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

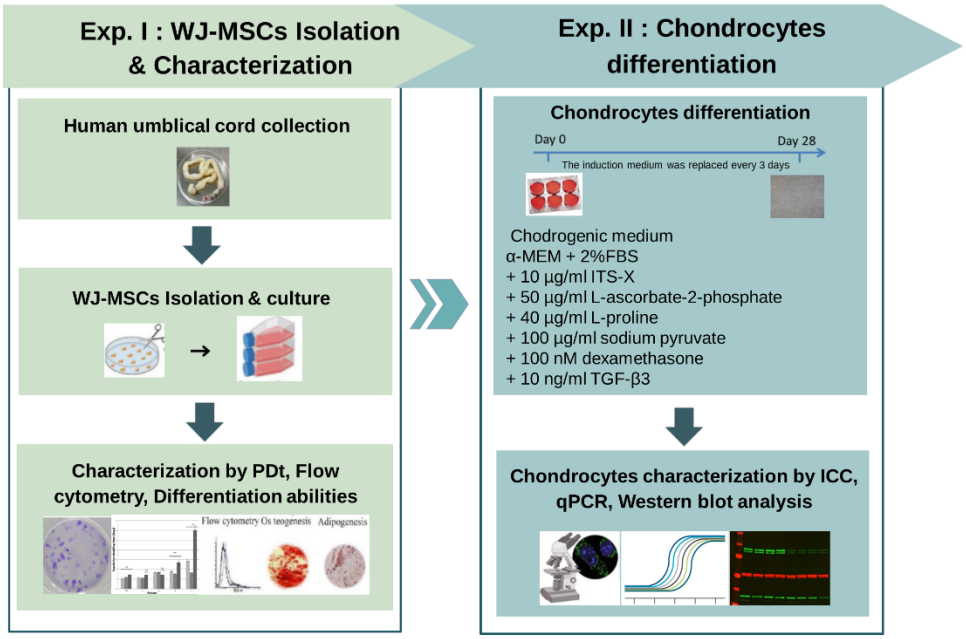
3.3.1 Ethics Statement

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	111	Bwalya et al.,
		R: TCTCGTGCCAGATCATCACC		2018
Sox9	000346.4	F: ACACACAGCTCACTCGACCTTG	103	Tao et al., 2016
		R: GGGAATTCTGGTTGGTCCTCT		
Col2a1	001844.4	F: GAGACAGCATGACGCCGAG	67	Tanthaisong et
		R: GCGGATGCTCTCAATCTGGT		al., 2017
Col10a1	000493.4	F: CCCTCTTGTTAGTGCCAACC	155	Tanthaisong et
		R: AGATTCCAGTCCTTGGGTCA		al., 2017
Runx2	001015051.4	F: ATACCGAGTGACTTTAGGGATGC	131	Tanthaisong et
		R: AGTGAGGGTGGAGGGAAGAAG		al., 2017
ß-catenin	001330729.2	F: AATGCTTGGTTCACCAGTG	176	Tanthaisong et
		R: GGCAGTCTGTCGTAATAGCC		al., 2017
GAPDH[*]	002046.7	F: TGCACCACCACCTGCTTAGC	87	Kee et al., 2018
		R: GGCATGGACTGTGGTCATGAG		

[*]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, ß-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.

