2018

Systems biology approach for the establishment of veterinary bone tissue engineering: the proteomics of cBM-MSCs and cDPSCs osteogenic models

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SYSTEMS BIOLOGY APPROACH FOR THE ESTABLISHMENT OF VETERINARY BONE TISSUE ENGINEERING: THE PROTEOMICS OF cBM-MSCs AND cDPSCs OSTEOGENIC MODELS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Veterinary Biosciences
Department of Veterinary Anatomy
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2018

ชีววิทยาเชิงระบบเพื่อพัฒนาวิศวกรรมเนื้อเยื่อกระดูกในทางสัตวแพทย์: การศึกษาโปรตีโอมิกส์ของต้นแบบเซลล์กระดูกจากเซลล์ต้นกำเนิดมีเซ็นไคม์จากไขกระดูกและเนื้อเยื่อในโพรงประสาทฟันของสุนัข

นางศิริรัตน์ นันทวิสัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวศาสตร์ทางสัตวแพทย์ ภาควิชากายวิภาคศาสตร์
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2561

Thesis Title	SYSTEMS BIOLOGY APPROACH FOR THE ESTABLISHMENT OF VETERINARY BONE TISSUE ENGINEERING: THE PROTEOMICS OF cBM-MSCs AND cDPSCs OSTEOGENIC MODELS
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เซลล์ต้นกำเนิดมีเซ็นไคม์ของสุนัขซึ่งเป็นเซลล์ต้นกำเนิดมัลติโพเทนต์แสดงความเป็นไปได้ในการนำมาใช้เพื่อการรักษาโรคกระดูกผิดปกติ แม้ว่าเซลล์ต้นกำเนิดมีเซ็นไคม์จากเนื้อเยื่อหลายแหล่งที่มาจะแสดงคุณลักษณะที่คล้ายคลึงกันก็ตาม การแสดงศักยภาพในการเปลี่ยนแปลงจากเซลล์ต้นกำเนิดมีเซ็นไคม์ไปเป็นเซลล์กระดูกนั้นมีความแตกต่างกันตามชนิดของเนื้อเยื่อเซลล์ต้นกำเนิด การศึกษานี้ได้ถูกออกแบบที่จะค้นหาและวิเคราะห์ศักยภาพในการเปลี่ยนแปลงเซลล์ต้นกำเนิดมีเซ็นไคม์จากไขกระดูกสุนัขและเซลล์ต้นกำเนิดมีเซ็นไคม์จากเนื้อเยื่อในโพรงประสาทฟันสุนัขไปเป็นเซลลล์กระดูกในระดับห้องปฏิบัติการ การศึกษานี้เริ่มต้นด้วยการเปรียบเทียบความสามารถการทำงานของเอนไซม์อัลคาไลน์ฟอสฟาเทสและการย้อมสีวอนคอตซ่า ต่อไปเซลล์กระดูกที่แปรสภาพมาจากเซลล์ต้นกำเนิดทั้ง 2 ชนิดจากการถูกเหนี่ยวนำให้เป็นเซลล์กระดูกในวันที่ 7 และ 14 ถูกวิเคราะห์ด้วยวิธีชีววิทยาเชิงระบบในระดับโปรตีนด้วยวิธีโปรตีโอมิคส์ร่วมกับวิธีการติดฉลากไดเมททิลซึ่งถูกนำมาเปรียบเทียบกับเซลล์ต้นกำเนิดที่ไม่ได้ถูกเหนี่ยวนำให้แปรสภาพ ผลการศึกษาพบว่าเซลล์ต้นกำเนิดมีเซ็นไคม์จากไขกระดูกสุนัขและเซลล์ต้นกำเนิดมีเซ็นไคม์จากเนื้อเยื่อในโพรงประสาทฟันสุนัขสามารถถูกเหนี่ยวนำเพื่อเปลี่ยนแปลงเป็นเซลล์กระดูกได้แต่ศักยภาพต่างกัน การศึกษาโปรติโอมิคส์พบว่าเซลล์ต้นกำเนิดจากเนื้อเยื่อทั้งสองชนิดนี้แสดงระดับโปรตีนแตกต่างกันทั้งในด้านการส่งสัญญาณ , สารเคลือบเซลล์, วงจรเซลล์, เมทาบอลิซึมและการขนส่งโมเลกุล ซึ่งล้วนแล้วแต่มีความเกี่ยวข้องในกระบวนการสร้างกระดูก นอกจากนี้ผลจากการวิเคราะห์ข้อมูลและผลการยืนยันการทำหน้าที่ของเซลล์แสดงให้เห็นว่ามี 4 สัญญาณที่สำคัญต่อการเปลี่ยนแปลงไปเป็นเซลล์กระดูกของเซลล์ต้นกำเนิดมีเซ็นไคม์จากไขกระดูกสุนัขและเซลล์ต้นกำเนิดมีเซ็นไคม์จากเนื้อเยื่อในโพรงประสาทฟันสุนัขได้แก่ สัญญาณวินต์, สัญญาณนอท, สัญญาณโบนมอร์โฟจินิติกโปรตีนและสัญญาณทรานสฟอร์มมิ่งโกรทแฟคเตอร์ ผลการศึกษานี้สามารถนำมาใช้เป็นข้อมูลความรู้ของการเปลี่ยนแปลงเป็นเซลล์กระดูกจากเซลล์ต้นกำเนิดมีเซ็นไคม์จากไขกระดูกสุนัขและเซลล์ต้นกำเนิดมีเซ็นไคม์จากเนื้อเยื่อในโพรงประสาทฟันสุนัข นอกจากนี้ยังสามารถนำมาใช้เป็นความรู้พื้นฐานและแนวโน้มในการนำเซลล์ต้นกำเนิดมีเซ็นไคม์มาใช้เพื่อการพัฒนาเนื้อเยื่อวิศวกรรมกระดูกเพื่อใช้ในการรักษาในการทดแทนเนื้อเยื่อกระดูกต่อไป

สาขาวิชา ชีวศาสตร์ทางสัตวแพทย์ ลายมือชื่อนิสิต .....

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5775522731 : MAJOR VETERINARY BIOSCIENCES

KEYWORD: canine bone-marrow derived mesenchymal stem cells (cBM-MSCs), canine dental pulp stem cells (cDPSCs), osteogenic differentiation, systems biology

Sirirat Nantavisai : SYSTEMS BIOLOGY APPROACH FOR THE ESTABLISHMENT OF VETERINARY BONE TISSUE ENGINEERING: THE PROTEOMICS OF cBM-MSCs AND cDPSCs OSTEOGENIC MODELS. Advisor: Dr. Chenphop Sawangmake, D.V.M., M.SC., Ph.D. Co-advisor: Asst. Prof. Dr. SIRAKARNT DHITAVAT, D.V.M., M.SC., Ph.D.,Assoc. Prof. CHANIN KALPRAVIDH, D.V.M.

The utilization of canine mesenchymal stem cells (cMSCs) with multipotent capabilities has been regarded for possible therapy of incorrigible bone disease. Although various sources of cMSCs show similar characteristics, they are different in osteogenic potential due to their original cellular sources. This study was designed to globally explore and analyze the *in vitro* differentiation potential and behavior of canine bone-marrow derived mesenchymal stem cells (cBM-MSCs) and canine dental pulp stem cells (cDPSCs) toward osteogenic lineage. An *in vitro* osteogenic differentiation potential of the cells was preliminarily compared in terms of alkaline phosphatase activity assay and *Von Kossa* staining. Global study of an *in vitro* osteogenic differentiation potential of the isolated cells was performed using proteomic-based analysis through mass spectrometry with dimethyl labelling method at day 7 and 14 post-induction, comparing with undifferentiated cells. The result presented that cBMSCs and cDPSCs contained osteogenic differentiation potential but had differences in their alkaline phosphatase activity level and mineralization. Proteomics profiling revealed that cBM-MSCs and cDPSCs showed the differences in their protein expression of signaling pathways, extracellular matrix organization, cell cycle, metabolism, transport of small molecules, and vesicle-mediated transport which have been shown to involve in bone regeneration mechanisms. Basing on database analysis and functional assay confirmation, there were four potential osteogenic-regulating pathways; Wnt signaling, Notch signaling, bone-morphogenetic protein (BM-related signaling and transforming growth factor (TGF-related signaling, which played the crucial regulating of cBM-MSCs and cDPSCs toward osteogenic lineage. The obtained results could be used as a comprehensive data and principal knowledge of the osteogenic differentiation potential of cBM-MSCs and cDPSCs *in vitro* and the trend of MSC-based tissue engineering for osteogenic regenerative therapy, concentrating on cMSCs application.

Field of Study:	Veterinary Biosciences	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....
		Co-advisor's Signature .....
		Co-advisor's Signature .....

ACKNOWLEDGEMENTS

This research work was financially supported by the 100th Anniversary of Chulalongkorn University Doctoral Scholarship and the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund), Chulalongkorn University.

I would like to acknowledge the chairman of thesis committee, Professor Marrissak Kalpravich (D.V.M., M.Sc., Ph.D.), members of thesis committee, Professor Thanaphum Osathanon (D.D.S., Ph.D.), Assistant Professor Theerawat Tharasanit (D.V.M., Ph.D.), and Associate Professor Pakpoom Kheolamai (M.D., Ph.D.), for kindly providing useful comments to the study.

For the success of this research work, I would like to express my deepest gratitude to my thesis advisor and co-advisors, Associate Professor Chanin Kalpravich (D.V.M., M.Sc.), Assistant Professor Sirakarnt Dhitavat (D.V.M., Ph.D.), and Dr. Chenphop Sawangmake (D.V.M., Ph.D.). Their advice and care helped me navigate all the problems until this successful day.

For the genuine friendship, I would like to thank all staffs and graduate students of the Veterinary Bioscience Graduate Program, Faculty of Veterinary Science; the Veterinary Stem Cell and Bioengineering Innovation Center (VSCBIC), Faculty of Veterinary Science; the Oral Biology Graduate Program, Faculty of Dentistry; and the Research Unit of Mineralized Tissue (RUMT), Faculty of Dentistry, Chulalongkorn University for all help and support.

For my lovely family, I would like to express my deep gratitude to my father, mother, siblings, husband, and kid for the endless love, support, understanding, and encouragement.

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CHAPTER I

INTRODUCTION

Importance and Rationale

Surgical reconstruction of bone defects is a significant challenge for orthopedic surgeon, especially a non-union or fibrous tissue forming in a large bone defect. The standard method to treat bone defects are bone grafts using autogenic, allogenic, or xenogenic transplant (1). However, the treatment result is varied and leads to donor morbidities (2) . From this reason, molecular and cellular studies have been introduced and intensively studied to solve the problems.

Study of bone tissue engineering comprises various aspects of osteogenic cell resources, biomaterials and scaffolds, and signaling molecule enhancing osteogenesis. The previous study found that canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) exhibited potential in bone engineering for repairing canine mandibular bone defects (3, 4). Moreover, alternative stem cell source

derived from dental tissues, namely canine dental pulp stem cells (cDPSCs) has been isolated and investigated. In this regard, stem cells from dental pulp showed the desired abilities of osteogenic differentiation and bone tissue regeneration (5). Thus, the properties of stem cells derived from bone marrow and dental pulp suggest the possibility of application in bone defect regeneration. However, studies regarding osteogenic differentiation potential of cBM-MSCs and cDPSCs are still lacking. This creates a gap in the research establishing MSC-based bone tissue engineering in veterinary practice.

Systems biology is one of the most powerful biological disciplines and it has already contributied to a radical transformation to the field of of molecular life science and biomedicine. It provides a global and less biased view of bone tissue engineering. The large-scale analysis of proteins by mass spectrometry-based proteomics has been continuously developed continuously for deep biology researches. Many studies demonstrated the importance of proteomics on bone tissue engineering, by which proteomics experiments and computational analyses thoroughly explain the signaling

dynamics and the internal cellular responses (6, 7). However, the veterinary field still lacks studies regarding extensive clarification of osteogenic differentiation paths of cBM-MSCs and cDPSCs toward osteogenic lineages. Thus, this study aimed to globally explore and analyze the *in vitro* differentiation potential and behavior of cBM-MSCs and cDPSCs toward osteogenic lineage using the systems biology approach.

Objectives of the study

Regarding the lacked knowledge described above, this study was directed to elucidate the potential and behavior of two important cMSCs (cBM-MSCs and cDPSCs) on the differentiation toward osteogenic lineage *in vitro*. In addition, the crucial of osteogenic-regulating pathways *in vitro* were evaluated.

Objective 1) "To isolate and characterize canine bone-marrow derived mesenchymal stem cells (cBM-MSCs) and canine dental pulp stem cells (cDPSCs) using for veterinary bone tissue engineering establishment". Strategies: 1a) To isolate canine bone-marrow derived mesenchymal stem cells (cBM-MSCs) and canine dental pulp stem cells (cDPSCs). **1b)** To characterize canine bone-marrow derived mesenchymal stem cells (cBM-MSCs) and canine dental pulp stem cells (cDPSCs) using for veterinary bone tissue engineering establishment.

Objective 2) "To globally explore and analyze the *in vitro* differentiation potential and behavior of canine bone-marrow derived mesenchymal stem cells (cBM-MSCs) and canine dental pulp stem cells (cDPSCs) toward osteogenic lineage using systems biology approach". Strategies: 2a) Preliminary comparison of an *in vitro* osteogenic differentiation potential by canine bone-marrow derived mesenchymal stem cells (cBM-MSCs) and canine dental pulp stem cells (cDPSCs). **2b)** Globally explore, analyze, and compare the *in vitro* osteogenic differentiation potential and behavior of canine bone-

marrow derived mesenchymal stem cells (cBM-MSCs) and canine dental pulp stem cells (cDPSCs).

Objective 3) "To confirm the selected potential osteogenic-regulating pathways that govern the *in vitro* osteogenic differentiation paths by canine bone-marrow derived mesenchymal stem cells (cBM-MSCs) and canine dental pulp stem cells (cDPSCs)".

Strategies: 3a) Selection of potential osteogenic-regulating pathways that govern the *in vitro* osteogenic differentiation path by canine bone-marrow derived mesenchymal stem cells (cBM-MSCs) and canine dental pulp stem cells (cDPSCs). **3b)** Confirmation of the selected potential osteogenic-regulating pathways that govern the *in vitro* osteogenic differentiation path by canine bone-marrow derived mesenchymal stem cells (cBM-MSCs) and canine dental pulp stem cells (cDPSCs).

