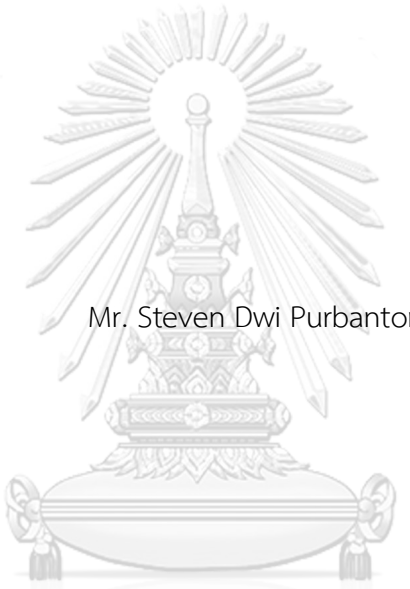
# OSTEOGENIC EFFECTS OF OSTEOGENIC GROWTH PEPTIDE ON HUMAN PERIODONTAL LIGAMENT STEM CELLS

Mr. Steven Dwi Purbantoro





A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Veterinary Science and technology
Common Course
FACULTY OF VETERINARY SCIENCE
Chulalongkorn University
Academic Year 2021

ผลต่อการสร้างกระดูกของออสทีโอเจนิกโกรทเปปไทด์ในเซลล์ต้นกำเนิดจากเอ็นยึดปริทันต์มนุษย

นายสตีเฟน ดวิ เบอร์บันโทโร



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี ไม่สังกัดภาควิชา/เทียบเท่า
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2564

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| Thesis Title | OSTEOGENIC EFFECTS OF OSTEOGENIC GROWTH PEPTIDE ON HUMAN PERIODONTAL LIGAMENT STEM CELLS |
| By | Mr. Steven Dwi Purbantoro |
| Field of Study | Veterinary Science and technology |
| Thesis Advisor | Assistant Professor Dr. CHENPHOP SAWANGMAKE, D.V.M., M.Sc., Ph.D |
| Thesis Co Advisor | Professor Dr. THANAPHUM OSATHANOON, D.D.S., Ph.D |

Accepted by the FACULTY OF VETERINARY SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the FACULTY OF VETERINARY SCIENCE

(Professor Dr. SANIPA SURADHAT, D.V.M., Ph.D)

THESIS COMMITTEE

..... Chairman

(Associate Professor Dr. NOPADON PIRARAT, D.V.M., Ph.D., DTVBP)

..... Thesis Advisor

(Assistant Professor Dr. CHENPHOP SAWANGMAKE, D.V.M., M.Sc., Ph.D)

..... Thesis Co-Advisor

(Professor Dr. THANAPHUM OSATHANOON, D.D.S., Ph.D)

..... Examiner

(Assistant Professor Dr. THEERAWAT THARASANIT, D.V.M., Ph.D)

..... Examiner

(Dr. NUTTHA KLINCUMHOM, D.V.M., Ph.D)

..... External Examiner

(Associate Professor Dr. Pakpoom Kheolamai, M.D., Ph.D)

สตีเฟน ดวิ เบอร์บันโทโร : ผลต่อการสร้างกระดูกของออสทีโอเจนิกโกรทเปปไทด์ในเซลล์ต้นกำเนิดจากเอ็นยึดปริทันต์มนุษย. ( OSTEOGENIC EFFECTS OF OSTEOGENIC GROWTH PEPTIDE ON HUMAN PERIODONTAL LIGAMENT STEM CELLS) อ.ที่ปรึกษาหลัก : เจนภพ สว่างเมฆ, อ.ที่ปรึกษาร่วม : ธนภูมิ โอสถานนท์

ด้วยผลกระทบต่อภาวะด้านสุขภาพจากโรคกระดูกพรุนและโรคปริทันต์อักเสบในปัจจุบัน การรักษาที่มีประสิทธิภาพจึงถูกมองหาเพื่อนำมาใช้รักษาผลจากการสลายของกระดูก ออสทีโอเจนิกโกรทเปปไทด์เป็นเปปไทด์สายสั้นๆ ที่สามารถเพิ่มการสร้างเนื้อกระดูกได้ วัตถุประสงค์ของการศึกษานี้คือการศึกษาผลของการสร้างกระดูกจากออสทีโอเจนิกโกรทเปปไทด์ในเซลล์ต้นกำเนิดเอ็นยึดปริทันต์ของมนุษย์ การศึกษานี้พบว่าออสทีโอเจนิกโกรทเปปไทด์ปริมาณต่ำ สามารถเพิ่มศักยภาพการแบ่งตัวเพิ่มจำนวนโดยไม่แสดงความเป็นพิษต่อเซลล์ นอกจากนี้ออสทีโอเจนิกโกรทเปปไทด์ปริมาณต่ำยังแสดงศักยภาพในการเพิ่มการเปลี่ยนแปลงเป็นเซลล์กระดูก โดยในการศึกษาการส่งสัญญาณกระตุ้น ได้แก่ สัญญาณโบนมอร์โฟจินิติกโปรตีน สัญญาณเฮดจ์ฮอก สัญญาณทรานสฟอร์มมิ่งโกรทแฟคเตอร์ และสัญญาณวินต์ ผลการศึกษาพบว่าออสทีโอเจนิกโกรทเปปไทด์มีแนวโน้มที่จะส่งสัญญาณกระตุ้นการสร้างกระดูกผ่านสัญญาณทรานสฟอร์มมิ่งโกรทแฟคเตอร์ในเซลล์ต้นกำเนิดเอ็นยึดปริทันต์ของมนุษย์ โดยสรุปออสทีโอเจนิกโกรทเปปไทด์ปริมาณต่ำมีศักยภาพในการกระตุ้นการสร้างกระดูกโดยกระตุ้นผ่านสัญญาณทรานสฟอร์มมิ่งโกรทแฟคเตอร์ ดังนั้นออสทีโอเจนิกโกรทเปปไทด์เป็นสารที่มีศักยภาพในการรักษาโรคกระดูกพรุนและ / หรือโรคปริทันต์อักเสบ



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| สาขาวิชา | วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี | ลายมือชื่อนิสิต ..... |
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# # 6278504031 : MAJOR VETERINARY SCIENCE AND TECHNOLOGY

KEYWORD: osteogenic growth peptide (OGP), human periodontal ligament stem cells (hPDLSCs), osteogenic differentiation, bone regeneration

Steven Dwi Purbantoro : OSTEOGENIC EFFECTS OF OSTEOGENIC GROWTH PEPTIDE ON HUMAN PERIODONTAL LIGAMENT STEM CELLS. Advisor: Asst. Prof. Dr. CHENPHOP SAWANGMAKE, D.V.M., M.Sc., Ph.D Co-advisor: Prof. Dr. THANAPHUM OSATHANOON, D.D.S., Ph.D

With the current global health burden of osteoporosis and periodontitis, an effective treatment is looked for to treat the bone resorption effect. Osteogenic growth peptide (OGP) is a short peptide that is known able to increase osteogenesis. The objective of this study is to investigate thoroughly the osteogenic effect of OGP on human periodontal ligament stem cells (hPDLSCs). This study found that low-dose OGP can increase the proliferation without cytotoxic effect. Enhanced osteogenic differentiation potential was also found at low concentration of OGP. Several signaling pathways, including bone morphogenetic protein (BMP), Hedgehog, transforming growth factor-beta (TGF-β), and Wnt, are regulated by OGP to regulate osteogenic differentiation of hPDLSCs. OGP was also found partially regulating TGF-β signaling pathway toward osteogenic lineage of hPDLSCs. Collectively, low-dose OGP has osteogenic differentiation potential on hPDLSCs by regulating TGF-β signaling pathway. Thus, OGP is potent candidate to treat osteoporosis and/or periodontitis.



