Disease (Prospective Cohort Study). **Int. J. Gen. Med.** 14: 8573– 8581. (Q2 Scopus Indexed).

Satishkumar, C.S.C., Nair, S.J., Joseph, A.M., Suresh, S., Muthupandian, I., Kumaresan, S., Ashekhi, A., and **Gulrez, N**. (2021). Relationship between Perceived Chewing Ability, Oral Health Related Quality of Life and Depressive Symptoms among Completely Edentulous Individuals. **Indian J. Dent. Res.** 32: 211-215. (Q3 Scopus Indexed)

Samuel, S.R., Kuduruthullah, S., Khair, A.M.B., Shayeb, M.A., Elkaseh, A., Varma, S.R., **Gulrez, N**., Elkhader, I.A., and Ashekhi, A. (2021). Impact of pain, psychological-distress, SARS-CoV2 fear on adults'OHRQOL during COVID-19 pandemic. **Saudi J Biol Sci.** 28: 492–494. (Q1* Scopus Indexed)

Kaseh, A.E., Shayeb, M.A., Kuduruthullah, S and **Gulrez, N**. (2021). The Retromolar Space and Wisdom Teeth in Humans: Reasons for Surgical Tooth Extraction, European Journal of Dentistry. **Saudi J Biol Sci.** 15(1): 117-121. (Q2 Scopus Indexed)

Khan, Z., **Gulrez, N**., Khan, H., and Khair, A-M.B. (2021). An anatomical study of orbital dimensions and its utility in orbital reconstructive surgery. **Oncology and Radiotherapy.** 15(3): 1-9. (Q4 Scopus Indexed)

Shayeb, M.A., Fathy, E., **Gulrez, N**., El-Sahn, N.A., Elsahn, H., Khader, I.E., and Habbal, A.W.A. (2020). Prevalence of most common tongue lesions among a group of UAE population: Retrospective study. **Oncology and Radiotherapy.** 1(46): 01-05. (Q4 Scopus Indexed)

Gulrez, N. (2018). A Morphometric Analysis of the Shape, Position, Number and Size of Mental Foramen in dry Human Mandibles. **Int. j. anat. res.** 6(3.1):5424-5430. (Index Copernicus)

Gulrez, N. (2016). A Study of the Clinico-Anatomical Variations in the Shape and Size of Gallbladder. **J. Morphol. Sci.** 33(2): 62-67. (Q4 Scopus Indexed)

Gulrez, N. (2014). Importance of Knowing the Level of Sacral hiatus for Caudal Epidural Anesthesia. **J. Morphol. Sci.** 31(1): 9-13. (Q4 Scopus Indexed)

Gulrez, N. (2013). Can Fetal Ossicle be used as Prosthesis in Adults- A Morphometric Study. **Int. J. Exp. & Clin. Anat.** 7(1): 52-57. (Q4 Scopus Indexed)

Gulrez, N. (2010). Anatomical Variations in the Extra-Hepatic Biliary Apparatus. **Int. J. Exp. & Clin. Anat.** 4(1):86. (Q4 Scopus Indexed)

Gulrez, N. (2004). Digital Anatomy- Its Utility and Future. **Indian J. Bio-Res.** 56 (2): 98-101.

Gulrez, N., and Faruqi, F. (2003). Morphometric Analysis of Malleus in Human Fetuses during Prenatal Period. **Indian J. Bio-Res.** 53(2): 93-101.

Gulrez, N., Faruqi, F., and Ahmad, A. (2003). Dimentional Analysis of Incus in Human Fetuses during Prenatal Period. **Indian J. Bio-Res.** 50(3): 99-105.

Gulrez, N., Faruqi, F., and Ahmad, A. (2003). Studies on Morphometry of Stapes in Human Fetuses during Prenatal period. **Indian J. Bio-Res.** 50(3): 81-89

BIOGRAPHY

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