Keywords (Thai): เซลล์ต้นกำเนิดมีเซ็นไคม์จากไขกระดูกสุนัข เซลล์ต้นกำเนิดมีเซ็นไคม์จากเนื้อเยื่อในโพรงประสาทฟันสุนัข การเปลี่ยนแปลงเป็นเซลล์กระดูก ชีววิทยาเชิงระบบ

Keywords (English): canine bone-marrow derived mesenchymal stem cells (cBM-MSCs), canine dental pulp stem cells (cDPSCs), osteogenic differentiation, systems biology

Hypothesis

Canine bone-marrow derived mesenchymal stem cells (cBM-MSCs) and canine dental pulp stem cells (cDPSCs) possess distincts *in vitro* osteogenic differentiation potential relying on their unique osteogenic differentiation paths.

CHAPTER II

LITERATURE REVIEW

Bone tissue engineering in veterinary medicine

Currently, the repair critical-sized bone defects in animal cases is challenging for the veterinary orthopedic surgeon. Autogenous bone graft is the gold standard to treat critical-sized bone defects. In this regard, iliac crest is regularly considered as a bone harvesting site. However, critical complications in autogenous bone graft have been reported, including donor site morbidity, chronic pain, and the risk of infection (8, 9). Although the various technical reports have suggested the possibility of employing allograft and xenograft, the risks of recipient infection and immunogenicity are still widely debated. Therefore, bone tissue engineering (BTE) has been introduced and developed to synthesize the biomimetic bone tissue. The fundamental components of BTE are osteogenic cell resources, signaling molecules, and bioactive scaffold. All of the three core elements propose to develop the bioactive and biomimetic bone tissue.

Nowadays, the promising future of BTE research is attractive for the researcher to deeply explore its probable use in humans and animals. In the veterinary field, BTE has implemented progressively both *in vitro* and *in vivo*. The previous studies reported the success for bone regeneration by using the tissue-engineered scaffold (10, 11). Moreover, the advancement of enhancing molecules has been evolved from the last decade (12, 13). In addition, the isolation of osteogenic stem cells from several parts of the body are employed to provide a regenerative regimen with promising innovations. Due to their ability to differentiate toward several cell types, ESCs and induced pluripotent stem cells (iPSCs) are attractive for osteogenic cellular therapies. However, ESCs and iPSCs require the issue for collecting and the trouble for culture condition. In addition, the ability of ESCs and iPSCs are lacking to dedicate the differentiation pathway resulting the tumorigenicity that is the critical consideration of ESCs and iPSCs. Then, the MSCs from adult tissue are prospects and interesting to address and overcome these problems.

MSC-based bone tissue engineering

To obtain an efficient MSC-based tissue engineering, three main components are experimentally optimized i.e. scaffolds, osteogenic cells, and signaling or enhancing molecules (14). In this regard, osteogenic cell resources are of attention. Adult stem cells (ASCs), which are derived from mature tissues, are of interest due to their plasticity and availability. Normally, the standard of osteogenic cell source is BM-MSCs that are multipotent cells isolated from bone marrow aspiration and found in multiple species including humans, mouses and canines. The characterization of human BM-MSCs have been illustrated as *CD10+*, *CD13+*, *CD29+*, *CD44+*, *CD59+*, *CD71+*, *CD73+*, *CD90 (Thy1)+*, *CD105+*, *CD106 (VCAM)+*, *CD146+*, *CD166 (ALCAM)+*, *STRO-1+*, *CD11a-*, *CD14-*, *CD19-*, *CD31 (PECAM)-*, *CD34 (C-18)-*, *CD45-*, *CD48-*, *CD135-*, and *HLA-DR-* (15-17). To compare with canine resources, the previous studies found that canine BM-MSCs (cBM-MSCs) express stemness markers (e.g. *Rex1*, *Nanog*, and *Oct3/4*, etc.) and surface markers (e.g. *CD44*, *CD73*, *CD90*, *CD105*, *CD146*, and *STRO-1*, etc.) referring to their characteristics of MSCs (18, 19). In addition, cBM-MSCs

exhibited potential property for bone tissue engineering, as illustrated in canine mandibular bone defects repair (3). As previously described, cBM-MSCs have been proposed as a potential cell resource for using in MSC-based bone tissue engineering (14). However, bone marrow collecting technique is considered as an invasive protocol that may cause donor morbidity (20).

To address the problem, other MSCs types have been introduced as the candidates for a replacement technique. Among these, alternative stem cell sources derived from dental tissues have been isolated and investigated. The previous studied illustrated that human dental-MSCs including human dental pulp stem cells (hDPSCs), human periodontal ligament stem cells (hPDLSCs), and stem cells from human exfoliated deciduous teeth (SHED) can be isolated (21). Remarkably, human dental-MSCs showed the ability to self-renewal and potential to differentiate toward osteogenic, chondrogenic, adipogenic, pancreatic, and neurogenic lineages (21-24). Currently, canine dental pulp stem cells (cDPSCs) are another interesting resource due to their properties in self-renewing, multipotentiality, cell availability, and tissue accessibility

(25). Besides, extraction of tooth due to minor dental problems and use them as cell resource are clinically practical (26). In aspect of canine-derived cells, cDPSCs showed the differentiation potential toward osteogenic, odontogenic, adipogenic, and neurogenic lineages *in vitro*. Additionally, the expression of cell surface marker of cDPSCs reported different from hDPSCs. These cells also expressed mesenchymal stem cell surface protein markers e.g. *STRO-1*$^{+}$ (relative low), *CD73*$^{-}$, *CD45*$^{-}$ and *CD90*$^{+}$ (relative low), while *Nanog* and *CD146* were detected in mRNA level (27). Moreover, the previous result demonstrated that stem cells from canine dental pulp have the potential to generate bone tissue (5). Thus, this preclinical study of cBM-MSCs and cDPSCs could pave the way for MSC-based tissue engineering in orthopedics and oral maxillofacial reconstruction for clinical application and suggested the possibility of application in bone defect regeneration.

However, studies regarding osteogenic differentiation potential by cBM-MSCs and cDPSCs are still lacking. This makes an unmet gap of knowledge for establishing MSC-based bone tissue engineering in veterinary practice. Thus, a differentiation

potential toward osteogenic lineage by cBM-MSCs and cDPSCs will be extensively explored and compared to distinguish the possibility for clinical application in bone tissue regenerative therapy. The result will fulfill knowledge regarding stem cell-based treatment for bone tissue regeneration in both pre-clinical and clinical approaches.

Systems Biology platform for bone tissue engineering

Systems biology has emerged from integration omics approach and developed into an approach of understanding the biological system (28). Generally, the theory of systems biology has been purposed to resolve a challenging biological solution that requires the development of new technologies in order to explore the new data type. Currently, progress in systems biology approach is often driven by advanced omics technology. The high-throughput experimental techniques are genomics, transcriptomics, proteomics, and metabolomics. These tools can be combined with computer-based bioinformatics equipment to quickly describe and analyze large-scale data or detect molecular interactions of DNA, mRNA, protein, and metabolite levels.

Building on the previous studies, systems biology and the interdisciplinary fields of tissue engineering have been developed independently. For the past two decades, high-throughput methods have been used to analyze the relevant components on bone development and BTE. Recently, the large-scale analysis of proteins by mass spectrometry-based proteomics have been developed continuously for deep biology researches. Many studies demonstrated the importance of proteomics on BTE, as proteomics experiments and computational analyses help thoroughly explain the signaling dynamics and the internal cellular responses (6, 7). Moreover, this technology has elucidated biological functions for the newly identified proteins in the cellular context (7). However, the veterinary field still lacks studies regarding extensive clarification of osteogenic differentiation paths of MSCs toward osteogenic lineages. Thus, to fulfill the information in this regard and to evaluate the possibility of MSCs for clinical application in veterinary bone tissue regenerative therapy, a differentiation potential toward osteogenic lineage by cBM-MSCs and cDPSCs will be extensively explored and compared, which may provide a more global view of bone regulatory networks and

leading to more understand the interaction during osteogenesis to develop the strategies for establishment of bone tissue engineering.

Signaling transduction and bone tissue regeneration

Bone formation or osteogenesis have involved of the two major pathways. First, Intramembranous ossification is the direct conversion of mesenchymal stem cells toward bone tissue that occurs in craniofacial region. In another pathway, the mesenchymal stem cells differentiate toward cartilage and replaced to form bone tissue called endochondral ossification (29). Both pathways are related with various factors that have been illustrated as the key factors influencing the differentiation potential of MSCs *in vitro* and *in vivo*. Among those, signaling transductions have been widely studied and proposed as interesting key processes toward osteogenic differentiation. Generally, there are various steps of MSCs during differentiation toward osteogenic lineage starting from multipotent mesenchymal stem cells, osteoprogenitor cells, preosteoblast, early osteoblast, late osteoblast, mature osteoblast, and osteocyte, respectively, that various

signaling pathways are also critical and *integrated* during osteogenic differentiation. The important osteogenic signaling pathways including Wnt signaling, Notch signaling, transforming growth factor (TGF)-beta receptor complex, and bone morphogenetic protein (BMP)-2 signaling, etc. are still extensively explored for advancement of veterinary orthopedics research. In this regard, the attractive signaling pathways are Wnt signaling and Notch signaling that are highly influential during bone formation.

Wnt signaling pathway

Wnt signaling has been widely studied and proposed as one of interesting key pathway. Interestingly, Wnt signaling protein is related to bone tissue development during embryogenesis as described in various studies (30, 31). Wnt signaling directly enhances endochondral ossification, especially the differentiation of osteoblast and development of axial and appendicular skeletons (31).

There are two pathways of the Wnt pathway including the canonical pathway and the noncanonical pathway. Regarding Wnt signaling cascade, this complex

pathway is activated by the binding of Wnt protein ligand family to a membrane-bound, seven-pass transmembrane spanning receptors termed frizzled (Fz) receptors (32, 33). For canonical pathway, this pathway is also called the Wnt/β-catenin pathway starting by binding of Wnt ligand with Fz receptors. Next, the signal transduction triggers the translocation of cytoplasmic negative Wnt regulator to bind a destruction complex of β-catenin e.g. axin, glycogen synthase kinase (GSK)-3β, and adenomatosis polyposis coli (APC) (34). These inhibit β-catenin phosphorylation and degradation by proteasome, so the level of nucleus-accumulated β-catenin is increased (33). β-catenin acts as a co-activator of gene transcription factors e.g. T-cell factor/lymphoid enhancing factor (TCF/LEF) (35). For noncanonical pathways, two major pathways are the Wnt-planar cell polarity pathway (Wnt-PCP pathway) and the Wnt-calcium pathway (Wnt-Ca2+pathway). Non-canonical pathway involves various signaling molecules e.g. G-protein, RhoA/Rho-associated protein kinase (15), and inositol-1,4,5-trisphosphate (IP3)-dependent intracellular calcium etc. (36). These regulate cell cytoskeleton, adhesion, and migration (37).

To explore Wnt signaling pathway, many Wnt regulators are employed. Secreted frizzled-related proteins (sFRPs) and Dickkopf (DKK)-1 are used as Wnt negative regulators (38). Cysteine-rich domain of sFRPs is used to block the binding of Wnt ligands with Fz receptor or co-receptor LRP5/6 on membrane surface (39). DKK-1 inhibits Wnt pathway by formation of a complex of Wnt ligands, LRP5/6, and Kremen (Krm) (40). These lead to Wnt ligand destruction (33, 38).

Notch signaling pathway

Notch signaling pathway is a regulation signaling pathway of cell-to-cell signal transduction and communication. This signaling is important for cell proliferation, migration and apoptosis. Notch signaling is started when Notch ligands bind with their receptors, then the Notch intracellular domain (NICD) is cleaved and released. Next, the NICD translocate from the cellular membrane to the nucleus to bind CSL family and regulates downstream targets. Lately, various studies have illustrated that Notch signaling also plays a crucial role in the process of skeletal remodeling. Moreover,

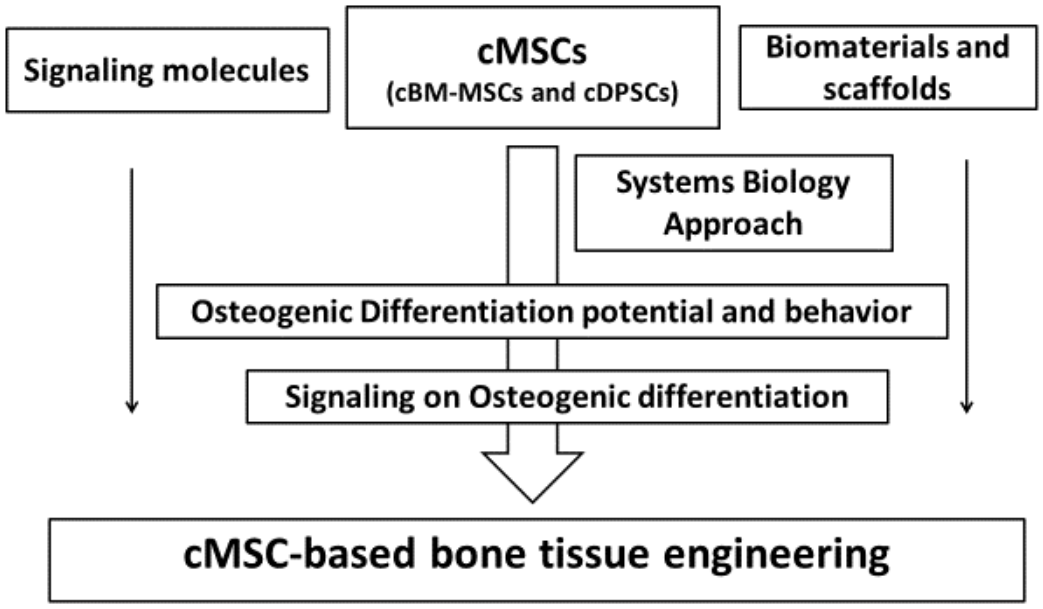
Notch signaling showed the potential to activate of BMP-9 via BMP/Smad pathway and enhanced the osteogenic gene expression (41). In addition, previous study showed enhancing of Jagged/Notch signaling had relatively involved to increase the osteogenic differentiation potential of human BM-MSCs and human periodontal ligament stem cells (42, 43). However, the knowledge of how to differentiate toward osteogenic lineage from this signaling is still lacking in animal stem cells to.