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| Field of Study: | Veterinary Science and technology | Student's Signature ..... |
| Academic Year: | 2021 | Advisor's Signature ..... |
| | | Co-advisor's Signature ..... |

# ACKNOWLEDGEMENTS

This research work was financially supported by The Graduate Scholarship Program for ASEAN countries of Chulalongkorn University, National Research Council of Thailand (NRCT, N41A640135), and Thailand Research Fund (TRF, RTA6180001).

In this chance, I would like to acknowledge to the Chairman of thesis committee, Associate Professor Dr. Nopadon Pirarat, DVM., MSc., PhD., members of the thesis committee Assistant Professor Dr. Theerawat Tharasanit DVM., PhD., Dr. Nuttha Klincumhon, DVM., PhD., and Associate Professor Dr. Pakpoom Kheolamai, MD., PhD. for kindly providing valuable and useful comments to improve this study.

My deepest gratitude goes to my thesis advisor and co-advisor, Assistant Professor Dr. Chenphop Sawangmake, DVM., MSc., PhD. and Professor Dr. Thanaphum Osathanon, DDS., PhD. for their advice and care in helping for many aspects until this study has become successful.

For all the scientific knowledge I gained during my study, I would like to express my highly gratitude to my mentors: Dr. Sirirat Nantavisai, DVM., PhD., Dr. Watchareewan Rodprasert, BSc., PhD., Dr. Suryo Kuncorojakti, DVM., MVet., PhD., and Dr. Chutirat Torshakul, DVM., PhD who have always been supporting and giving advice for the success of this work

I also feel grateful for the genuine friendship and lovely and kindly support from the members of Veterinary Stem Cell and Bioengineering Innovation Center (VSCBIC), Faculty of Veterinary Science, Chulalongkorn University. To all graduate students of the International Graduate Course of Veterinary Science and Technology (VST), Faculty of Veterinary Science, Chulalongkorn University, I thank you all for encouragement for each other.

To Research Unit of Mineralized Tissue (RUMT), Faculty of Dentistry, Chulalongkorn University, Mr. Noppadol Sa-ard-iam, Immunology Research Center, Faculty of Dentistry, Chulalongkorn University, and Assoc. Prof. Sayamon Srisuwatanasagul and K. Earth, Department of Anatomy, Faculty of Veterinary Science, Chulalongkorn University for all help and support.

Last but not least, I would like to express my warmest gratitude to my father, mother, grandmother, sister, brother-in-law, niece, and nephew for the endless love, encouragement, and understanding.

Steven Dwi Purbantoro

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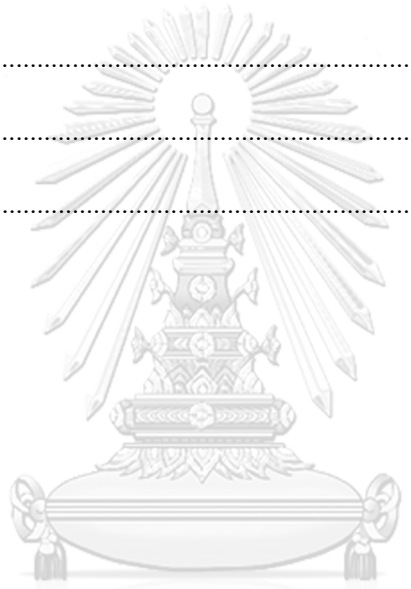

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# CHAPTER I

## INTRODUCTION

### IMPORTANCE AND RATIONALE

Osteoporosis and periodontitis are known to cause resorption of bone tissue (Hienz et al., 2015; Iñiguez-Ariza and Clarke, 2015). These two conditions have been mentioned to be one of global health concerns (Lems and Raterman, 2017; Nazir, 2017). It has been suggested that there is a relationship between these two conditions that may be occurred due to the involvement of systemic bone resorption, hormonal balance, inflammation, and bone homeostasis (Wang and McCauley, 2016). Hence, osteoporosis and periodontitis may be treated with the same treatment.

Common treatments that are currently used for these conditions are supplementation of calcium and vitamin D, teriparatide as hormonal replacement therapy (HRT) and bisphosphonates as anti-resorptive agent, also, having a behavior change in reducing smoking to periodontal disease risk (Wang and McCauley, 2016). Unfortunately, using anti-resorptive agents in treating osteoporosis may not be enough to achieve a successful result. To this matter, anabolic agents may be needed to combine with the anti-resorptive agents, and vice versa, to enhance osteogenesis that will increases the bone mass density (BMD) (Chen et al., 2011). Therefore, as protein therapy is trendily studied, researchers are looking for a highly potent bone-related protein to be the candidate for osteoporosis and/or periodontitis treatment.

As several bone-related proteins have been widely introduced and elaborated, one protein, namely osteogenic growth peptide (OGP), has the potential to cope with the problem. OGP is only a fourteen-amino acid peptide that was firstly isolated in the serum of post-ablation rat bone marrow in 1992 (Bab et al., 1992). Interestingly, they found that OGP is identical with C-terminus of histone 4 (H4). Pountos et al. (2016) described that OGP is getting involved in bone regeneration process due to its osteoinductive property.

To enhance osteogenic differentiation, there is, indeed, an involvement of several molecules and pathways, such as heme oxidase 1 (HO-1) (Vanella et al.,

2010), CXC chemokine ligand 13 (CXCL13) via histone 4 (H4) acetylation (Li et al., 2015), osteoprotegerin (OPG) (Spreafico et al., 2006), and RhoA/Rho-associated protein kinase (Chen et al., 2011) pathway by the OGP treatment has been reported in several kinds of cells. Moreover, not only regulate osteogenic differentiation, but they also address that OGP regulates adipogenic differentiation. With these reports, it may support the current treatment solution that OGP may act work as an anabolic and, also, anti-resorptive agent. Hopefully, by the treatment of OGP, osteoporotic and/or periodontic patients are then expected to have a higher BMD and balanced-bone homeostasis.

Although OGP has been studied on several kinds of cells, there are still inadequate basic science knowledge either on another kind of cells, especially dental-derived mesenchymal stem cells (MSCs) or specific molecules or pathways involved in OGP treatment. Currently, dental-derived MSCs become an interest object for researchers to explore anything due to its easiness to obtain and isolate (Dave and Tomar, 2018), especially human periodontal ligament stem cells (hPDLSCs). Up to date, hPDLSCs have been suggested to be used for periodontal regeneration (Seo et al., 2004; Huang et al., 2009; Song et al., 2015). Several studies have shown that hPDLSCs showed the properties of MSCs, such as proliferation, multilineage differentiation, and immunomodulatory capacity (Seo et al., 2004; Nagatomo et al., 2006; Tang et al., 2014; Li et al., 2019; Liu et al., 2020). A report of osteogenic differentiation potential of OGP on hPDLSCs has been recorded by Xu et al. (2009). However, their study only evaluated proliferation and alkaline phosphatase activity. In another words, the osteogenic differentiation potential of hPDLSCs by OGP treatment is still yet fully understood.

In final, hPDLSCs can be described as a model and, even, potential cells to repair bone defect-associated with periodontitis within OGP treatment. To overcome the matter mentioned before, in this study, we would like to investigate the osteogenic differentiation potential and its potential signaling involved on hPDLSCs by the treatment of OGP. The result of this study may complete the basic science knowledge of OGP that can be used for further application for osteoporosis and/or periodontitis treatment.