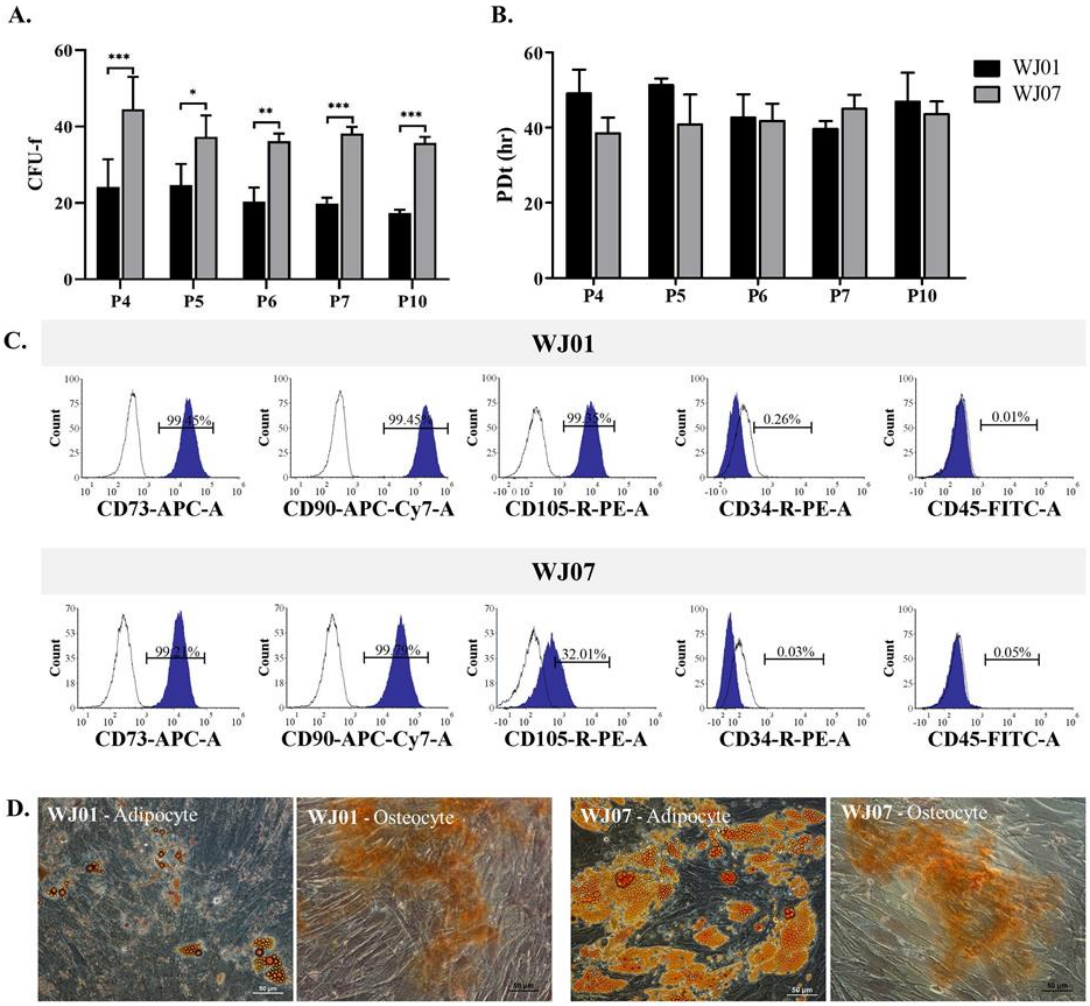


Figure 3.1 Characterization of hWJ-MSCs. (A) Colony forming unit results from WJ01 and WJ07 cell line at passage 4, 5, 6, 7 and 10 (* $P< 0.05$, ** $P< 0.01$, *** $P< 0.001$). (B) Population doubling time results of WJ01 and WJ07 cell line. (C) Cell surface marker expression of WJ01 and WJ07 cell lines.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

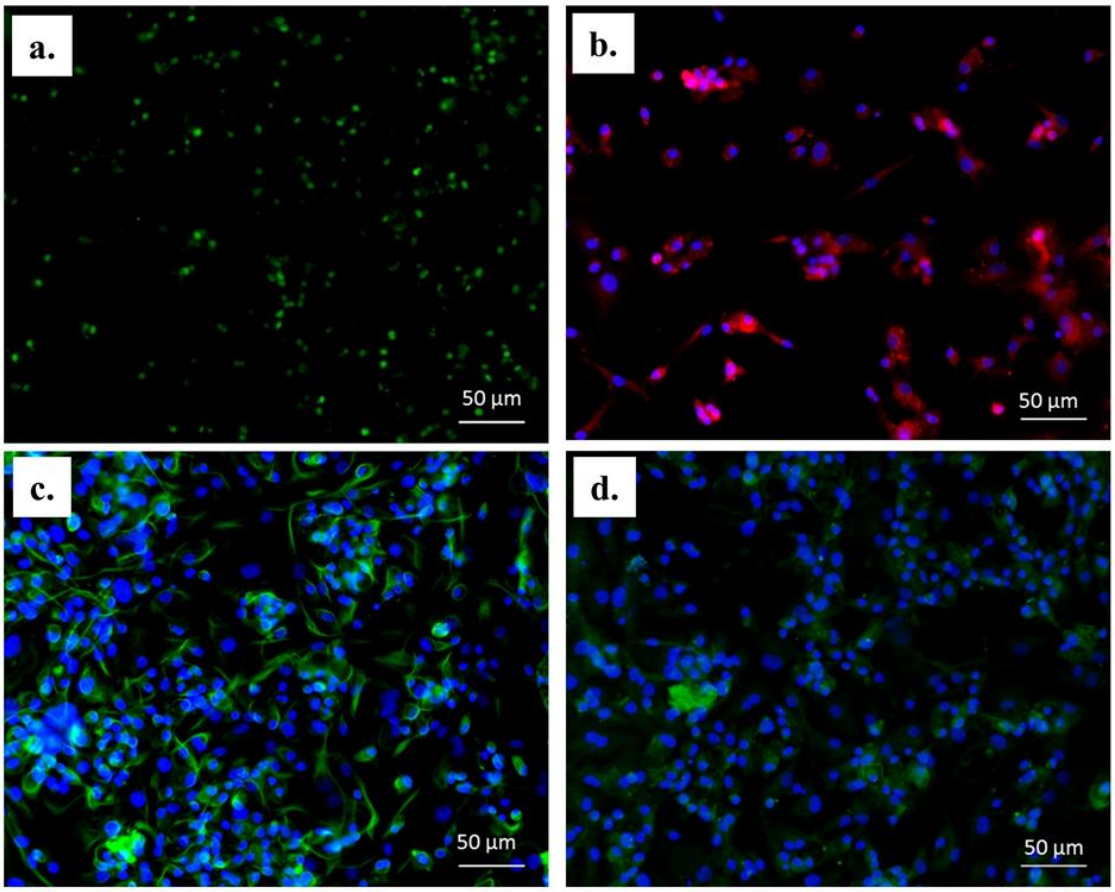


Figure 3.2 Chondrocyte characterization by ICC. (A) Sox9 (green; nuclear marker). (B) Aggrecan (red). (C) type II collagen (green). (D) type X collagen (green). Scale bar = 50 µm.