Although, the evidences suggest the possibility of MSC-based tissue engineering for bone tissue regeneration and support an importance of signaling pathways in osteogenic differentiation, the information regarding comparative osteogenic differentiation potential between cBM-MSCs and cDPSCs is still lacking and necessary for establishing the practical therapeutic regimen.

Thus, the objectives of the study are pointed out into main aspects which comprise of exploring and analyzing the *in vitro* differentiation potential and behavior of cBM-MSCs and cDPSCs toward osteogenic lineage using the systems biology approach.

The obtained results will fulfill the gap in knowledge regarding MSC-based bone tissue engineering and its establishment in veterinary practice.

Conceptual Framework



CHAPTER III

METHODOLOGY

Materials and methods

Cell isolation, culture, and expansion

This study was approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Science, Chulalongkorn University (Animal Use Protocol No.1531038). For cBM-MSCs isolation, canine bone marrow was obtained from healthy dog aged 3-10 years old. The obtained bone marrow was washed with Hank's Balanced Salt Solution (HBSS, Thermo Fisher Scientific, USA). The mixture was centrifuged at 300 g for 15 minutes and 1,000 g for 5 minutes. Pellet was gently resuspended and seeded onto T-75 culture flasks (Corning, USA) and maintained in Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA), 1% Glutamax (Thermo Fisher Scientific, USA), and 1% Antibiotics-Antimycotic (Thermo Fisher Scientific, USA). Cells were incubated in 5%

CO_2 and 95% air at 37 °c with every 48 hours media substitution. Cells were subcultured when 90% confluence reached. For cDPSCs isolation, cells were obtained from healthy permanent teeth of dog aged 3-10 years old with aseptic technique, the pulp tissue was collected and cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA), 1% Glutamax (Thermo Fisher Scientific, USA), and 1% Antibiotics-Antimycotic (Thermo Fisher Scientific, USA). Cells were incubated in 5% CO_2 and 95% air at 37°C with every 48 hours media substitution. Cells were subcultured when 90% confluence reached. cBM-MSCs and cDPSCs in passage 2-5 were used for the experiments.

Osteogenic differentiation

The osteogenic differentiation protocol was performed according to previously published reports (24, 44). Briefly, cells were seeded onto 24-well culture plate (Corning, USA) in a concentration of 3.5 x 10^4 cells/well and maintained in osteogenic induction medium for 14 days with routine 48-hour substitution. Osteogenic medium was

growth medium supplemented with 50 mg/mL ascorbic acid, 100 nM dexamethasone, and 10 mM β-glycerophosphate. Cells cultured in growth medium were utilized as the control.

Alkaline phosphatase activity

The alkaline phosphatase (ALP) activities were measured 14 day after osteogenic induction. Cells in 24-well plates were gently washed with warm phosphate buffered solution (PBS). The cell layers were lysed with 0.1% Triton X-100, 1 M Tris-HCl 500 L and 5 mM $MgCl_2$. The lysate samples were incubated with 2 mg/mL of *p*-nitrophenol phosphate (Thermo Fisher Scientific, USA), 0.1 M of 2-amino-2-methyl-1-propanol (Sigma, USA), and 2 mM of $MgCl_2$. The samples were incubated for 15 mins at 37°C, and 50 mM of NaOH were added to stop the reaction. The absorbances were read immediately after incubation at a wavelength of 410 nm. Protein concentrations were measured using Qubit according to manufacturer's protocol (Invitrogen, USA). The enzyme activities were expressed as U/mg protein

Von Kossa staining

After 14-day osteogenic induction, cells seeded in the well plates were gently washed with warm PBS and fixed in 500 µl methanol for 10 min. Next, cells were gently washed with distilled water and incubated with 1% silver nitrate solution under UV light for 30 min. After several washes using distilled water, unreacted silver was removed with 5% sodium thiosulfate for 5 min, and the cells were rinsed with distilled water. Images of the black stain in the plate was obtained using an inverted microscope.

Protein extraction and in-solution digestion

Cells were washed with 1X PBS, All Samples were lysed with lysis buffer containing the protease inhibitor (Thermo Fisher Scientific, USA) and 5% sodium deoxycholate (SDC). Samples were homogenized by sonicator. Protein concentrations were measured using BCA Protein Assay (Thermo Fisher Scientific, USA). Protein samples (400 µg per sample) were mixed in 100 mM TEAB (Thermo Fisher Scientific, USA) and incubated at 56°C at 300 rpm for 1 hr. Next, these samples were alkylated

with Iodoacetamide (IA) in a dark room for 30 mins, mixed with 200 mM TCEP, and added cold methanol, and incubated overnight at -20°C. After that, the samples were centrifuged at 8,000 rpm for 10 mins and resuspended with 100 mM TEAB. The protein samples were incubated with trypsin at a ratio of 1:50 at 37°C for 16 hrs. The quantity of tryptic peptides was measured with the Pierce Quantitative Fluorometric Peptide Assay (Thermo Fisher Scientific, USA). The peptide samples were collected at -80°C.

In-solution dimethyl labeling and fractionation

The digested samples were reconstituted in 100 mM TEAB. The peptide samples of control group (cBM-MSCs and cDPSCs) and osteogenic induction groups (cBM-MSCs and cDPSCs) at day 7 and 14 were labeled with formaldehyde isotope including light reagents (formaldehyde and cyanoborohydride, medium reagents (formaldehyde-d2 and cyanoborohydride), and heavy reagents (deuterated and 13C-labeled formaldehyde and cyanoborodeuteride), respectively, at room temperature for an hour. Each isotope labeled sample was quenched by adding ammonia solution and

formic acid. Three labeled-peptide samples were mixed. To reduce complexity, the complex mixture samples were separated into 10 fractions using the Pierce High pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher Scientific, USA). Elution samples of each fraction were evaporated the liquid content to dryness using vacuum centrifugation. Dry samples were re-suspended in formic acid before LC-MS/MS analysis.

LC-MS/MS and analysis

Before MS injection, the fractionated peptides were resuspended to a final volume of 15 µl in 0.1% formic acid (12) (Sigma, USA). The samples were analyzed by an EASY nLC1000 system (Thermo Fisher Scientific, USA) connected to a Q-Exactive Orbitrap Plus mass spectrometer (Thermo Fisher Scientific, USA) supplied with a nano-electrospray ion source (Thermo Fisher Scientific, USA). Next, the peptide samples were eluted in 5-40% acetonitrile for 70 mins and 40-95% acetonitrile for 20 mins in 0.1% FA by using flow rate 300 nl/min. The full MS1 scan procedures employed a resolution at

70,000 and MS2 scan at 17,500. To select the target peak, range from 350 to 1,400 m/z from MS scan was identified by using Proteome Discoverer™ Software 2.1 (Thermo Fisher Scientific, USA). The measures were set including digestion enzyme (trypsin), maximum miss cleavage (45), maximum modification (4), fixed modification (carbamidomethylation of cysteine, +57.02146 Da), dimethylation of N-termini and lysine (light, +28.031300 Da, medium, +32.056407 Da and heavy, +36.075670 Da), and variable modifications (oxidation of Methionine, +15.99491 Da). The relative MS signal intensities of dimethyl labeled peptides were analyzed by Proteome Discoverer™ Software. The mean and standard deviation of fold change from five replicates were calculated to Log2 value of the normalized ratio.

Bioinformatics

The listed proteins were implemented to analyze by the online resource database for annotation, Reactome (https://reactome.org/) and DAVID (https://david.ncifcrf.gov/). These databases provided intuitive bioinformatics tools to

categorize and interpret the proteins from the control group and osteogenic induction on day 7 and 14. The analyzed results are shown as acknowledgement of pathway-relating proteins expressed by cBM-MSCs and cDPSCs during osteogenic differentiation.

Level expression of protein expression

On day 7 and 14 post-induction, the protein expression levels were calculated as Log2 normalized ratio, by normalizing with undifferentiated control group (day 0). The relevant proteins were excluded when they were found in less than 3 from 5 replicates. Then, the mean and standard deviation of fold change across all 5 biological replicates were determined. The levels of candidate protein expression were reported as fold-change number and color scale reflecting protein upregulation (red) and downregulation (blue).

Hierarchical Clustering

The significant protein calling was performed by Instant Clue Software. The proteins were clustered and showed as the heatmap or cluster map with Row Dendrogram describing the performed clustering and Column Dendrogram describing the experiment groups. Color scale was used for reflecting upregulation and downregulation of protein after osteogenic induction at day 7 and 14, by normalizing the data with the undifferentiated control (day 0).

Validation assay for potential signaling

To validate the relevance of potential signaling on osteogenic differentiation potential by cBM-MSCs and cDPSCs, specific inhibitors regarding each signaling pathway were employed at day 14 post-induction including Wnt canonical inhibitor (Dkk-1, 100 ng/ml), Notch inhibitor (DAPT, 25 μM), TGF-beta receptor complex inhibitor (SB-431542, 4 μM), and BMP-2 signaling inhibitors (noggin, 0.2 μg/ml and dorsomorphin, 4

μM). Semi-quantitative evaluation of matrix mineralization by *Von Kossa* staining was utilized at day 7 and 14 post-induction, and it is compared with osteogenic control.

Statistical analyses

To statistically analyze ALP activity and gene expression, four biological replicates were used for particular cells (n=4). The statistical analysis was performed using SPSS Statistics (IBM, USA). To compare two independent groups, the Mann Whitney U test was employed. Statistical difference was recognized when p-value < 0.05.

To statistically analyze proteomics data, five biological replicates were used for particular cells (n=5). The mean and standard deviation of fold change from five replicates in each cell were presented as Log2 value of the normalized ratio. The significant proteins were called when they expressed at least 3 from 5 replicates. Significant difference between groups was determined by Mann–Whitney U test and unpaired t-tests with p-value < 0.05.

CHAPTER IV

RESULTS AND DISCUSSION

Isolation and characterization of cBM-MSCs AND cDPSCs

The isolated cBM-MSCs and cDPSCs were characterized by the expression of mRNA relating stemness marker, proliferative marker, and MSC-related marker (Fig 1A and B). The results presented that cBM-MSCs and cDPSCs expressed mRNA markers relating stemness property (*Rex1* and *Oct4*), proliferative marker (*Ki67*), and MSC-related marker (*CD44*, *CD73*, and *CD90*), while hematopoietic cell marker (*CD45*) was not detected. mRNA markers were differently expressed between the cBM-MSCs and cDPSCs.

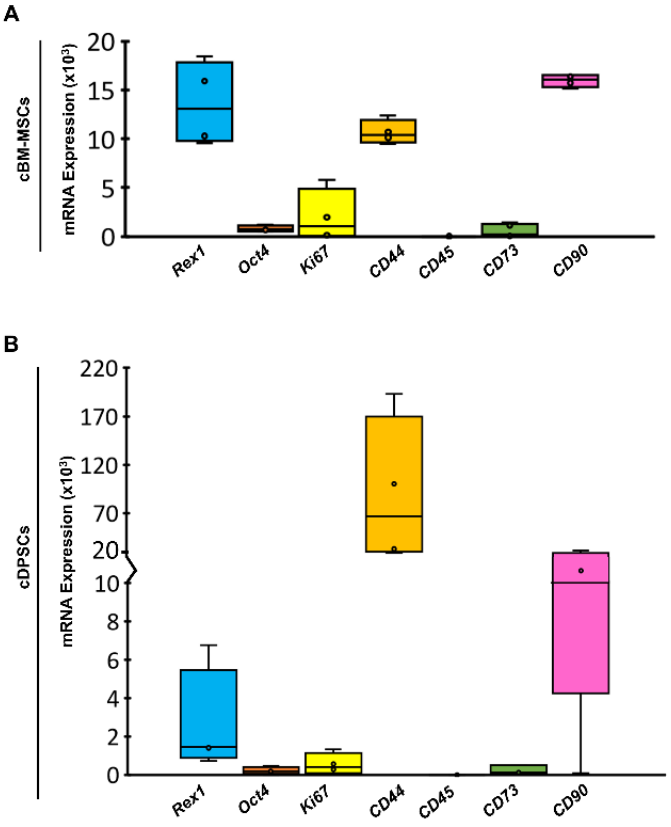


Figure 1 Characterization of the isolated cBM-MSCs and cDPSCs. The isolated cBM-MSCs (A) and cDPSCs (B) were characterized for the expression of mRNA related stemness marker, proliferative marker, and MSC-related marker (n=4). Relative mRNA expression was normalized with the reference gene, *Gapdh*.

Different osteogenic differentiation potential by canine MSCs derived from bone marrow and dental pulp

Although, as illustrated in Figure 2, both cBM-MSCs and cDPSCs were able to differentiate toward osteogenic lineage *in vitro*, the differentiation potential of them was distinct in regard of ALP activity (Fig 2A) and matrix mineralization (Fig 2B). cDPSCs showed superior ALP activity and mineralized nodule formation as detected by *Von Kossa* staining at day 14 post-induction. Additionally, osteogenic mRNA marker expression analyses illustrated that cBM-MSCs significantly upregulated *Osx*, while cDPSCs significantly upregulated *Runx2*, *Alp*, *Opn*, *Ocn*, and *Osx*, at day 14 post-induction (Fig 2C). These finding preliminarily suggested a different superior osteogenic differentiation potential of cDPSCs compared with cBM-MSCs *in vitro*.