**OBJECTIVE OF THE STUDY**

1. To isolate and characterize human periodontal ligament stem cells (hPDLSCs) for bone tissue engineering application
2. To investigate the effects of osteogenic growth peptide (OGP) on human periodontal ligament stem cells (hPDLSCs) toward osteogenic lineage
3. To explore the potential osteogenic-regulating pathways that engage the *in vitro* osteogenic differentiation commitment of osteogenic growth peptide (OGP) on human periodontal ligament stem cells (hPDLSCs) toward osteogenic lineage

**KEYWORDS**: Bone regeneration, human periodontal ligament stem cells (hPDLSCs), osteogenic growth peptide (OGP)

**HYPOTHESIS**

Osteogenic growth peptide (OGP) consists of the potential to enhance the osteogenic differentiation on human periodontal ligament stem cells (hPDLSCs) *in vitro*.

# CHAPTER II

# LITERATURE REVIEW

## Bone resorption disorders

In a balanced condition between bone formation and bone resorption, bone remodeling will be occurred. However, if there is an impairment between one of those two, it may lead into abnormal bone resorption. Such disorders with abnormal bone resorption include osteoporosis, periodontitis, Paget's disease, osteodystrophy, osteopetrosis, and due to cancers (Russell et al., 2001; Hienz et al., 2015). As osteoporosis and periodontitis have been highlighted as two of many global health burdens, these diseases are keen to be elaborated more.

Osteoporosis is a multifactorial and metabolic bone disease that results in extensively bone resorption due to less compensation of bone formation (Föger-Samwald et al., 2020). It can be classified in primary and secondary osteoporosis, whereas primary osteoporosis can be divided into two subgroups: type I (post-menopausal osteoporosis) and type II (senile osteoporosis) (Sözen et al., 2017). Shortly, primary osteoporosis is known for its bone mass loss due to hormone deficiency and aging, while secondary osteoporosis is because of secondary causes, such as lifestyle, genetic, endocrine, gastrointestinal, hematological, neurological and musculoskeletal, rheumatologic and autoimmune diseases, and other miscellaneous causes. These causes increase the production of receptor activator of nuclear factor kappa beta ligand (RANKL) and tumor necrosis factor alpha (TNF-$\alpha$) that lead bone resorption (Flegar et al., 2015).



It is known that fracture is a direct cause of osteoporosis, whereas known as osteoporotic fracture. The fracture may cause disability, morbidity, and mortality for the osteoporotic patients. Therefore, this condition has an impact on quality of life. Patients may develop the imbalance of mental, emotional, and physical health. Moreover, osteoporosis has been considered as one of public health major concerns. Not only affecting the health of patients, but also it affects financial aspect. This will occur due to hospitalization, outpatient, and long-term care for osteoporotic fracture patients (Pongchaiyakul et al., 2008).

By World Health Organization (WHO) criteria, osteoporosis can be diagnosed when an individual has a T-score of more than -2.5 standard deviation below of the reference (Kanis et al., 2002). The value of T-score can be calculated by measuring bone mass density (BMD) using dual X-ray absorptiometry (DXA) by comparing between BMD of patient and young females aged 20-29 years (Sözen et al., 2017). Moreover, T-score can be supported by evaluation of biochemical bone turnover marker (BMT) that may include serum C-terminal telopeptide type-I collagen (s-CTX) and urinary N-telopeptide (NTX), and formation marker, serum procollagen type-I N-terminal propeptide (s-PNIP).

Generally, drugs for osteoporosis can be classified as two big groups: anti-resorptive and anabolic agents. Anti-resorptive drugs are meant to reduce bone resorption through osteoclast detachment and apoptosis, or, even, to inhibit the function of osteoclasts. There are several common anti-resorptive agents that are used by osteoporotic patients, such as bisphosphonates, denosumab, and odanacatib. On the other hand, anabolic agent is used to stimulate bone formation. This agent may include parathyroid hormone (PTH), PTH-related protein (PTHrP), Dickkopf 1 (Dkk1), soluble activin A type II receptor (ActRIIA), bone morphogenic proteins (BMPs), and nitroglycerin (Iñiguez-Ariza and Clarke, 2015; Föger-Samwald et al., 2020). It has been mentioned that these drugs may have limitations including the involvement of the drug in other tissues, signaling pathways, and has a short half-life. Moreover, calcium and vitamin D supplementation can be used also as a basic therapy of osteoporosis (Föger-Samwald et al., 2020).

On another hand, periodontitis is a condition which periodontal tissue is getting inflamed. In advanced stage, alveolar bone loss due to extensive bone resorption will be occurred (Hienz et al., 2015). Similarly to osteoporosis, it is also known as a silent disease due to its unnoticed progress and has no pain (Bostanci and Belibasakis, 2017). Based on severity, periodontal disease can be classified from type I to V, that is from gingivitis, mild to severe periodontitis (McLeod, 2000). Based on cause, periodontitis can be classified into inflammatory and non-inflammatory periodontitis. Inflammatory periodontitis is caused by bacterial infection, whereas *Porphyromonas gingivalis* is one of major periodontal pathogens (How et al., 2016).

While, non-inflammatory periodontitis, also known as presenile osteoporosis, has been associated with systemic osteoporosis although it is not well defined yet (Wang and McCauley, 2016). In this matter, systemic bone resorption may disrupt bone hemostasis that leads local bone resorption in alveolar bone. Several molecules, including RANKL, osteoprotegerin (OPG), TNF-$\alpha$, interleukin 1 (IL-1), and prostaglandin E2 ($PGE_2$), have the involvement in alveolar bone destruction (Hienz et al., 2015), same molecules as osteoporosis.

Nazir (2017) reported that risk factors of periodontitis can be classified in big groups, such as modifiable (i.e., smoking lifestyle, poor oral hygiene, hormonal imbalance in women, diabetes mellitus, medications, and stress), non-modifiable (i.e., age and genetic), and other medical conditions-associated with periodontal disease (i.e., cardiovascular, metabolic, respiratory, chronic kidney diseases, pregnancy, rheumatoid arthritis, and cognitive function impairment) risk factors. Diagnosis of periodontitis can be done by assessing periodontal examinations (i.e., clinical probing depth (PD), attachment levels (CAL), and tooth loss) and radiographic examination (i.e., DXA) (Wang and McCauley, 2016).

Periodontitis treatment can be divided into four phases: 1) Phase I (control risk factors and inflammation); 2) Phase II (surgical correction); 3) Phase III (rehabilitative periodontal care) (McLeod, 2000); and 4) Phase IV (preventive care) (Tonetti et al., 2017). Prevention can be done to avoid periodontitis by improving oral hygiene practices and lifestyle, consuming healthy diet, and using fluoride and antimicrobial agents (Nazir, 2017).

**Dental-derived mesenchymal stem cells (MSCs) for bone tissue engineering (BTE)**

Several kinds of stem cells have been reported that can be used for cell-based therapy, such as embryonic stem cells (ESCs), induced-pluripotent stem cells (iPSCs), and adult stem cells (ASCs) (Aly, 2020). Mesenchymal stem cells (MSCs) are one of many types of ASCs that are plastic-adherent, differentiated into several lineages *in vitro*, and expressing specific surface antigen stem cells (Dominici et al., 2006; Ullah et al., 2015). Dental tissues are one of the MSCs sources that can be

obtained. Comparing to bone marrow-derived mesenchymal stem cells (BM-MSCs) collection that is invasive, dental-derived MSCs are known easier to obtain, may use the waste of teeth extraction, and non-invasive. It is known that dental-derived MSCs is from the development of neural crest (Dave and Tomar, 2018).