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). *ß-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 *ß-catenin* gene expression between differentiated cells at day 3, 7 and 14. However, level of *ß-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.

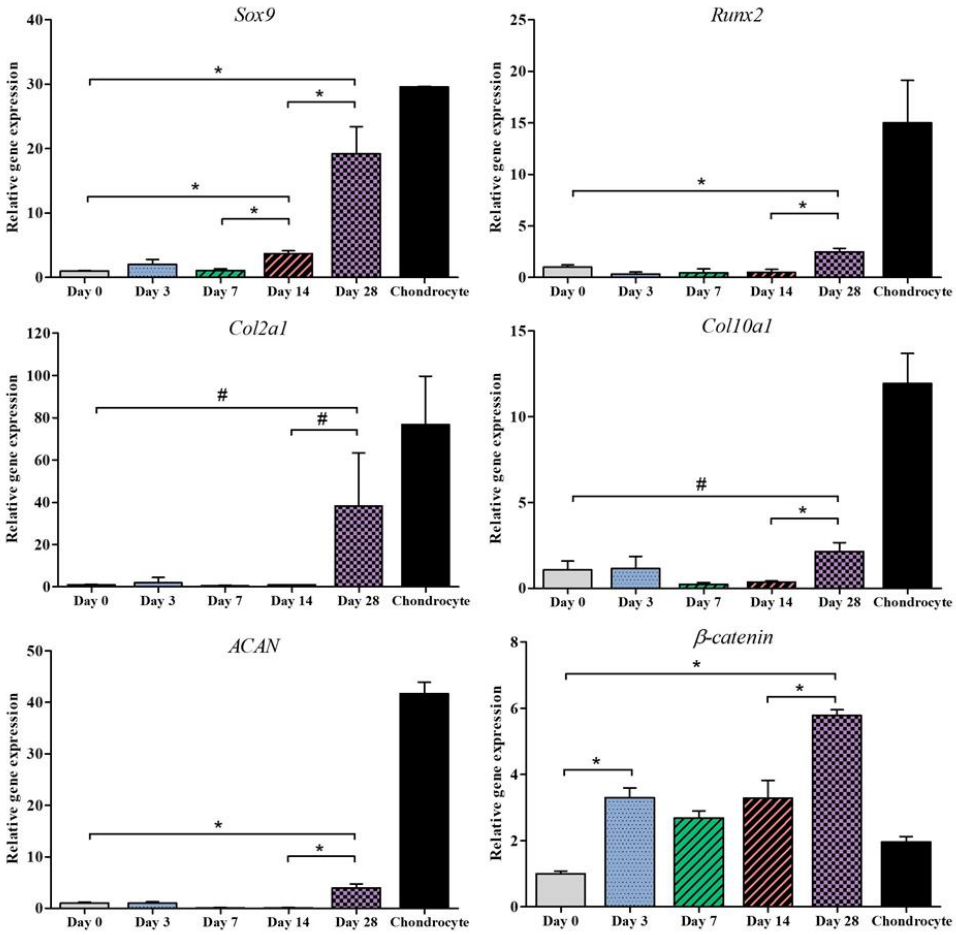


Figure 3.3 Gene expression analysis of chondrocyte by qPCR, *Sox9*, *Runx2*, *Col2a1*, *Col10a1*, *ACAN* and *ß-Catenin* genes. The targeted gene was normalized to *GAPDH* as a reference gene and calculated the relative expression compared to each group. (*$P< 0.01$, #$P< 0.05$).

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).

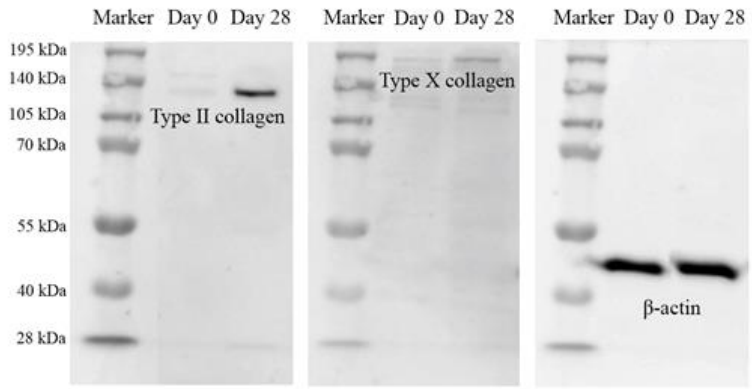


Figure 3.4 Type X collagen protein expression analysis of chondrocyte differentiated cells on day 28 by immunoblot and β-actin protein was used as an internal control.

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 i*n 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.