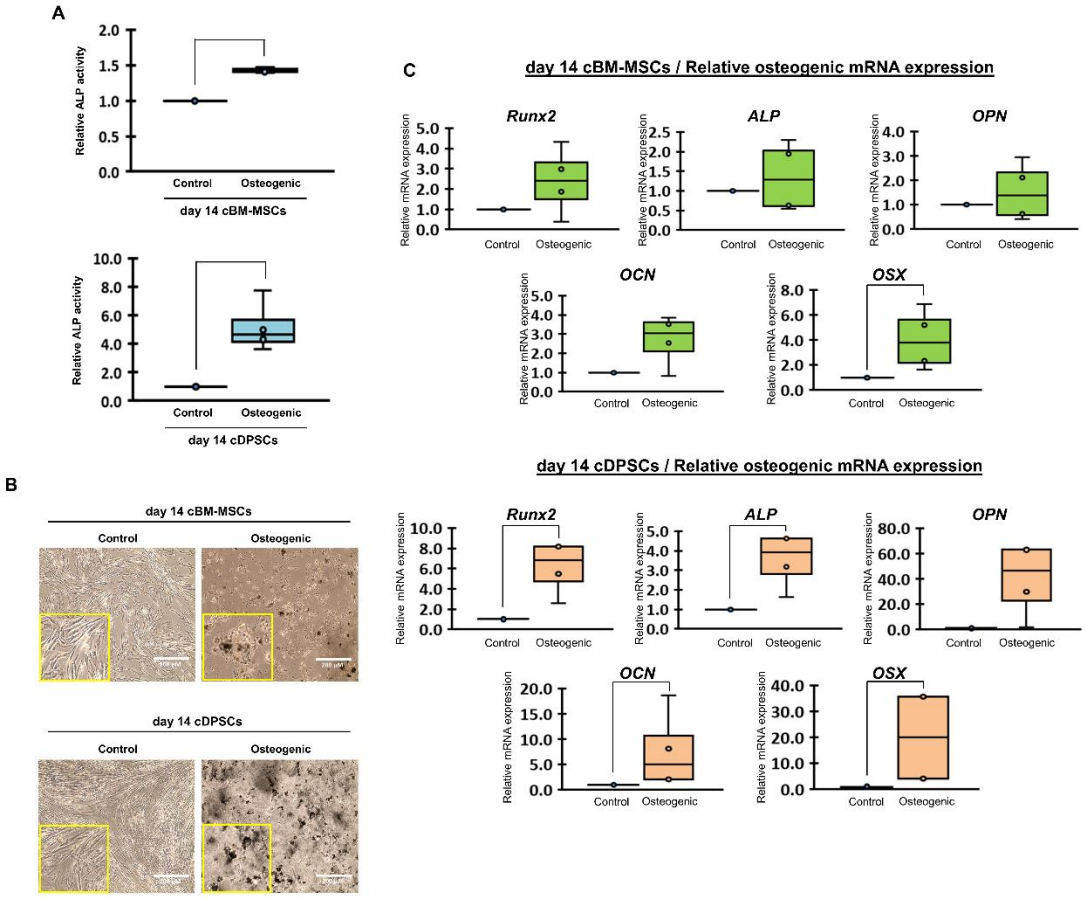


Figure 2 An *in vitro* osteogenic differentiation by cBM-MSCs and cDPSCs. ALP activity (A), *Von Kossa* staining (B), and osteogenic mRNA marker expression (C) of the cBM-MSCs and cDPSCs at day 14 post-osteogenic induction were investigated (n=4). The result of ALP activity was normalized with undifferentiated cells. Relative mRNA expression was normalized with the reference gene, *Gapdh*, and undifferentiated control. Bars indicate the significant difference between groups (p-value < 0.05).

Different protein expression pattern upon an in vitro osteogenic differentiation

To thoroughly understand the potential mechanisms underlying a different osteogenic differentiation, global protein expression pattern of the cells undergone osteogenic induction was analyzed using proteomics platform and bioinformatics analysis at day 7 and 14 post-induction. As shown in volcano plot, protein expression patterns of the cells upon osteogenic induction were differently distributed (Fig 3). Trend of protein upregulation upon osteogenic induction at day 7 and 14 was found in cBM-MSCs, while trend of protein expression by cDPSCs during osteogenic induction was slightly toward downregulation at day 14.

Heatmaps with Row and Column Dendrogram of the significant proteins expressed during an *in vitro* osteogenic induction by the cells at day 7 and 14 were showed in Figure 4. 2-D clustering analysis revealed 5 different clusters for each cell. For osteogenic cBM-MSCs, 2 clusters (cluster 1 and 2) were related to downregulated proteins at both day 7 and 14, while cluster 3 and 4 suggested trend of upregulation

(Fig 4A). For osteogenic cDPSCs, trend of protein upregulation in both day 7 and 14 was found in cluster 1 and 2, whereas cluster 5 showed downregulation at both timepoint. Interestingly, cluster 3 showed a contrasting trend of protein expression characterized by first a upregulation then followed by a downregulation (Fig 4A). According to the Four-Circle Venn Diagram, there were 359 and 201 identifiable proteins expressed by cBM-MSCs and cDPSCs during an osteogenic induction, respectively. However, only 10 proteins overlapped. The result showed that there were numerous proteins uniquely expressed by each cell at specific timepoint: 163 and 58 proteins were uniquely expressed by osteogenic cBM-MSCs at day 7 and 14, respectively; and 47 and 86 proteins uniquely expressed by osteogenic cDPSCs at day 7 and 14, respectively (Fig 4B). These suggested a distinct protein expression pattern by cBM-MSCs and cDPSCs at each timepoint during an *in vitro* osteogenic differentiation.

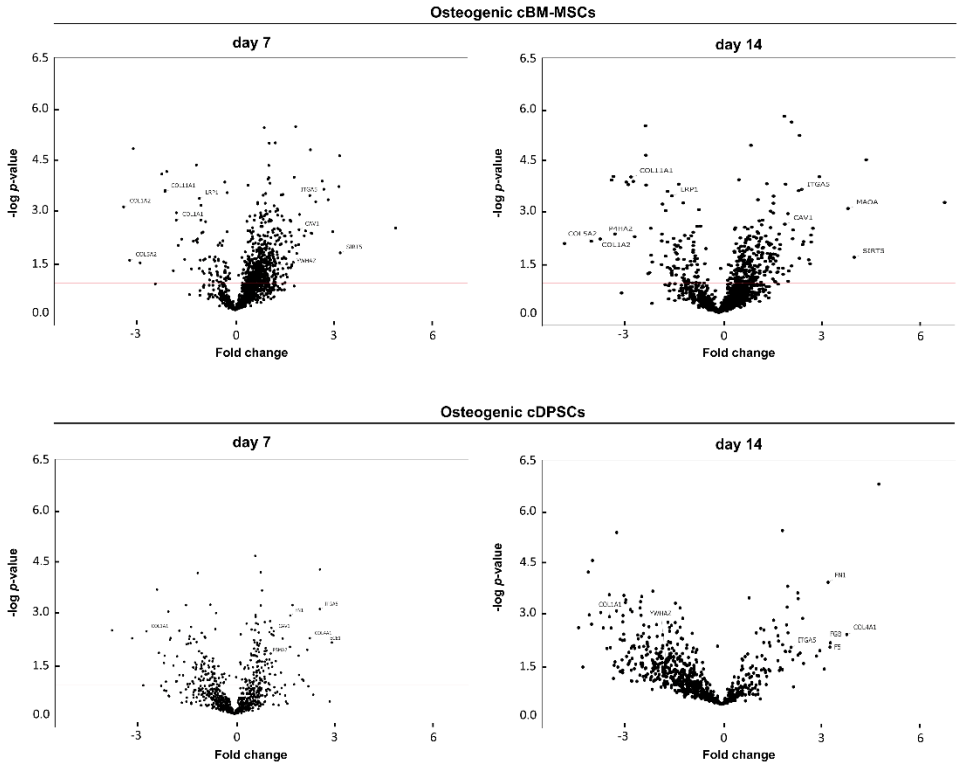


Figure 3 Volcano plots of expressed proteins by cBM-MSCs and cDPSCs upon an *in vitro* osteogenic differentiation.Volcano plots reflecting the distribution of expressed proteins by osteogenic cBM-MSCs and cDPSCs at day 7 and 14 post-induction were illustrated. The results were represented as -log *p*-value and fold change. Red lines indicate *p*-value < 0.05.

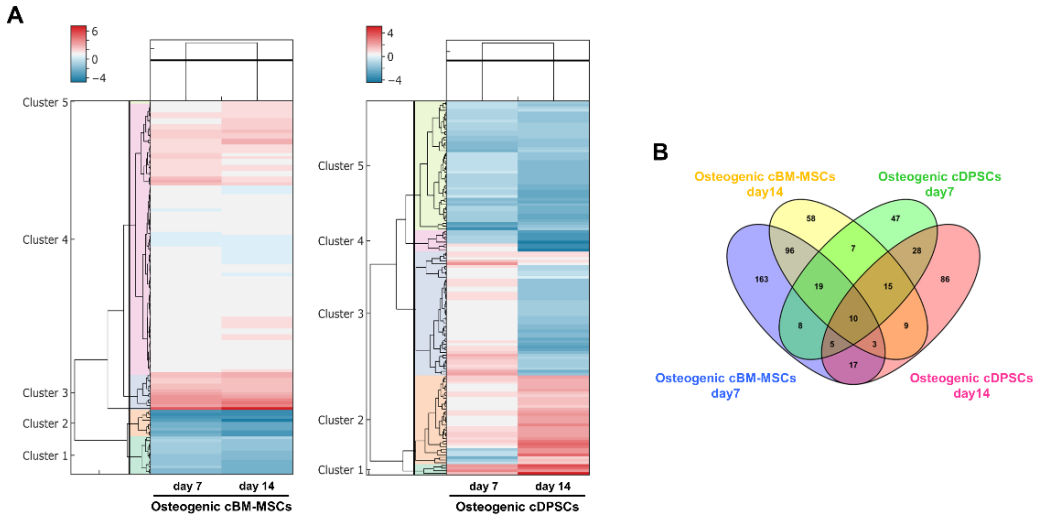
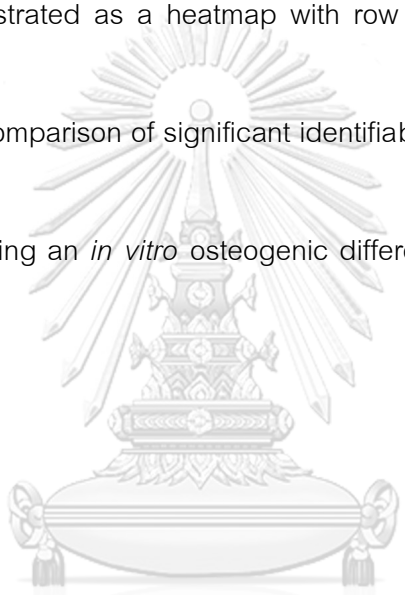


Figure 4 Heatmaps and Four-Circle Venn Diagram of expressed proteins by cBM-MSCs and cDPSCs upon an *in vitro* osteogenic differentiation. Heatmaps with Dendrogram (A) were illustrated for showing the clustering of significant expressed proteins by cBM-MSCs and cDPSCs upon an in *vitro* osteogenic differentiation at day 7 and 4 post-induction. Color scale represents fold-change of protein upregulation (red) or downregulation (blue). Four-Circle Venn Diagram (B) illustrated the number of proteins expressed by cBM-MSCs and cDPSCs upon an *in vitro* osteogenic differentiation at day 7 and 4 post-induction.

Quantitative proteomics analysis of cBM-MSCs and cDPSCs upon an in vitro osteogenic differentiation

Based on the protein analyses using the online resource for annotation and the pathway database, DAVID and Reactome, the quantitative protein expression profile in each category was illustrated as a heatmap with row and column dendrogram. The heatmaps provided a comparison of significant identifiable proteins expressed by cBM-MSCs and cDPSCs during an *in vitro* osteogenic differentiation at day 7 and 14 post-induction, respectively.



Signaling pathways

Proteins involving signaling pathways were analyzed based on three categories including kinase signaling cascades, development signaling pathways, and miscellaneous signaling. Different trends of signaling protein expression by each cell at specific timepoint were found. For kinase signaling cascades, quantitative expression of proteins involving receptor tyrosine kinases (RTKs), G-protein-coupled receptors

(GPCRs), mitogen-activated protein kinase (MAPK) family, and non-receptor tyrosine kinases (non-RTKs) were analyzed (Fig 5A). Some of proteins involving RTKs were upregulated in both cells (CAV1, ATP6V0D1, and FN1). Interestingly, FN1 was strongly upregulated in osteogenic cDPSCs, while CAV1 was dominantly expressed in osteogenic cBM-MSCs. Downregulated RTK-related proteins were mostly different between osteogenic cBM-MSCs (COL5A2, YES1, COL11A, and SH3KBP1) and osteogenic cDPSCs (ACTB, CD37, YWHAB, PRKACB, SH3KBP1). For GPCR-related proteins, LRP1 was downregulated, while ECE1 was upregulated in both cells. Some unique proteins were also upregulated (ROCK1, APOB, NRAS, and PRKAR2A) or downregulated (PRKACB, PSAP, and ANXA1) by each of cell. For MAPK family and non-RTKs, there were many unique proteins that were upregulated (CAMK2G, PSMA7, PSMD4, PSMD9, NRAS, CAMK2D, PSMC2, PSMD13, and PSMA5) or downregulated (SEPT7, ACTB, PSMD6, YWHAB, VCL, PRKACB, TLN1, and PSMB6) by each cell in each timepoint (Fig 5A).

The quantitative profile of protein involved in developing signaling pathways revealed the distinct expression pattern between cBM-MSCs and cDPSCs during osteogenic induction (Fig 5B). SERPINE1, a protein in TGF-beta receptor complex, was upregulated in osteogenic cBM-MSCs, but downregulated in osteogenic cDPSCs. Notch-related proteins were also expressed in different fashion. Most of them were upregulated in osteogenic cBM-MSCs (ATP2A2, YWHAZ, PSMD4, PSMD9, PSMC2, PSMD13, ACTA2, and PSMA5), but downregulated in osteogenic cDPSCs (YWHAZ, PSMD6, ACTA2, and PAMB6). These trends were also found in Wnt-, hippo-, and hedgehog-related protein expression. Set of upregulated proteins were mostly found in cBM-MSCs that had undergone osteogenic induction, whereas most of identified proteins in cDPSCs were downregulated (Fig 5B).

Further analysis on miscellaneous signaling showed that the correlated upregulation pattern only occur in integrin signaling-related proteins, FN1, while TLN1 downregulation and FGB upregulation were found in osteogenic cDPSCs (Fig 5C). Other proteins relating with Rho GTPases, nuclear receptors, mTOR signaling, and death

receptor were contrastingly expressed between the two osteogenic cells as illustrated by the downregulation of all identifiable proteins in osteogenic cDPSCs and the upregulation of the most proteins in osteogenic cBM-MSCs. There was only DYNC1H1 that showed constant downregulation in both cells (Fig 5C).