Dental-derived MSCs can be derived from tooth germ (TGSCs), exfoliated deciduous teeth, gingiva (GMSCs), dental pulp (DPSCs), alveolar bone (ABM-MSCs), dental follicle (DFSCs), apical papilla (SCAPs), and, lastly, periodontal ligament (PDLSCs).

PDLSCs were firstly introduced in 2004 as one of dental-derived MSCs (Seo et al., 2004). Previously, human PDLSCs (hPDLSCs) have been characterized for its expression of MSCs specific surface antigens including positive results of STRO-1, CD146 CD90, CD105, and CD73 and negative result of CD45 as haematopoietic stem cells (HSCs) surface marker (Seo et al., 2004; Zhu and Liang, 2015). It has been reported that PDLSCs are able to differentiate into osteogenic, adipogenic, chondrogenic, and neurogenic lineages (Osathanon et al., 2013; Zhu and Liang, 2015). Moreover, hPDLSCs also have the immunomodulatory property (Wada et al., 2009), and even osteogenic-differentiated PDLSCs have been reported that still maintain the property (Tang et al., 2014).

As an *in vitro* study has showed that hPDLSCs are able to differentiate into osteogenic, fibrogenic, and cementogenic lineages (Liu et al., 2020), confirmational of *in vivo* has also been done (Seo et al., 2004). Their study found that PDLSCs formed cementum-, PDL-like, and collagen fibres at the periodontal area in immunocompromised rats. They concluded that PDLSCs transplantation in the mice can generate reparation in periodontal tissue by involving the PDL attachment to surface of the tooth and orchestrating alveolar bone and PDL repair. Therefore, up to date, PDLSCs have been suggested to be applicable for periodontal regeneration (Lin et al., 2008; Song et al., 2015; Zhu and Liang, 2015).



**Osteogenic-related peptide for bone regeneration**

Biomolecules, such as peptides, are one of many choices to establish bone tissue engineering (BTE). Several peptides including thrombin (TP508), PTH/PTHrP,

calcitonin gene-related peptide (CGRP), Pepgen P15, arginyl-glycyl-aspartic acid (RGD) containing peptide, and osteogenic growth peptide (OGP) have been mentioned to orchestrate bone regeneration (Pountos et al., 2016). It is known that not only to be used as a single therapy, but also can be delivered by combining it with scaffold, stem cells, or drugs (Bose et al., 2012). To date, OGP has been addressed to be one of many potential peptides that may improve bone regeneration. Unfortunately, there is still lack of information about the peptide.

Osteogenic growth peptide, or commonly abbreviated as OGP, is a tetradecapeptide that is identic with the C-terminal 90-103 of histone 4 (H4). Historically, OGP was first isolated in 1992 and found highly abundant in the post-ablation bone marrow rat serum (Bab et al., 1992). To be modified into a tetradecapeptide, OGP is translated from the H4 mRNA and further proteolyzed becoming preOGP (H4(85-103)). Next, preOGP will be proteolyzed to become into OGP. Moreover, proteolysis is occurred again to modify OGP into OGP(10-14), which is known as the active form of OGP (Bab and Chorev, 2002). Pigossi et al. (2016) mentioned the protease that is important to cleavage OGP is neprilysin (NEP).

Classically, OGP has two domains: 1) Accessory domain (Sq 1-9) and 2) Active domain (Sq 10-14) (Policastro and Becker, 2016). In serum, accessory domain will bind with OGP-binding protein (OGPBP), namely alpha-2 macroglobulin ($\alpha_2$M), to form OGP-OGPBP complex and the active domain will be dissociated from the accessory domain. To be active, OGP(10-14) binds with the receptor, namely $G_i$ protein cell receptor (Policastro and Becker, 2016), on the mesenchymal stem cells (MSCs) surface (Pigossi et al., 2016). Subsequently, OGP will enhance the osteogenic differentiation. Thus, the involvement of OGP in bone healing is important due to its property of osteoinductivity (Pountos et al., 2016).

Many studies have reported that there are several molecules and pathways that getting involved in inducing osteogenic differentiation by the treatment of OGP. (Vanella et al., 2010) mentioned that the protein expression of heme oxidase 1 (HO-1) is upregulated during OGP treatment in human bone marrow MSCs (hBM-MSCs). In their study, they also found that OGP decreases the adipogenic differentiation. Another study using the same cells found that there is an involvement of RhoA/Rho-

associated protein kinase pathway (Chen et al., 2011). Moreover, OGP treatment increases the mRNA expression level of CXC chemokine ligand 13 (CXCL13) through H4 acetylation (Li et al., 2015). Their studies were supported by their increased results of several bone markers, such as phosphorylated-adenosine monophosphate-activated protein kinase (pAMPK), alkaline phosphatase, osteonectin, osteocalcin (OCN), BMP-2, and runt-related transcription factor 2 (Runx2). Interestingly, Spreafico et al. (2006) mentioned that there is an increased production of osteoprotegerin (OPG), a decoy receptor of receptor of activator of nuclear factor-kappa B ligand (RANKL). It has been suggested that OGP interchanges with OPG that will promote MSCs differentiation (Fei et al., 2010).

Also, OGP also has been reported to coat scaffolds. For instances, titanium (Chen et al., 2015) and hydroxyapatite surfaces (Polini et al., 2014; Zhang et al., 2017) have been mentioned to be coated by the peptide. Their studies found that the coating of OGP on such surfaces enhances osteogenic differentiation. Moreover, not only *in vitro*, but also OGP has been observed in *in vivo* studies. Several animal models, such as rats (Bab et al., 1992), mice (Chen et al., 2000), and rabbits (Sun and ASHHURST, 1998; Zhao et al., 2011), have been used to disseminate the osteogenic potential of OGP with satisfying results of enhanced bone healing. Therefore, OGP is likely has the potential to be used as a therapy for osteoporosis and/or periodontitis because of it regulates osteogenic and adipogenic differentiation.



**Signaling cascades orchestrating in bone regeneration**

As MSCs can differentiate into several lineages, especially osteogenic lineage, they must have commitment into such lineage. That commitment is known to be controlled by the activities of signaling pathways. In bone regeneration, several signaling pathways have been reported to be involved in osteogenic differentiation, including wingless-related integration site (Wnt), transforming growth factor beta (TGF-β), bone morphgenic protein (BMP), Notch, and Hedgehog.

Wnt signaling pathway

Generally, the activation of Wnt signaling pathway regulates cell growth, differentiation, function, and death (Soltanoff et al., 2009). Wnt ligands bind with its receptor, frizzled (FZD), and co-receptor, low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6). This binding transduces a signal via Disheveled (Dsh) to the destruction complex of Axin, β-catenin, adenomatous polyposis coli (APC), caseine kinase 1-α (CK1α), and glycogen synthase kinase-3 β (GSK3β). The signal will disrupt the the complex by inhibiting GSK3β to phosphorylate β-catenin. This results in an increased of post-translational modification (PTM) to stabilize β-catenin that leads to accumulation in cytosol and translocation to nucleus. In the nucleus, β-catenin replaces the co-repressor of lymphoid enhancer-binding factor/T-cell factor (Lef/Tcf) transcription factors, Groucho, and initiates transcription of target genes. This cascade is also known as Wnt/β-catenin or Wnt canonical pathway (Soltanoff et al., 2009; Houschyar et al., 2019). This pathway has been mentioned to get involved in skeletal development through MSCs differentiation and function (Regard et al., 2012). On another hand, Wnt signaling pathway that is β-catenin-independent is called Wnt non-canonical pathway. This pathway can be divided into $Ca^{2+}$-dependent and planar cell polarity (PCP) pathways (Katoh and Katoh, 2007). However, its fully mechanisms are still unclear.



A small molecule, namely Dickkopf 1 (Dkk1), has been considered as a potent inhibitor of Wnt/β-catenin pathway. It has been reported that this Wnt antagonist takes a major role in bone formation (Regard et al., 2012). Inhibition of Wnt signaling starts from the binding of Dkk1 LRP5/6 co-receptor (Soltanoff et al., 2009). Sequentially, Dkk1 forms a complex of LRP5/6 with kremen (Krm) and further inhibits the Wnt transduction (Krishnan et al., 2006).

TGF-β/BMP signaling pathway

As bone morphogenic proteins (BMPs) are members of TGF-β superfamily, its signaling is quite similar with TGF-β transduction. These two cascades are each can be divided into canonical (mothers against decapentaplegic homolog (Smad)-

dependent) and non-canonical (Smad-independent) pathway. It is known that all TGF-β superfamily members transduce signals through type I and type II transmembrane serine/threonine kinases, a dual receptor system. Signaling activation by ligand induces type II receptor to phosphorylate and activate type I receptor. Further, type I receptor phosphorylates receptor-regulated Smads (R-Smads), i.e., Smad 1, 5, and 8 in BMP pathway and Smad 2 and 3 in TGF-β pathway. Phosphorylation of Smads then forms a complex with Co-Smad (Smad 4). This R-Smad/Co-Smad complex translocates into nucleus and starts inducing target genes transcription with help of its co-factors CREB-binding protein (CBP) and p300 (Hayrapetyan et al., 2015; James et al., 2016; Wu et al., 2016a).

BMP signaling is known to regulate MSCs differentiation into osteoblast lineage via its indirect expression of Runx2, a transcription factor important in osteogenic differentiation, through distal-less homeobox 5 (Dlx5) expression (Soltanoff et al., 2009). On another hand, TGF-β also regulates proliferation of osteoprecursor, commitment to osteoblast lineage, and osteogenic differentiation (Chen et al., 2012). Moreover, TGF-β controls BMP signaling through its expression of BMP-2 and tomoregulin-1 (Tmeff1) (Xu et al., 2020). Therefore, these two pathways take a major role in bone development (Wu et al., 2016b).

Regulation of TGF-β and BMP signaling can be controlled by several inhibitors, such as SB-431542 and Dorsomorphin, respectively. SB-431542 is a small molecule that has been reported to specifically inhibit TGF-β type I receptor, activin receptor-like kinases (ALKs) (Inman et al., 2002). Similarly to SB-431542, Dorsomorphin, as one of BMP signaling inhibitors, inhibits the function of BMP type I receptor (Paul et al., 2008). In that study, they supported the data by evaluating Dorsomorphin on bone mineralization and formation *in vitro* in C2C12 cells and *in vivo* in zebrafish embryos.



Notch signaling pathway

Notch signaling is known to be simple yet evolutionary well-conserved in regulating embryonic development, differentiation, cell commitment, proliferation, differentiation, and homeostasis (Teodorczyk and Schmidt, 2015; Zieba et al., 2020).

In brief, Notch ligands (i.e., JAG1-2, DLL1, 3 and 4) interacts with Notch receptor (Notch1-4) through cell-to-cell contact. This interaction results in cleavage of Notch receptors, where, firstly, metalloproteinase tumor necrosis factor-a converting enzyme (TACE) cleavages the extracellular domain (NECD) and further its intracellular domain (NICD) is cleaved by **γ**-secretase complex. The cleaved NICD then translocates into nucleus to replace co-repressor and starts inducing Notch target genes transcription (Zieba et al., 2020). Inhibition of Notch signaling can be occurred in several targets, such as **γ**-secretase, by N-[N-(3,5-difluorophenacetyl)-L-ananyl]-S-phenylglycine t-butyl ester (DAPT). Inhibition of **γ**-secretase results in there is no cleavage of intracellular domain of Notch receptor, therefore no NICD translocates into nucleus and transcription at all (Teodorczyk and Schmidt, 2015).

Hedgehog signaling pathway

Similarly to Notch signaling, Hedgehog (Hh) pathway is also highly conserved that have an important role in bone formation, from embryonic limb development until intramembranous and endochondral ossification (Yang et al., 2015). In mammals, there are several Hh ligands, including Sonic Hh, Indian Hh (Ihh), and Desert Hh (Dhh). Currently, regulation of Hh pathway is known can be controlled by Robotnikinin that target specifically to Shh (Hitzenberger et al., 2017). The activation of Hh pathways begins when Hh ligands interact with Patched (Ptch) receptor that release and phosphorylate Smoothened (Smo) to be activated in the cilium of cell. Phosphorylation of Smo disrupts the function of protein kinase A (PKA) and allows the complex of glioma-associated oncogene family members (Gli), suppressor of fused homologue (Sufu) and kinesin family member 7 (Kif7) translocate from base to the top of the cilium. During the translocation, Kif7 sets apart Gli and Sufu binding that forms an active form of Gli. This activated Gli translocates to the nucleus and activate the transcription of Hh signaling target genes (Yang et al., 2015).