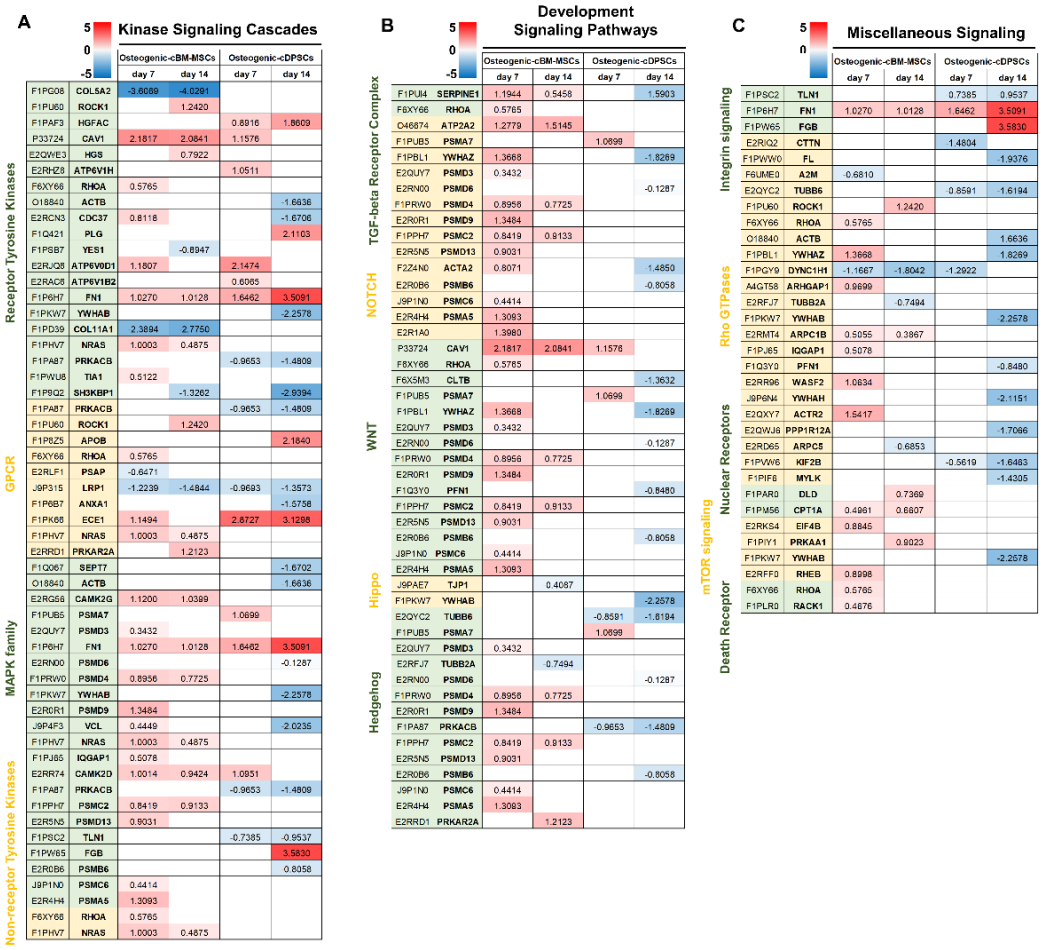


Figure 5 Quantitative proteomics analysis for expressed proteins involving signaling pathways by cBM-MSCs and cDPSCs upon an *in vitro* osteogenic differentiation. Heatmaps were illustrated for providing a comparison of significant proteins expressed by cBM-MSCs and cDPSCs upon an *in vitro* osteogenic differentiation at day 7 and 14 post-induction. Intensifying color scale represents fold-change of protein upregulation (red) or downregulation (blue). Proteins involving kinase signaling cascades (A), development signaling pathways (B), and miscellaneous signaling (C) were indicated.

Cellular components and processes

Additional analyses on cellular component- and process-related proteins included extracellular matrix (ECM) organization, cell cycle, DNA replication, gene expression, organelle biogenesis, metabolism of RNA, cell-cell communication, and cellular response to external stimuli. Analyses on ECM organization revealed that almost collagen formation-related proteins were downregulated in both cells upon an osteogenic induction, especially COL1A2, COL5A2, COL1A1, PLOD2, P4HA1, and P4HA2. Interestingly, COL4A1 was predominantly upregulated in osteogenic cDPSCs (Fig 6). Almost identifiable proteins relating to fibronectin matrix formation, elastic fiber, and laminin interactions were upregulated in both cells like ITGA5, FN1, and VTN. However, further analyses on proteins corresponding with non-integrin membrane-ECM interactions, ECM proteoglycans, degradation of the ECM, and integrin cell surface interactions showed a distinct expression patterns between two cells. Most of relevant proteins in osteogenic cDPSCs were upregulated (COL4A1, VTN, FN1, PLG, and FGB),

while those in osteogenic cBM-MSCs seemed to be downregulated (COL5A2, COL1A1, and A2M) (Fig 6).

Analyses on cell cycle-related proteins demonstrated a trend of active cell cycle in osteogenic cBM-MSCs, but not in the osteogenic cDPSCs (Fig 7A). Most of identifiable proteins connected to cell cycle checkpoints, cell cycle (mitotic), chromosome maintenance, and meiosis were predominantly upregulated in osteogenic cBM-MSCs (YWHAZ, PSMD4, PSMD9, PSMC2, PSMD13, UBE2V2, RPA1, PSMA5, PCNA, and NPM1), whereas those in osteogenic cDPSCs were downregulated (YWHAZ, PSMD6, YWHAB, YWHAH, PSMB6, KIF2B, TUBB6, and NUDC) (Fig 7A). Additional analyses on identifiable proteins involving DNA replication (M/G1 transition and synthesis of DNA), gene expression (RNA polymerase I transcription, RNA polymerase II transcription termination, and epigenetic regulation), and metabolism of RNA (capped intron-containing pre-mRNA processing, capped intronless pre-mRNA processing, mRNA stability, deadenylation-dependent mRNA decay, nonsense-mediated decay, rRNA processing in mitochondria, and tRNA processing on mitochondria) illustrated the

trend of upregulation in osteogenic cBM-MSCs (PSMC2, PSMD13, RPA1, PSMD4, PSMA5, PSMD9, PCNA, PSMD3, PSMC6, CAVIN1, SRSF6, DDX39B, SRSF3, RBM8A, PABPN1, NA, TRA2B, RBMX, PABPN1), but illustrate the trend of downregulation in osteogenic cDPSCs (PSMB6, PSMD6, ACTB, TRA2B, YWHAZ, YWHAB, and HSD17B10) (Fig 7B, C, and E). Interestingly, analyses on organelle biogenesis-related proteins revealed an upregulation of proteins involving mitochondrial biogenesis in osteogenic cBM-MSCs (NA, SIRT5, ATP5B) and downregulation of proteins involving cilium assembly in both osteogenic cBM-MSCs and cDPSCs (TUBB6, DYNC1H1, and TUBB2A) (Fig 7D).

Proteins relating to cell-cell communication (cell junction organization and nephrin family interactions) and cellular responses to external stimuli were further analyzed. The results showed a trend of upregulation in osteogenic cBM-MSCs (FERMT2, ILK, IQGAP1, CAMK2G, GPX8, IGFBP7, PSMD4, PSMD9, CAMK2D, PSMC2, PSMD13, RPA1, and PSMA5), but downregulation in osteogenic cDPSCs (FLNA, ACTB,

ACTN3, DCTN1, TUBB6, ERO1A, STIP1, PSMB6, and TXNRD1). DYNC1H1 was downregulated in both osteogenic cells (Fig 8A and B).

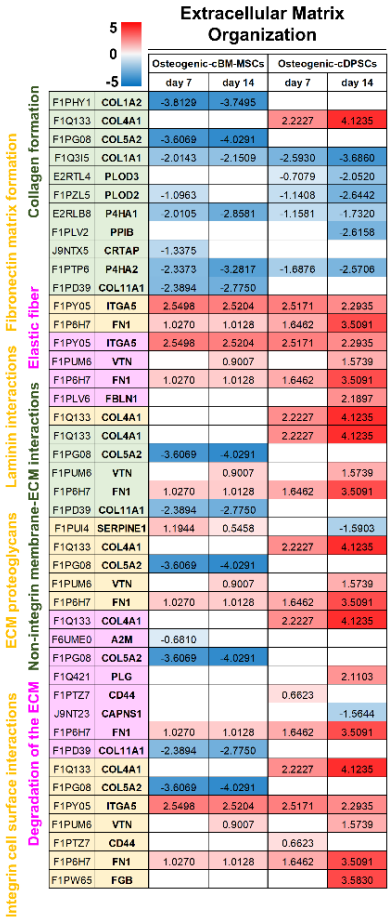


Figure 6 Quantitative proteomics analysis for expressed proteins involving extracellular matrix organization by cBM-MSCs and cDPSCs upon an *in vitro* osteogenic differentiation. Heatmaps were illustrated for providing a comparison of significant proteins expressed by cBM-MSCs and cDPSCs upon an *in vitro* osteogenic differentiation at day 7 and 14 post-induction. Intensifying color scale represents fold-change of protein upregulation (red) or downregulation (blue). Proteins involving extracellular matrix organization were indicated.

Figure 7 Quantitative proteomics analysis for expressed proteins involving cell cycle, DNA replication, gene expression, organelle biogenesis, and metabolism of RNA by cBM-MSCs and cDPSCs upon an *in vitro* osteogenic differentiation. Heatmaps were illustrated for providing a comparison of significant proteins expressed by cBM-MSCs and cDPSCs upon an *in vitro* osteogenic differentiation at day 7 and 14 post-induction. The intensifying color scale represents fold-change of protein upregulation (red) or

downregulation (blue). Proteins involving cell cycle (A), DNA replication (B), gene expression (C), organelle biogenesis (D), and metabolism of RNA (E) were indicated.

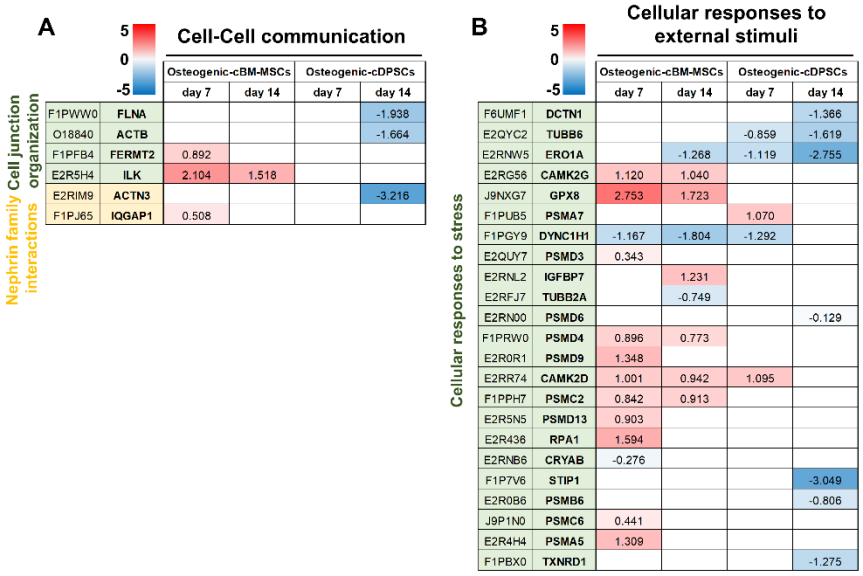


Figure 8 Quantitative proteomics analysis for expressed proteins involving cell-cell communication and cellular responses to external stimuli by cBM-MSCs and cDPSCs upon an *in vitro* osteogenic differentiation. Heatmaps provide a comparison of significant proteins expressed by cBM-MSCs and cDPSCs upon an *in vitro* osteogenic differentiation at day 7 and 14 post-induction. The intensifying color scale represents fold-change of protein upregulation (red) or downregulation (blue). Proteins involving cell-cell communication (A) and cellular responses to external stimuli (B) were indicated.

Cellular metabolisms, programmed cell death, and transports

Additional analyses of identifiable proteins were expanded to cover proteins relating to metabolism, program cell death, transport of small molecules, and vesicle-mediated transport. For metabolism-related proteins, almost proteins in metabolism of carbohydrates were downregulated in both osteogenic cBM-MSCs and cDPSCs (PRKACB, PCK2, NA, ALDOC, PYGB, TPI1, PGLS, PGAM1, VCAN, and TKT), while proteins in the metabolism of lipids (CPT1A, CAV1, ACAT2, KPNB1, ESYT1, HSD17B4, PON2, ACADM, and CPNE1), the integration of energy metabolism (SLC25A6, SLC25A5, IQGAP1, and PRKAR2A), the metabolism of nitric oxide (CAV1 and SPR), the citric acid (TCA) cycle and respiratory electron transport (CS, DLD, SUCLA2, NDUFS8, NDUFA2, ETFDH, ACO2, ATP5B, FH, SUCLG2, and UQCRC1), and metabolism of nucleotides (PAICS, ATIC, ADA, and ADK) were mostly upregulated in osteogenic cBM-MSCs. Analyses of proteins involving metabolism of vitamins and cofactors showed that LRP1 and SHMT2 were downregulated in both osteogenic cells. Some of unique

proteins were upregulated in cBM-MSCs (PC and SPR) and cDPSCs (APOB and IDH1) (Fig 9A).

For proteins in post-translational protein modification, most of identifiable proteins were uniquely upregulated in cBM-MScs (ETFB, SEC22B, PSMD3, IGFBP7, RAB5C, RPS2, CALR, RHOA, USP14, PSMD4, SCFD13, NA, UBE2V2, RPA1, PCNA, LAMC1, DDX5, PSMA5, COPA, ARCN1, PSMC2, SEC31A, NPM1, RAB10, HGS, PSMD9, MFGE8, and PSMC6) or cDPSCs (ITIH2, FGG, SERPINC1, AFP, CYR61, DNAJC3, LMAN1, RAB7A, COPS4, RAB14, LOC477072, APOB, RAB6A, PSMA7, and AHSG). Some proteins were both upregulated in both osteogenic cells (F5, CKAP4, RAB2A, FN1, and VDAC2) (Fig 9B).

It has been showed in analyses that trend of upregulated proteins by osteogenic cBM-MSCs is mostly related to the metabolism of amino acids and their derivatives (RSP3, HSD17B10, RSP15, PSMD3, PSMD4, PSMD9, RPS11, PSMC2, DLD, PSMD13, SQOR, DLST, NA, RPS4X, PSMC6, and PSMA5), biological oxidations (MAOA, UGDH, and SULT1C4), translation (GSPT1, RPS11, RPS3, EIF3A, RPS15, EIF3C, PABPC1,

RPS4X, and EIF3E), unfolded protein response (UPR) (SEC31A, CALR, and ATP6V0D1), protein repair (PCMT1), surfactant metabolism (CKAP4 and LMCD1), amyloid fiber formation (MFGE8), and mitochondrial protein import (CS, ACO2, ATP5B, and SLC25A6). Contrastingly, most of the identifiable proteins in osteogenic cDPSCs were downregulated and related to metabolism of amino acids and derivatives (HSD17B10, PSMD6, ALDH9A1, PSMB6, NQO1, and TXNRD1), biological oxidations (CNDP2), protein folding (TUBB6 and ACTB), UPR (DCTN1 and TLN1), and peptide hormone metabolism (ERO1A). For proteins in peroxisomal protein import, HSD17B4 was upregulated in osteogenic cBM-MSCs, while IDH1 was upregulated in osteogenic cDPSCs (Fig 9C).