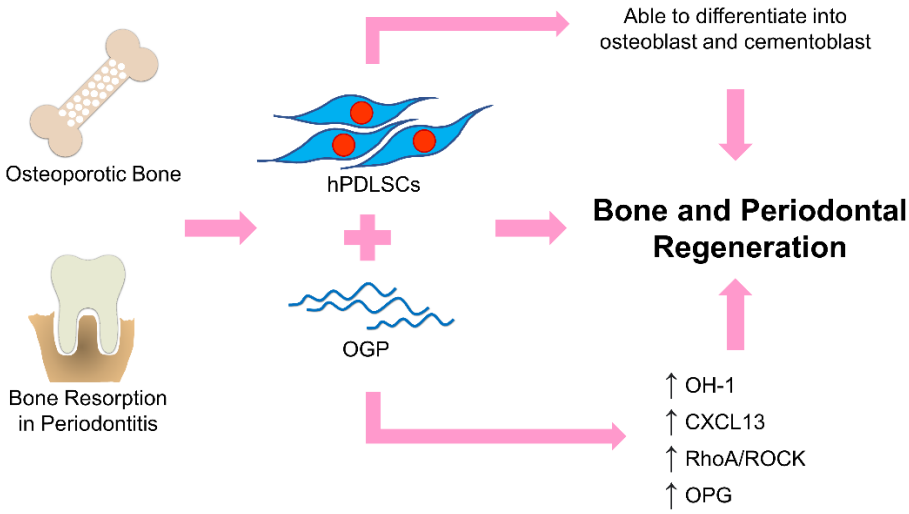


Figure 1 Potential of hPDLSCs and OGP for bone and periodontal regeneration

CONCEPTUAL FRAMEWORK

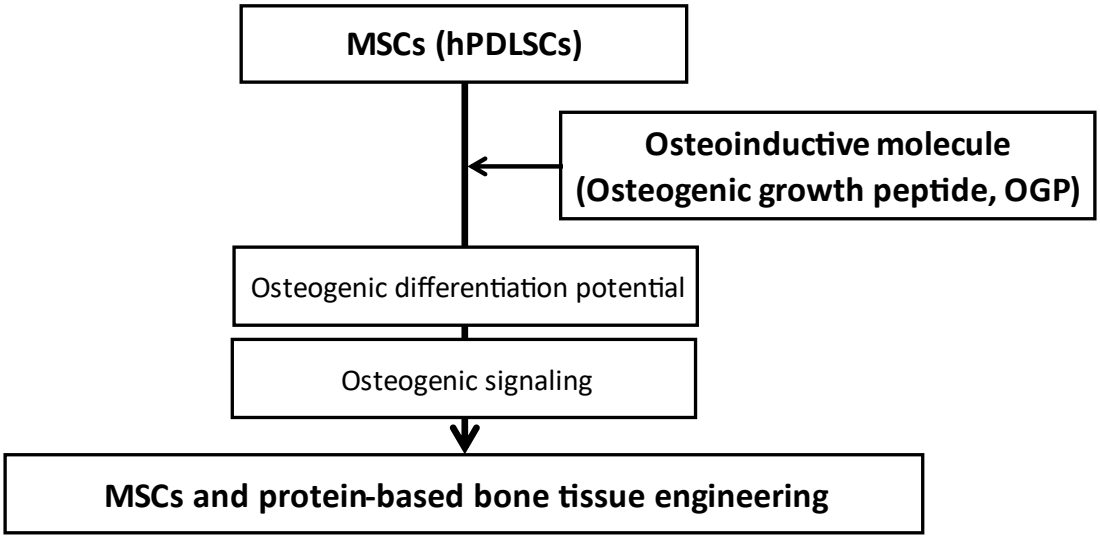


Figure 2 Experiment conceptual framework

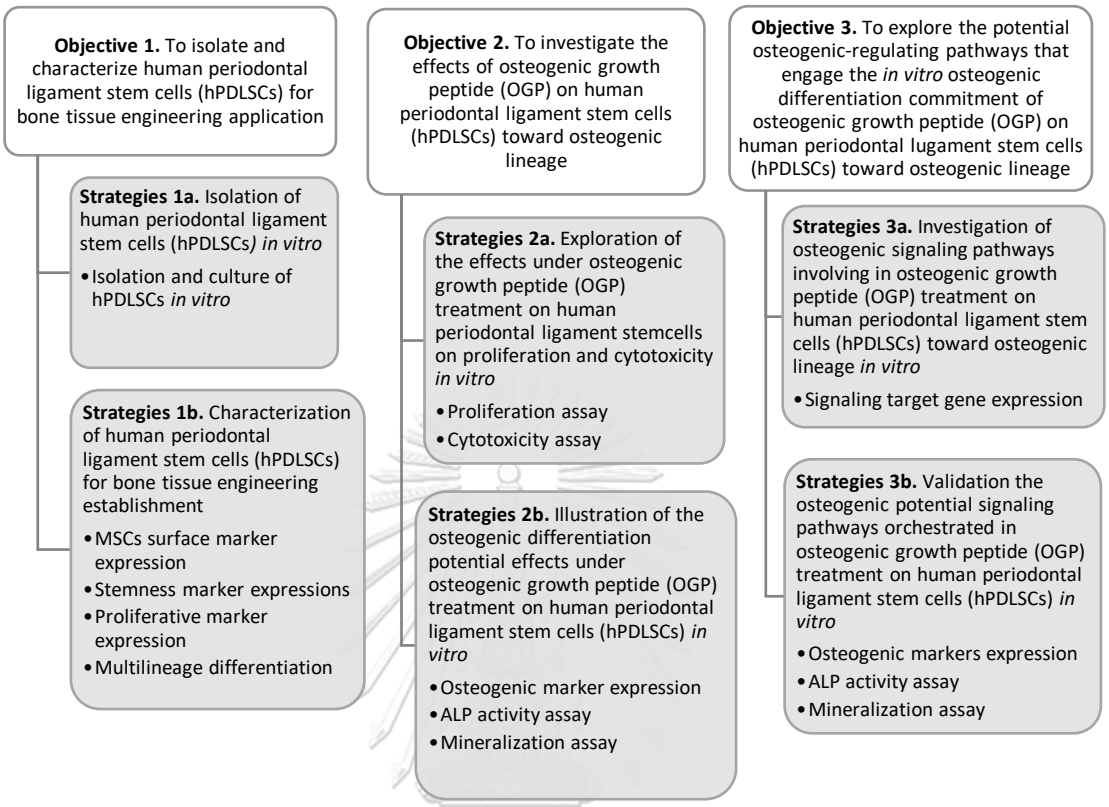


Figure 3 The schematic experiment plan

# CHAPTER III
# MATERIALS AND METHODS

Materials and methods were set up according to the proposed objective as following below:

**Objective: 1) To isolate and characterize human periodontal ligament stem cells (hPDLSCs) for bone tissue engineering application**

**Strategies: 1a)** Isolation of human periodontal ligament stem cells (hPDLSCs) *in vitro*

The use of hPDLSCs in this study has been approved by the Human Research Ethic Committee, Faculty of Dentistry, Chulalongkorn University (HREC-DCU 2018/054) with informed consents of donors. hPDLSCs were harvested from healthy extracted third molar teeth. The protocol was modified from the previous report (Sawangmake et al., 2014a). The isolated hPDLSCs in passage 2-5 were used in the experiments.

**Strategies: 1b)** Characterization of human periodontal ligament stem cells (hPDLSCs) for bone tissue engineering establishment

Isolated hPDLSCs were characterized to evaluate the MSCs poluripotency properties. MSCs surface markers (CD105, CD73, CD90, CD44) and hematopoietic stem cells (HSCs) marker (CD45) were explored using flow cytometry. mRNA expressions of pluripotency markers (*REX-1*, *NANOG*, and *OCT-4*) and proliferative marker *(KI67*) was performed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Next, multilineage differentiation was observed. Osteogenic, adipogenic, and chondrogenic differentiation potential of hPDLSCs were investigated following to previous published method (Sawangmake et al., 2014a; Sawangmake et al., 2014b; Nantavisai et al., 2020). RT-qPCR for osteogenic, adipogenic and chondrogenic mRNA expressions was performed. Alizarin Red S staining, Oil Red O staining and Alcian Blue staining were employed for evaluation of osteogenic, adipogenic and chondrogenic differentiation, respectively.

**Objective: 2) To investigate the effects of osteogenic growth peptide (OGP) on human periodontal ligament stem cells (hPDLSCs) toward osteogenic lineage**

**Strategies: 2a)** Exploration of the effects under osteogenic growth peptide (OGP) treatment of human periodontal ligament stem cells (hPDLSCs) on proliferation and cytotoxicity *in vitro*

After reaching passage 2-5, hPDLSCs were seeded at $5x10^3$ cells per well and assessed for proliferation and cytotoxicity on day 1, 5, and 7 after the treatment of OGP (Cat. No. HY-P1563, MedChemExpress, USA) with concentration 0.01, 0.1, 1, 10, and 100 nM (Figure 4). This evaluation was employed by performing alamarBlue™ assay and immunofluorescence staining. Immunofluorescence staining in this study used calcein-AM and propoidium iodide (PI) dyes to stain the live and dead cells, respectively.

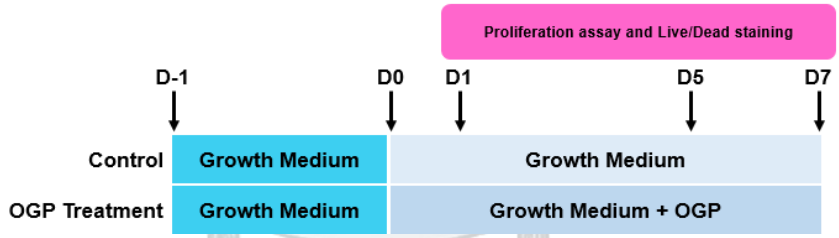


**Figure 4 Experiment plan of OGP treatment on proliferation and cytotoxicity**



**Strategies: 2b)** Illustration of the osteogenic differentiation potential effects under osteogenic growth peptide (OGP) treatment on human periodontal ligament stem cells (hPDLSCs) *in vitro*

hPDLSCs were cultured in a 24-well plate at $3x10^4$ cells per well supplemented with osteogenic medium (OM) containing all components mentioned above plus dexamethasone, ascorbic acid, and β-glycophosphate and allowed the cells to adhere for 24 h. Groups were divided into control and treatment groups and incubated for 3 w at 37 °C in a humid atmosphere 5% $CO_2$. Treatment groups consisted of various concentrations (0.01, 0.1, 1, 10, and 100 nM) of OGP. On another hand, control groups were divided into undifferentiated group in growth medium,

osteogenic-differentiated in OM, and osteogenic-differentiated positive control using OM supplemented with bone morphogenic protein 2 (BMP-2). Evaluation of bone regeneration potential was conducted on day 1, 3, 7, 14, and 21. Bone potential testing was performed to determine osteogenic differentiation, alkaline phosphatase activity, and mRNA expressions for Runt-related transcription factor 2 (*RUNX2*), osterix (*OSX*), osteocalcin (*OCN*), osteopontin (*OPN*), alkaline phosphatase (Nantavisai et al.), and collagen type 1 alpha 1 (*COL1A1*) genes using Alizarin Red S staining, ALP assay, and RT-qPCR, respectively. This experiment plan is illustrated in Figure 5.

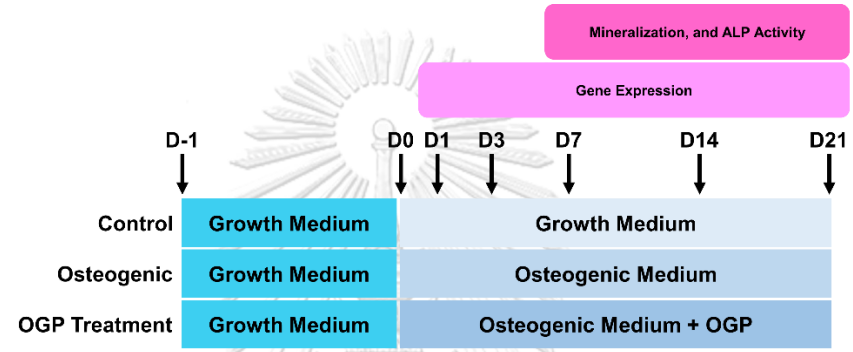


**Figure 5 Schematic plan of OGP treatment on osteogenic effects**



**Objective: 3) To explore the potential osteogenic-regulating pathways that engage the in vitro osteogenic differentiation commitment of osteogenic growth peptide (OGP) on human periodontal ligament stem cells (hPDLSCs) toward osteogenic lineage**

**Strategies: 3a)** Investigation of osteogenic signaling pathways involving in osteogenic growth peptide (OGP) treatment on human periodontal ligament stem cells (hPDLSCs) toward osteogenic lineage in vitro

Further evaluation was explored to conduct the osteogenic potential signaling pathways that relate to OGP treatment at 0.1 nM as the best concentration from previous experiment on hPDLSCs. The potential osteogenic-regulating pathways were selected based on the data from previous publications (Vanella et al., 2010; Chen et al., 2012; Houschyar et al., 2019; Manokawinchoke et al., 2020; Men et al., 2020). The potential pathways presented bone morphogenetic protein (BMP), Hedgehog, Notch,

transforming growth factor-beta (TGF-β), and Wnt signaling pathways. Day 1 and 3 post-osteogenic induction, target genes expression from Wnt signaling, TGF-β signaling, BMP signaling, Notch signaling, and Hedgehog signaling were evaluated using RT-qPCR (Figure 6). In this study, the best signaling was selected for the next experiment to be validated.

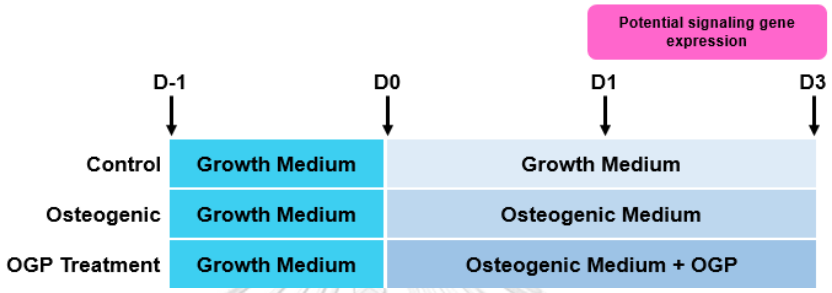


**Figure 6 Schematic plan of osteogenic signaling pathways investigation in OGP treatment**

**Strategies: 3b)** Validation the osteogenic potential signaling pathways orchestrated in osteogenic growth peptide (OGP) treatment on human periodontal ligament stem cells (hPDLSCs) *in vitro*



To validate the relevant of the TGF-β signaling pathway as the most potent signaling pathway in OGP treatment on hPDLSCs, one specific inhibitor of the selected signaling pathway, SB-43152, was employed upon an *in vitro* osteogenic induction. The selected potential pathway was explored at day 1, 3, 7, 14, and 21 post-inducton. Osteogenic gene expression (*RUNX2*, *OSX*, *OCN*, *OPN*, *COL1A1*, and *ALP*), ALP activity assay, and mineralization evaluation assay was analyzed and illustrated to present the confirmation of osteogenic-related pathway under OGP treatment (Figure 7).

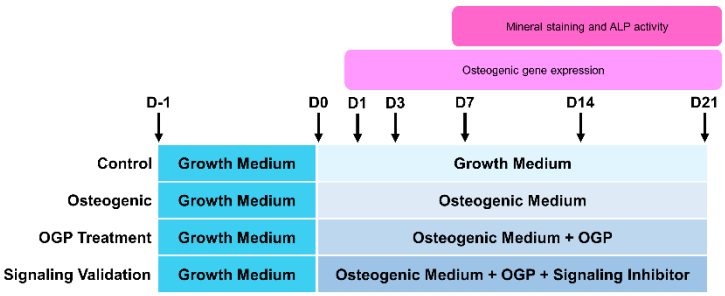


**Figure 7 Schematic plan of signaling pathway validation in OGP treatment**

## Detailed materials and methods

### hPDLSCs isolation, culture, and expansion in vitro

For hPDLSCs isolation, the tooth was washed with phosphate buffer saline (PBS) under sterile condition with aseptic technique. Periodontal ligament was collected and put onto a 35 mm culture dish according to previous publication technique (Manokawinchoke et al., 2020). Cells were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA), 1% Antibiotics-Antimycotic (Thermo Fisher Scientific, USA), and 1% Glutamax (Thermo Fisher Scientific, USA). Cells were incubated at 37°C with 5% $CO_2$. Culture medium was changed every 48 hours and subcultured at 80% confluence. hPDLSCS in passage 2-5 were employed in this study.