It seemed that insulin-like growth factor (IGF) also plays an important role in both osteogenic cells as illustrated in the upregulation of protein relating to regulation of IGF transport and uptake by IGF-binding proteins (IGFBPs) (CKAP4, F5, ITIH2, APOB, PLG, IGFBP7, FN1, AHSG, CYR61, FGG, MFGE8, LOC477072, SERPINC1, LAMC1, DNAJC3,

and AFP). Only CALU and VACN from the proteins relating to regulation og IGF that were downregulated (Fig 10).

Analyses on proteins that control the programmed cell death revealed an upregulation trend of apoptotic-related proteins in osteogenic cBM-MSCs (ROCK1, KPNB1, YWHAZ, PSMD3, PSMD4, PSMD9, PSMC2, PSMD13, PSMC6, PSMA5, and DNM1L), while those in osteogenic cDPSCs were mostly downregulated (VIM, YWHAZ, PSMD6, YWHAB, YWHAH, and PSMB6) (Fig 11).

Proteins regulating transport of small molecules and vesicle-mediated transport were further analyzed. For transport of small molecule, most of identifiable proteins in adenosine triphosphate (ATP)-binding cassette (ABC)-family protein-mediated transport (PSMC2, PSMD13, PSMD3, PSMC6, PSMD4, PSMA5, and PSMD9), aquaporin-mediated transport (PRKAR2A), and mitochondrial calcium ion transport (PHB2) were upregulated in osteogenic cBM-MSCs, whereas those regulating ABC-family protein-mediated transport (PSMB6 and PSMD6), aquaporin-mediated transport (PRKACB), and plasma lipoprotein assembly, remodeling, and clearance (PRKACB) were downregulated in

osteogenic cDPSCs. Some proteins involving a solute carrier (SLC)-mediated transmembrane transport (SLC1A5) and plasma lipoprotein assembly, remodeling, and clearance (A2M) were downregulated in osteogenic cBM-MSCs. Interestingly, proteins controlling iron uptake and transport (ATP6V1H, ATP6V0D1, LOC477072, and ATP6V1B2) and ion channel transport (ATP6V1H, ATP1A1, CAMK2G, ATP2A2, ATP6V0D1, ATP6V1B2, and CAMK2D) were mostly upregulated in both osteogenic cells (Fig 12A).

For vesicle-mediated transport, identifiable proteins regulating membrane trafficking were uniquely upregulated in osteogenic cBM-MSCs (COPA, ARCN1, SEC22B, YWHAZ, RAB5C, SEC31A, ACTR2, RAB10, HGS, MYO1C, SCFD1, and HIP1) or osteogenic cDPSCs (LMAN1, RAB7A, COPS4, TXNDC5, RAB14, LOC477072, APOB, and RAB6A). Some of those were also downregulated in osteogenic cBM-MSCs (TUBB2A, ARPC5, and TJP1) or osteogenic cDPSCs (CLTB, YWHAZ, DCTN1, ACTB, YWHAB, and YWHAH). F5 and RAB18 were upregulated in both osteogenic cells, while SH3KBP1, MAP1LC3B, and DYNC1H1 were downregulated in both cells also. For

protein regulating binding and uptake of ligands by scavenger receptors, LRP1 was downregulated in both osteogenic cells. CALR was upregulated in cBM-MSCs, and APOB was upregulated in cDPSCs (Fig 12B).

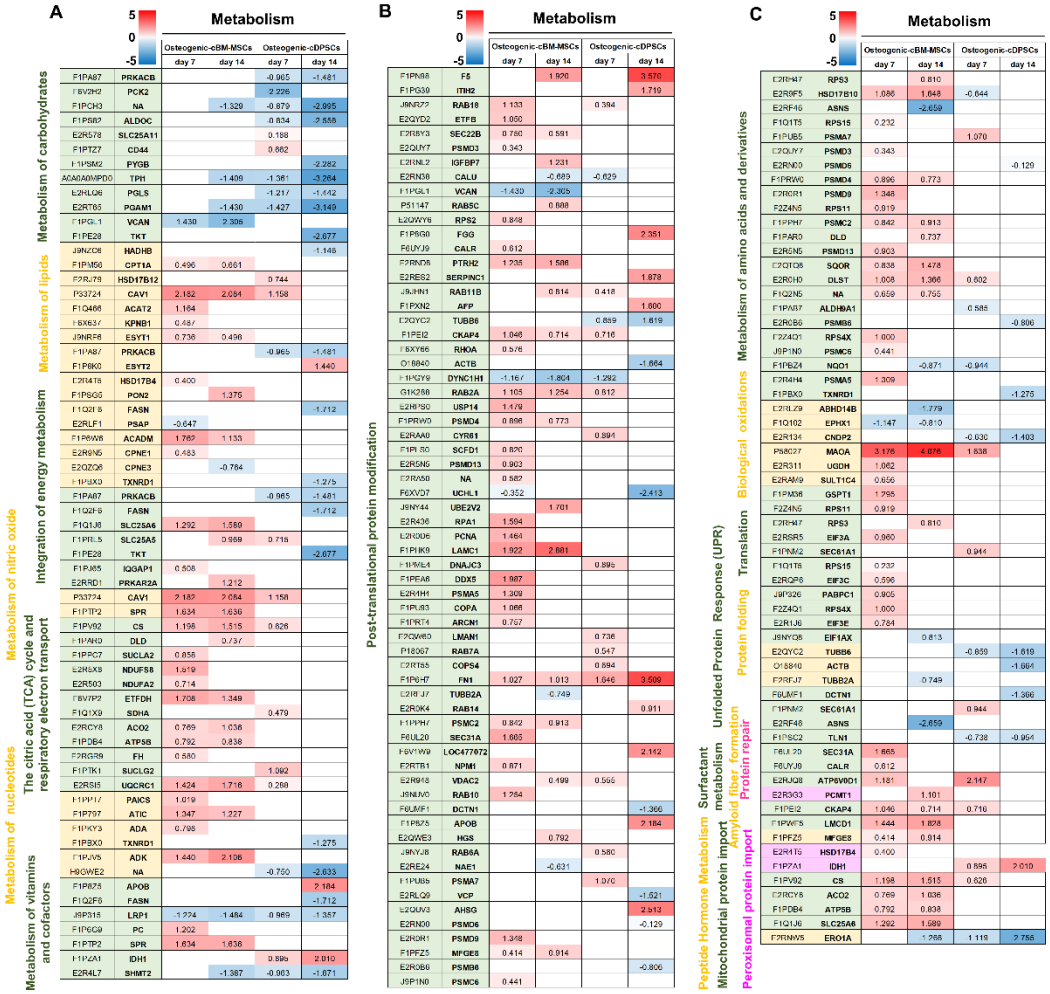


Figure 9 Quantitative proteomics analysis for expressed proteins involving cellular metabolisms by cBM-MSCs and cDPSCs upon an *in vitro* osteogenic differentiation. Heatmaps provide a comparison of significant proteins expressed by cBM-MSCs and cDPSCs upon an *in vitro* osteogenic differentiation at day 7 and 14 post-induction. The

intensifying color scale represents a fold-change of protein upregulation (red) or downregulation (blue). Proteins involving cellular metabolisms were indicated as well.

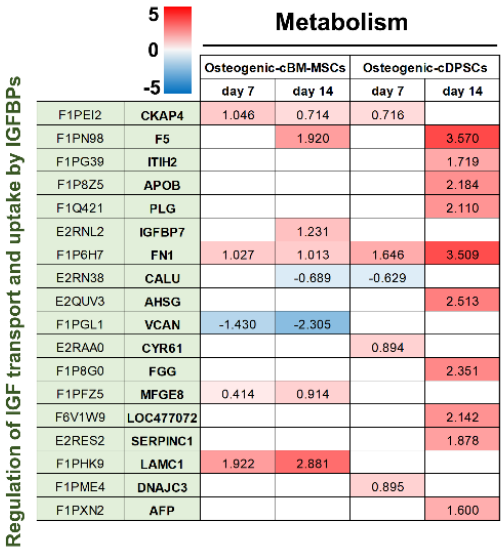


Figure 10 Quantitative proteomics analysis for expressed proteins involving regulation of IGF transport and uptake by IGFBPs by cBM-MSCs and cDPSCs upon an *in vitro* osteogenic differentiation. Heatmap provides a comparison of significant proteins expressed by cBM-MSCs and cDPSCs upon an *in vitro* osteogenic differentiation at day 7 and 14 post-induction. The intensifying color scale represents a fold-change of protein upregulation (red) or downregulation (blue). Proteins involving regulation of IGF transport and uptake by IGFBPs were indicated as well.

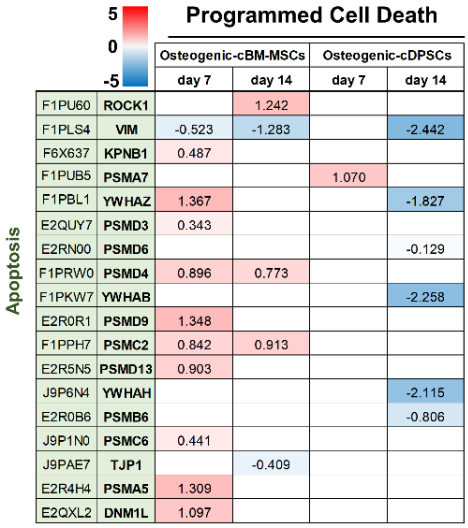


Figure 11 Quantitative proteomics analysis for expressed proteins involving programmed cell death by IGFBPs by cBM-MSCs and cDPSCs upon an *in vitro* osteogenic differentiation. Heatmap provides a comparison of significant proteins expressed by cBM-MSCs and cDPSCs upon an *in vitro* osteogenic differentiation at day 7 and 14 post-induction. The intensifying color scale represents a fold-change of protein upregulation (red) or downregulation (blue). Proteins involving programmed cell death were indicated as well.

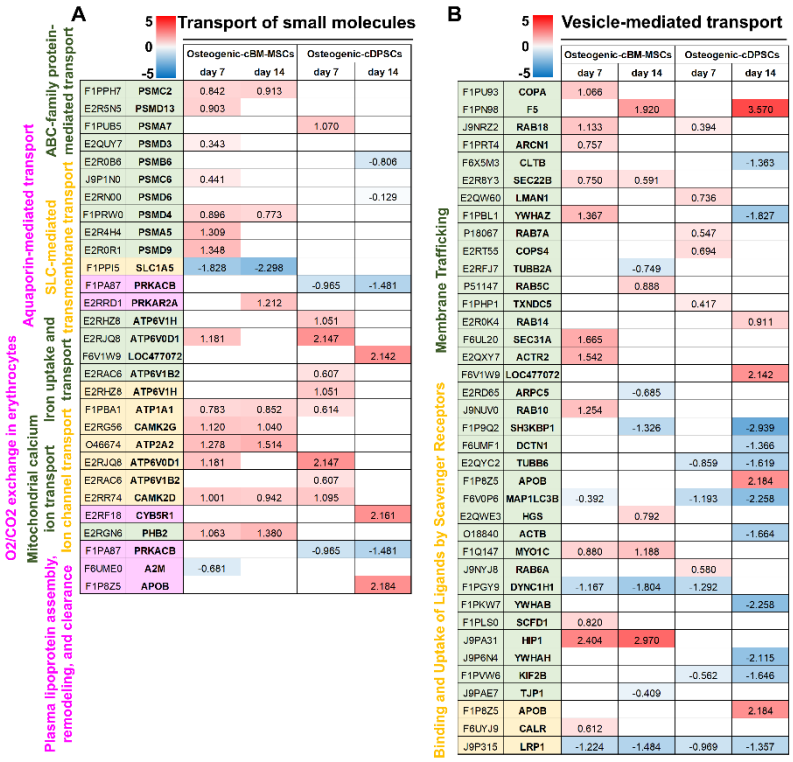


Figure 12 Quantitative proteomics analysis for expressed proteins involving transport of small molecules and vesicle-mediated transport by cBM-MSCs and cDPSCs upon an *in vitro* osteogenic differentiation. Heatmaps provide a comparison of significant proteins expressed by cBM-MSCs and cDPSCs upon an *in vitro* osteogenic differentiation at day 7 and 14 post-induction. The intensifying color scale represents fold-change of protein upregulation (red) or downregulation (blue). Proteins involving transport of small molecules and vesicle-mediated transport were indicated as well.

Confirmation of signaling involved an in vitro osteogenic differentiation by cBM-MSCs and cDPSCs

According to the quantitative proteomics analysis, a set of potential signaling involved an *in vitro* osteogenic differentiation by cBM-MSCs and cDPSCs were further analyzed and validated. Functional validations of the candidate signaling were analyzed based on mid- and late-state matrix mineralization of the cells upon treatment with specific signaling inhibitors. *Von Kossa* staining was used in this regard (Fig 13A and B).

As previously mentioned, cDPSCs showed a superior *in vitro* osteogenic differentiation potential comparing with cBM-MSCs at day 7 and 14 post-induction. Effects of Wnt signaling on an *in vitro* osteogenic differentiation were analyzed using canonical Wnt inhibitor, Dkk-1, which inhibits LRP5/6 interaction with Wnt ligand and forms a ternary complex with transmembrane protein KREMEN resulting the internationalization of LRP5/6 (47). The results showed that canonical Wnt interference will led to a strongly enhanced matrix mineralization by cDPSCs but not cBM-MSCs

upon an *in vitro* osteogenic differentiation at day 7 and 14 post-induction. These confirmed that the different roles of canonical Wnt signaling participated in the osteogenic differentiation by cBM-MSCs and cDPSCs *in vitro*.

Further validation on Notch signaling was studied using gamma secretase inhibitor (GSI), DAPT, which inhibits the formation of Notch intracellular domain (NICD). The results illustrated the dramatic enhancing effects of Notch interfering on an *in vitro* osteogenic differentiation by both cBM-MSCs and cDPSCs at day 14 post-induction. It could be suggested that Notch inhibition could enhance late-state matrix mineralization by cBM-MSCs and cDPSCs upon an osteogenic induction *in vitro*.

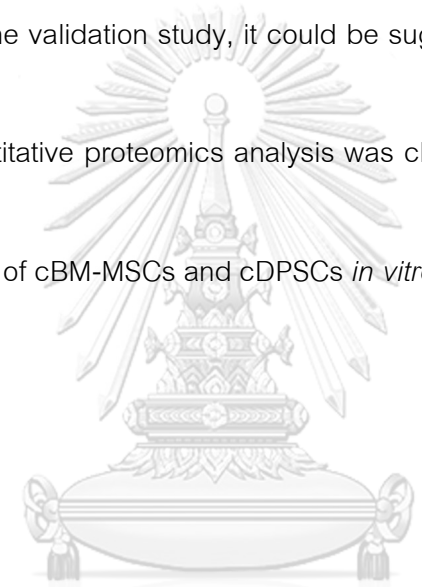
Additional validation on transforming growth factor (TGF)-beta was experimented using selective and potent inhibitor of the TGF-beta/activin/NODAL pathway, SB431542, which inhibits TGF-beta type I receptor, namely activin receptor-like kinase (ALK) (ALK5, ALK4, and ALK7), but not bone morphogenetic protein (BMP) type I receptor (ALK2, ALK3, and ALK6) and components of ERK, JNK, or p38 MAPK pathways (48). The results showed that TGF-beta/activin/NODAL pathway interfering attenuated matrix

mineralization by cBM-MSCs, but strongly enhanced matrix mineralization by cDPSCs upon an *in vitro* osteogenic differentiation at day 7 and 14 post-induction. These suggested the contrasting effects of TGF-beta/activin/NODAL pathway manipulation on mid- and late-state matrix mineralization by cBM-MSCs and cDPSCs upon an osteogenic induction *in vitro*.

Another validation on bone morphogenetic protein (BMP) was studied using two different BMP antagonists, noggin and dorsomorphin. The endogenous BMP antagonist, noggin, it binds and inactivates members of the TGF-beta superfamily signaling proteins, such as bone morphogenetic protein 4 (BMP4). Meanwhile, dorsomorphin, a selective BMP signaling inhibitor, selectively inhibits BMP type I receptors (ALK2, ALK3, and ALK6) and smad1/5/8 phosphorylation. Interfering of BMP signaling proteins by using noggin showed that the matrix mineralization by cDPSCs but not cBM-MSCs was enhanced upon an *in vitro* osteogenic differentiation at day 14 post-induction. However, inhibition of BMP signaling and smad1/5/8 phosphorylation by dorsomorphin caused the suppression of matrix mineralization by both cBM-MSCs and cDPSCs upon an *in vitro*

osteogenic differentiation at day 7 and 14 post-induction. These suggested the relevance and potential of strategic manipulation of BMP signaling on mid- and late-state matrix mineralization by cBM-MSCs and cDPSCs upon an osteogenic induction *in vitro*.

According to the validation study, it could be suggested that potential signaling derived from the quantitative proteomics analysis was closely related to the osteogenic differentiation potential of cBM-MSCs and cDPSCs *in vitro*.



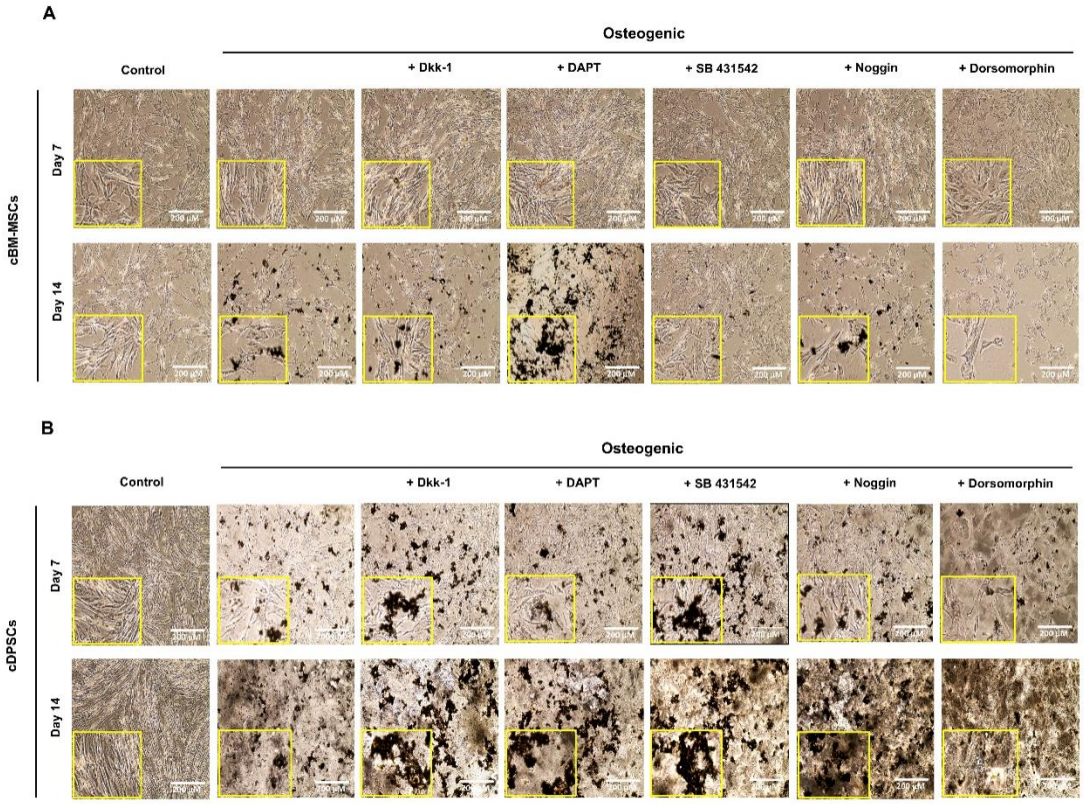


Figure 13 Validation assay of potential osteogenic signaling pathways related to an *in vitro* osteogenic differentiation potential by cBM-MSCs and cDPSCs. Functional validation of the potential signaling related to an *in vitro* osteogenic differentiation by cBM-MSCs (A) and cDPSCs (B) was analyzed by treatment with specific signaling inhibitors (canonical Wnt inhibitor: Dkk-1, Notch inhibitor: DAPT, TGF-beta inhibitor: SB431542 and BMP inhibitors: noggin and dorsomorphin). Semi-quantitative analysis of matrix mineralization by *Von Kossa* staining was utilized at day 7 and 14 post-induction.

Tree diagram analysis of potential signaling

Based on the hierarchical clustering and pathway analysis, tree diagrams of potential signaling derived from quantitative proteomics analysis and validation study were illustrated (Fig 14). Tree diagrams represent dynamic changing of particular signaling components in cBM-MSCs and cDPSCs upon an *in vitro* osteogenic differentiation by comparing protein expression level in three consecutive timepoints (day 7 vs day 0, day 14 vs day 0, and day 14 vs day 7).

To dissect Wnt signaling pathway, Wnt-related signaling components were categorized as 1) T-cell factor (TCF) dependent pathway or canonical pathway and 2) beta-catenin independent pathway (planar cell polarity (PCP)/convergent extension pathway) or non-canonical pathway (Fig 14A). It has been showed that TCF dependent and beta-catenin independent pathways play a different dynamic pattern in osteogenic cBM-MSCs and cDPSCs. For osteogenic cBM-MSCs, signaling components of both TCF dependent and beta-catenin independent pathways were significantly upregulated at day 7 and maintained until day 14 post-induction (#02), while some of those were only

significantly upregulated at day 7 post-induction (#05). However, CAV1, considered as TCF dependent signaling component, was downregulated at day 7 post-induction and maintained at that level until day 14 post-induction (#26). Analysis on osteogenic cDPSCs revealed that most of signaling components of both TCF dependent and beta-catenin independent pathways were significantly downregulated at day 14 post-induction comparing with undifferentiated control and not significantly different (#17) or significantly lower (#18) when compared with day 7 expression level. PSMA7 was significantly upregulated at day 7 post-induction, but the expression level at day 14 was significantly downregulated (#06) (Fig 14A). These illustrated a distinct dynamic expression pattern of Wnt-related signaling components by osteogenic cBM-MSCs and cDPSCs *in vitro*.

For Notch signaling pathway analysis, a different expression pattern of Notch signaling components was illustrated (Fig 14B). For osteogenic cBM-MSCs, most of Notch-related proteins were significantly upregulated at day 7 post-induction. Some of those upregulations were maintained until day 14 post-induction (#02) or downregulated

(#05 and #06). PSMA7, PSMD6, and PSMB6 were unchanged throughout the induction period (#14). For osteogenic cDPSCs, most of Notch-related components were downregulated at day 14 post-induction (#17 and #18), and some of those components were remained unchanged during the induction (#14). PSMA7 was significantly upregulated at day 7 post-induction but downregulated later (#06) (Fig 14B). These showed the unique expression pattern of Notch signaling components by osteogenic cBM-MSCs and cDPSCs *in vitro*.

According to BMP-related signaling pathway analysis, set of most relevance signaling pathways were categorized as 1) TGF-beta receptor complex, 2) non-receptor tyrosine kinase (non-RTK), and 3) hedgehog (Fig 14C). For osteogenic cBM-MSCs, some of proteins involving hedgehog and non-RTK were significantly upregulated at day 7 and maintained until day 14 post-induction (#02) or slightly decreased (#03). RHOA, a non-RTK-related protein, was significantly upregulated at day 7 post-induction (#05), and some of hedgehog-related protein were upregulated at day 7 or 14 post-induction (#05 and #11). There were TGF-beta receptor complex protein SERPINE1 and some of

hedgehog-related protein remained unchanged (#14), while hedgehog-related protein TUBB2A was continuously downregulated (#17). For osteogenic cDPSCs, most of proteins in non-RTK and hedgehog signaling were remained unchanged (#14) or downregulated since day 7 (#26) or at day 14 post-induction (#18). PSMA7 was significantly upregulated at day 7 post-induction but downregulated later (#06) (Fig 14C). These suggested the varied expression pattern of proteins involving BMP-related signaling pathway by osteogenic cBM-MSCs and cDPSCs *in vitro*.

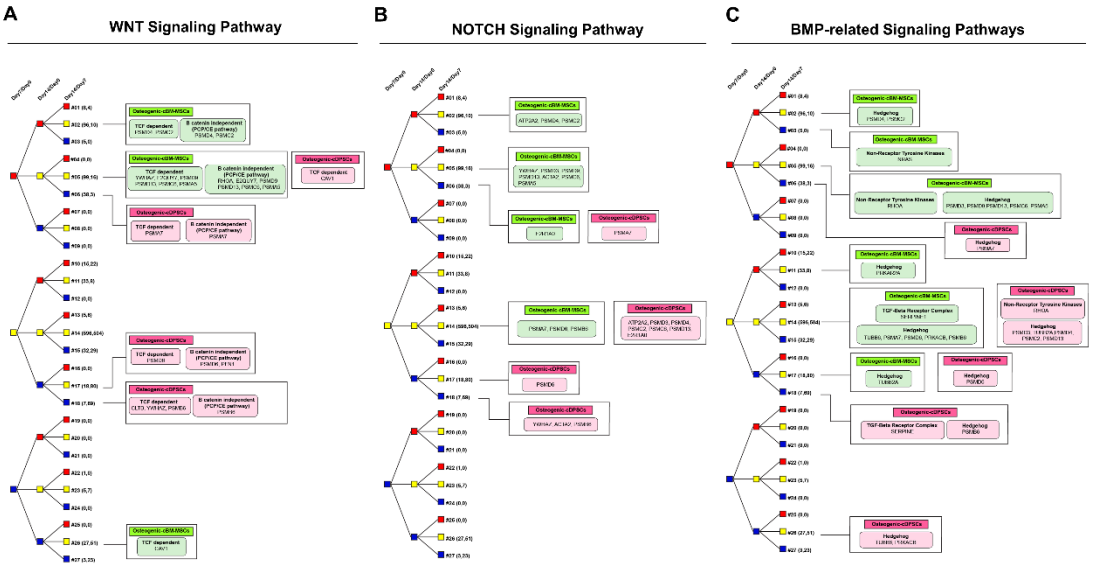


Figure 14 Tree diagram analysis for mapping and prediction of the influent proteins from potential signaling pathways by cBM-MSCs and cDPSCs upon an *in vitro* osteogenic differentiation. Tree diagrams of potential signalings derived from quantitative proteomics analysis and validation assay were illustrated including Wnt signaling pathway (A), Notch signaling pathway (B), and BMP-related signaling pathways (C). The diagrams represent dynamic changing of particular signaling components in cBM-MSCs (green box) and cDPSCs (pink box) upon an *in vitro* osteogenic differentiation by comparing protein expression level in three consecutive

timepoints (day 7 vs day 0, day 14 vs day 0, and day 14 vs day 7). In tree diagram connections, significant upregulation and downregulation are represented as red and blue square boxes, respectively, while yellow square box represents non-significant expression. All episodes are coded (#1 - #27). Numbers in the brackets refer to total protein numbers expressed by osteogenic cBM-MSCs and cDPSCs for each episode, respectively.

Discussion

In the present study, the isolated cBM-MSCs and cDPSCs were successfully isolated and characterized by verifying mRNA markers relating to stemness property, proliferative marker, and MSC-related marker. It has been illustrated that cBM-MSCs and cDPSCs are expressed a different level of those mRNA markers which suggests a unique characteristic for each cell. Although, several publications have suggested criterion for characterizing the isolated MSCs, there is still no consensus on cMSCs characterization (56, 57). In this study, we found that cBM-MSCs and cDPSCs can express mRNA markers relating to MSCs, but not hematopoietic cell markers. The mRNA expression analysis was used instead of flow cytometry analysis due to a limitation on specific antibody reactivity. It has been reported that MSCs from various species and sources expressed different level of markers which agree with our findings. For example, CD44 is an adhesion molecule that can interact with fibronectin, hyaluronan, selectins, and collagen (58). Some studies presented that *CD44* was apprized to be greatly expressed by MSCs isolated from mice and human (59, 60).

However, another study found that MSCs sourced from bone marrow naturally lacked CD44 protein expression (61). These finding suggest varieties of MSC-related marker expression.

In this study, an *in vitro* osteogenic differentiation potential by cBM-MSCs and cDPSCs was thoroughly explored and analyzed. Both cBM-MSCs and cDPSCs showed their ability to differentiate toward osteogenic lineage; however, cDPSCs exhibited a marked osteogenic status with higher level of ALP activity and greater mineralization compared with cBM-MSCs.

Previous studies on cBM-MSCs and cDPSCs have focused mainly on their potential toward osteogenic differentiation, rather than clarifying the exact behavior in the osteogenic lineage. Bearden et al. (18) studied the osteogenic characteristics of cBM-MSCs and concluded that these cells are mesenchymal stem cells in their morphology and they have the potential to differentiate toward osteogenic, chondrogenic, and adipogenic lineages in different conditions. Dissayanaka et al. (27) also studied the characteristic of cDPSCs which displayed stem cell-like capability and

ability to differentiate along the osteogenic, adipogenic, and chondrogenic lineages. However, the different characterization between cBM-MSCs and cDPSCs in protein expression which is the functional level during osteogenic differentiation is not clear.

To understand relevant proteins upon osteogenic differentiation of cBM-MSCs and cDPSCs, global analysis by mass spectrometry was utilized. The present study successfully used a method of dimethyl labelling with LC-MS/MS-based peptide sequencing to selectively label, purify, and identify proteins from osteogenic cBM-MSCs and cDPSCs at day 7 and day 14 post-induction, which were compared to undifferentiated cells (day 0) following previous study protocol (62). Recently, quantitative proteomic was also utilized for analysis of osteogenic differentiation of human MSCs (63). To analyze the data, the volcano plot, heatmaps, and the Four-Circle Venn Diagram were employed to show the different trends of protein expression between *in vitro* osteogenic cBM-MSCs and cDPSCs. Both osteogenic cBM-MSCs and cDPSCs illustrated a unique protein expression pattern as seen in different protein expression distribution in the volcano plot, protein clustering in heatmap, and non-

overlapping expressed protein in Four-Circle Venn Diagram. These suggested an importance of global analysis on particular conditions or diseases. Previous proteomics analysis of human osteoarthritis patients identified upregulation of complement proteins (64). In addition, 1,943, 2,084, and 2,274 of human BM-MSCs proteins were found from quantitative phosphoproteomics profile after day 1, day 3, and day 7 of osteogenic induction (65), while cBM-MSCs and cDPSCs found less when compared to osteogenic human BM-MSCs.

To further analyze significant similarities and differences on expressed proteins between osteogenic cBM-MSCs and cDPSCs, annotation and pathway databases were employed. Comprehensive analyses were performed based on insight categories including signaling pathways, cellular components and processes, cellular metabolisms, program cell death, and cellular transports. In this study, the results showed that, since MSCs are the sources of osteoblast precursors, their differentiation is rigorously controlled by an extremely sophisticated set of signaling molecules and pathways. Osteogenic paths of both cBM-MSCs and cDPSCs were closely related with Wnt, Notch,

TGF-beta, and BMP signaling, as confirmed by pathway functional validation. Previous research has shown that these signaling pathways play an important role in the differentiation, proliferation, and migration of osteoblasts in humans and animals (66). However, according to the quantitative proteomics analysis, other potential signaling pathways that might also play a pivotal role in MSCs osteogenicity, including RTKs, GPCRs, MAPK family, and non-RTKS.

It has been showed that upregulated RTK-related proteins play a crucial role in osteogenic path (67). Upregulating of RTK-related proteins including caveolin-1 (CAV-1), characterized as a putative tumor suppressor, had been reported to induce osteogenesis (68, 69). Previous study found that ATP6V0D1, a ATPase H^+ Transporting V0 Subunit D1, colocalized with CAV1, suggesting the possibly related function of both proteins (70). In addition, fibronectin-1 (FN1) is elaborated in remodeling of the ECM in several cellular processes, and the proteomic result illustrated a specific regulation of FN1 in human osteoblastic cells during osteoblast differentiation and the relation to CAV-1 (71, 72). Here we showed that CAV-1, ATP6V0D1, and FN1 were also expressed and

might regulate cBM-MSCs and cDPSCS during osteogenic differentiation imply the importance of RTKs on osteogenic path.

Previous proteomic analysis identified LRP1, the low-density lipoprotein-receptor-related protein 1, that improved fracture healing in old mice and controlled osteoclast activity (73, 74). However, this result found that LRP1 was downregulated in osteogenic cBM-MSCs and cDPSCs, but ECE1, endothelin-converting enzyme-1, become upregulated. The binding of ECE1 to GPCR triggers downstream pathway resulting in the activation of osteoblastic proliferation and new bone formation (75). Thus, ECE1 is an attractive GPCR-related target protein to stimulates bone formation of osteogenic cBM-MSCs and cDPSCs.

In eukaryotic cells, the proteasome (PSM) is a complex molecule constructed from large proteins, namely proteasome endopeptidase complex subunits, and relates to ubiquitin pathway which is the mechanisms controlling intracellular proteolysis (76). Previous research showed that ubiquitin-proteasome pathway involved in osteogenesis both *in vitro* and *in vivo*. It is suggested that inhibition of the proteome process by

specific inhibitors could enhance bone formation by an activation of BMP-2 expression (77). In this study, we found that proteins in PSM family were dynamically expressed in both cBM-MSCs and cDPSCs and closely related to set of proteins relating MAPK family as well as Notch, Wnt, and hedgehog signaling. Further study on PSM family protein on osteogenic differentiation of cBM-MSCs and cDPSCs is needed for more understanding on underlying mechanisms.

Based on cellular component and process analyses, collagen type I alpha 1 (COL1A1) and collagen type I alpha 2 (COL1A2) are proteins which support bone tissues in the body, and mutations of COL1A1 and COL1A2 are related to osteogenesis imperfecta (78). However, they were downregulated in cBM-MSCs and cDPSCs during an osteogenic induction, but integrin subunit alpha 5 (ITGA5), FN1, and vitronectin (VTN) were upregulated. ITGA5 promotes osteoblast differentiation in human MSCs by increasing Runx2 expression and activity (79). VTN is a multifunctional glycoprotein found and involved in various physiological processes and promotes cell attachment in bone and ECM (80). Though the expression of collagen type IV alpha 1 (COL4A1)

suggests an underlying molecular mechanisms for osteopenia (81), COL4A1 revealed that osteogenic cDPSCs were principally upregulated. Thus, further experiments are necessary to address the difference of ECM organization between osteogenic cBM-MSCs and cDPSCs.

Further analysis revealed that, among many of the differently expressed proteins, several proteins of osteogenic cBM-MSCs and cDPSCs involved with metabolism of carbohydrate were downregulated, while several proteins in metabolism of lipids were upregulated. Previous study suggested that bone mineral density rises with body fat mass, and obesity has a protective effect against osteoporosis (82). However, recent study from rat bone marrow found that low-carbohydrate with high-fat diets have negative influence during osteogenesis by reducing osteogenic transcription factors (Runx2, osterix, and C/EBPβ) (83). This indicates that osteogenesis from different cell sources may employ different metabolism for bone formation or resorption.

In addition to proteins in post-translational protein modification, recent studies have provided some evidence that IGFBP7, an insulin-like growth factor-binding protein

7, increases the osteogenic differentiation of BMSCs by the Wnt/β-catenin signaling pathway (84). In addition, the presence of Ras homolog gene family member A, RHOA, indicated its involvement in cytoskeleton rearrangement of BM-MSCs (85, 86). These findings suggest that proteins in post-translational protein modification are required for cBM-MSCs and cDPSCs osteogenic differentiation. Insulin-like growth factors, IGFs, play a role during fetal development and postnatal growth in several cell types (87). Upregulated protein related to IGFs including IGFBP7 and FN1 can enhance osteogenesis of MSCs (88). Therefore, cBM-MSCs and cDPSCs might utilize IGFs for osteogenic differentiation, which further requires study.

As mass spectrometry-based proteomics is a relatively new tool in veterinary stem cell research along with the limited information on specific databases, we needed to validate further a subset of LC-MS/MS observations using secondary validation method. Therefore, an *in vitro* functional validation assay using specific osteogenic signaling inhibitors of selected potential pathways were employed and confirmed osteogenicity with the semi-quantitative mineralization assay. When cultured in the

presence of osteogenic stimulation, the potential of mineralization revealed the obvious differences of the osteogenic regulating pathway between cBM-MSCs and cDPSCs in their osteogenic paths.

In relation to Wnt and TGF-β signaling, the inhibition of Wnt and TGF-β of cBM-MSCs and cDPSCs at day 7 and day 14 showed different results. Upon the interference of Wnt or TGF- β, the osteogenicity of cBM-MSCs was inhibited which was indicated by less mineralized nodules. Some previous studies found that the activation of Wnt and TGF-β pathways stimulate the differentiation of mouse MSCs and cBMSCs towards the osteoblastic lineage (89-91). However, after inhibiting Wnt and TGF-β signaling, a mineralization level of cDPSCs were greater than those of the control and cBM-MSCs on both at day 7 and 14. Considering the proteomics results which showed downregulation of Wnt- and TGF-β related proteins together with validation experiment, therefore, the inhibition of WNT or TGF-β exerts beneficial effects on cDPSCs osteogenic differentiation, but attenuates this process in cBM-MSCs.

Recent researches have demonstrated that Notch signaling promotes osteogenic differentiation of mesenchymal stem cells (41, 92). On contrary, findings of this study showed the opposite trends with the increase in calcium nodule formation of cBM-MSCs and cDPSCs when Notch signaling was blocked by DAPT. These results are consistent with previous findings suggesting Notch signaling has a negative effect on MSC differentiation (46, 93). These findings suggest that Notch signaling may maintain cMSCs proliferation but suppresses cMSCs osteogenic differentiation, and suggest that different Notch receptor subtypes have different influences on osteogenesis differentiation of cMSCs (94-96).

The importance of BMP signaling have widely recognized and promoted in bone formation of critical-size bone defects, which is useful in the field of bone tissue engineering and regeneration (97). Noggin and dorsomorphin, antagonists targeting BMP signaling, have been reported to negatively regulates BMP activities during osteogenesis (98, 99). Noggin binds to BMPs with high affinity and blocks BMPs' binding to the BMP receptor, while dorsomorphin inhibits Smad activation, a

downstream pathway (99, 100). In this study, the osteogenic cBM-MSCs were completely inhibited, and the osteogenic cDPSCs were partially inhibited to form calcium nodules after inhibiting BMP signaling with dorsomorphin. It is expected that BPM signaling with Smad dependent were the principle pathway of cBM-MSCs and cDPSCs to differentiate toward osteogenic lineage. In contrast, this result showed a increasing rate of mineralization of cDPSCs after treatment with noggin, suggesting that noggin facilitates osteogenic differentiation of cDPSCs, but does not affect cBM-MSCs to form mineralized nodule. Recent study showed noggin significantly increases ALP activities and simplifies osteogenic differentiation (101). Collectively, this study suggested that osteogenic differentiation of cBM-MSCs and cDPSCs mainly utilizes Smad pathway, but both may play a different role in BMP ligands.

To further map the candidate signaling on osteogenic differentiation potential, tree diagrams were employed to present Wnt, Notch, and BMP-related signaling pathways. Wnt signaling comprises two well-known pathways, canonical and non-canonical, which can modulate bone formation by activation from Wnt ligands (102).

This study showed a trend of upregulated proteins in both canonical and non-canonical Wnt signaling by osteogenic cBM-MSCs. Previous report showed supporting evidence that BM-MSCs utilized canonical and non-canonical Wnt signaling for regulating osteogenic differentiation (103). In contrast, at present, it is difficult to propose a clear model of how WNTs act on osteogenic cDPSCs. As discussed in detail on cBM-MSCs, several lines of evidence suggest that regulation and activation of Wnt signaling in osteoblasts is important for bone formation. Therefore, it is not unexpected that we would find the downregulation of protein related canonical and non-canonical Wnt signaling pathway for osteogenic cDPSCs, while cDPSCs showed osteogenic potential higher than cBM-MSCs. Some previous studies presented osteoblastic bone formation was not affected in mice after deletion of β-catenin in osteoblast (45). Thus, knowledge about the function of Wnt signaling has been broadened that it had different influence between on osteogenic cBM-MSCs and on cDPSCs, and further experiment for clarifying role of Wnt signaling on cMSCs osteogenic differentiation is indeed required.

Next, Notch signaling was another potential pathway involving osteoinductive effects on osteoblasts (104). Here, the tree diagram indicated Notch-related proteins of osteogenic cBM-MSCs that were significantly upregulated, while cDPSCs were downregulated. In addition, Notch signaling may interact with other signaling pathways such as Wnt and BMP to regulate skeletal development and homeostasis. One study showed that the expression of NICD blocked the differentiation of osteoblast precursors (105). Therefore, cBM-MSCs and cDPSCs were mapped with different pattern behavior of proteins in Notch signaling. Finally, due to the interesting result of the inhibition for BMP signaling of osteogenic cBM-MSCs and cDPSCs, we mapped and focused on BMP-related proteins. Proteins at day 7 and day 14 were expressed differently between cBM-MSCs and cDPSCs. Some studies showed the relation between hedgehog and BMP signaling (106, 107), BMP cross-talk with the RhoA (108), and BMP Signaling involving TGF-β (109). Interestingly, almost protein expression in hedgehog of osteogenic cBM-MSCs were upregulated, but cDPSCs were downregulated. Previous study showed that inhibition of hedgehog was a cause of lasting bone defects in mice

(110), whereas osteogenic cDPSCs presents an interesting osteogenic protein involving hedgehog which were downregulated. Thus, the behavior involving BMP-related protein for osteogenic differentiation of previous study appears to be specific for osteogenic cBM-MSCs but would not be expected to relate in osteogenic cDPSCs.

CHAPTER V

CONCLUSION

In conclusion, our study utilized an *in vitro* model of osteogenic differentiation, high-throughput quantitative proteomics, and a validation assay of candidate osteogenic signaling to obtain a comprehensive understanding of protein behavior upon osteogenic differentiation of cBM-MSCs and cDPSCs. To our knowledge, this study is the most comprehensive proteomics-based analysis of osteogenic cDPSCs and cDPSCs to date. The results of the present study indicated numerous different behaviors between cBM-MSCs and cDPSCs toward osteogenic lineage. These findings revealed the confirmation of regulating osteogenic signaling pathways to support the mass spectrometry analysis. This study data is useful for understanding of cMSCs osteogenic path and suggests the trend of MSC-based bone tissue engineering used for bone regeneration, concentrating on cBM-MSCs and cDPSCs application.

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