### Colony formation assay

The protocol of this assay was modifying from the previous published studies (Nantavisai et al., 2020; Nowwarote et al., 2020). hPDLSCs were seeded at 500 cells in a 60 mm culture dish (Corning, USA) and cultured for 14 days. After that, cells were washed with PBS and fixed with 100% methanol (Sigma-Aldrich, USA) for 20 min at 4 °C. Staining was performed by using crystal violet (Sigma-Aldrich, USA). More than 50 aggregated cells will be counted and considered as a colony.

**Flow cytometry**

Single cell suspension of hPDLSCs was combined with FITC-conjugated anti-human CD105 antibody (Bio Legend, USA), FITC-conjugated anti-human CD73 antibody (Bio Legend, USA), FITC-conjugated anti-human CD90 antibody (Bio Legend, USA), FITC-conjugated anti-human CD44 antibody (Bio Legend, USA), and FITC-conjugated anti-human CD45 antibody (Bio Legend, USA) for MSCs surface marker expression. FITC-conjugated Mouse IgG1**K** Isotype was employed as isotype control. FACS caliber flow cytometer (BD Biosciences, USA) was utilized to evaluate the stained cells.

**Multilineage differentiation**

***In vitro osteogenic differentiation***

The osteogenic differentiation protocol was performed and modified according to previously published reports (Sawangmake et al., 2014a; Sawangmake et al., 2014b). Cells were seeded in a concentration of $3x10^4$ per well of 24-well culture plate. Next, cells were maintained and cultured in osteogenic induction medium for 21 days. The osteogenic medium was containing growth media supplemented with 50 mg/mL ascorbic acid (Sigma-Aldrich, USA), 100 nM dexamethasone (Sigma-Aldrich, USA), and 10 mM β-glycerophosphate (Sigma-Aldrich, USA).



***In vitro chondrogenic differentiation***

Chondrogenic differentiation was performed according to the previous established protocol (Nantavisai et al., 2020). Briefly, $5 x10^4$ cells wereseeded per well in a 24-well culture plate and cultured in chondrogenic induction media for 21 days prior to alcian blue staining and chondrogenic gene expression evaluation. Chondrogenic medium was consisted of DMEM supplemented with 1% L-glutamine (Thermo Fisher Scientific, USA), 1% antibiotic-antimycotic (Thermo Fisher Scientific, USA), 50 mg/mL ascorbic acid (Sigma-Aldrich, USA), 40 mg/ml L-proline (Sigma-Aldrich, USA), 0.1 μM dexamethasone, 1% insulin-transferrin-selenium (Thermo Fisher Scientific, USA), 10 ng/mL transforming growth factor beta 3 (TGF-β3) (Sigma-Aldrich, USA), and 15% FBS.

***In vitro adipogenic differentiation***

According to the established adipogenic induction protocol (Nantavisai et al., 2020), $3x10^4$ cells per well were seeded in a 24-wells culture plate (Corning, USA) and maintained in adipogenic medium for 23 days. Adipogenic induction medium was containing growth media supplemented with 1 µM dexamethasone (Sigma-Aldrich, USA), 0.1 mM indomethacin (Sigma, USA), 1 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich, USA), and 1 µg/mL insulin (Sigma-Aldrich, USA) for 72 h. After that, cells were maintained in adipogenic maintain medium containing growth media supplemented with 1 µg/mL insulin for 24 hr. Induction cycle was repeated for 4 times. Finally, cells were cultured in adipogenic maintain medium until 23 days of induction prior to Oil Red O staining and adipogenic gene expression assessment.

**Alkaline phosphatase activity**

The alkaline phosphatase activity was evaluated at day 7, 14, and 21 after osteogenic induction according to the protocol from previous reports (Sawangmake et al., 2016; Nantavisai et al., 2020). In brief, cells were lysed in lysis buffer containing 0.1% Triton X-100, 1 M Tris-HCl 500 L and 5 mM $MgCl_2$. Lysate sample was incubated with *p*-nitrophenol phosphate (PNPP) (Life Technologies Corp., USA), 2-amino-2-methyl-1-propanolol (Sigma, USA), and 2 mM of $MgCl_2$ for 15 minutes at 37 ˚C. The reaction was stopped by adding 0.1 M NaOH. The absorbance was measured at wavelength of 410 nm. Next, total protein concentration was measured by Qubit® (Thermo Fisher Scientific, USA) following the manufacturer's protocol. The enzyme activity was calculated as U/mg protein.

**Alizarin red S staining**

Alizarin red S staining was performed at 7, 14, and 21 days after osteogenic induction. The following protocol was adapted from the previous report (Sawangmake et al., 2016). hPDLSCs were washed with PBS and fixed with cold methanol for 20 min at 4 °C. After that, cells were washed with distilled water (pH

4.2) for 3 times and stained with 2% Alizarin red (Sigma, USA) solution for 5 min at room temperature. Lastly, cells were washed 2-3 times with distilled water (pH 4.2).

**Alcian blue staining**

Cells were washed with PBS. Next, pellet of hPDLSCs was fixed with 4% paraformaldehyde and blocked with paraffin. 0.1% alcian blue (Sigma-Aldrich, USA) solution (pH 2.5) was used to stain cells. Observation was performed under a light microscope.

**Oil red O staining**

hPDLSCs were washed with PBS and fixed in 4% paraformaldehyde for 1 hr at room temperature. Next, cells were washed with PBS for 2 times and dehydrated using 60% isopropanol for 5 min. Further, cells were washed with PBS and stained with oil red O solution (Sigma, USA) for 1 hr. Stained cells were then rinsed with PBS. The result was observed under microscope.

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

This assay was performed following to the manufacturer's protocol. To collect RNA from hPDLSCs, the sample was extracted by using TRIzol® reagent (Invitrogen, USA) and Direct-Zol RNA isolation kit (ZymoResearch, USA). ImProm-II™ Reverse Transcription System kit (Promega, USA) was used to obtain complementary DNA (cDNA). Next, PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, USA) was utilized for the quantitative real-time PCR (qPCR) using Bio-Rad Real-Time PCR Detection System (Bio-Rad, USA). Finally, mRNA expression was normalized to 18S ribosomal RNA as the reference gene. The mRNA expression was shown as relative mRNA expression by comparing to control group.

**AlamarBlue™ assay**

A $5x10^3$ of OGP-untreated and -treated hPDLSCs were incubated in culture medium supplemented with 5% alamarBlue™ (Invitrogen, USA) for 3 hrs after 1, 5, and 7 days of culture in growth medium. After incubation, absorbance of reduced and oxidized from alamarBlue™ was measured by using spectrophotometer at 570 and 600 nm wavelength, respectively. Then, percentage of reduction was calculated by using the absorbance values, according to the manufacturer's protocol.

**Live/Dead assay**

This assay employed $5x10^3$ hPDLSCs and evaluated the result on day 1, 5, and 7 after culture in growth medium. Staining was performed following to the manufacturer's protocol. Result was evaluated using Carl Zeiss™ Apoptome.2 apparatus-equipped fluorescent microscope (Carl Zeiss, USA). Viable cells were visualized in green color by the calcein-AM. On another hand, dead cells were seen in red color by PI.

**Potential signaling pathway selection**

OGP concentration of 0.1 nM was chosen as the best concentration of OGP treatment on hPDLSCs based on osteogenic gene expression, mineralization assay, and ALP activity assay evaluations as had been compared with undifferentiated and differentiated control groups. The potential pathways presented Wnt signaling, Notch signaling, TGF- β signaling, BMP signaling and Hedgehog signaling. Target genes from bone morphogenetic protein (BMP) (*ID1*, *DLX5*, and *MSX2*), Hedgehog (*PTCH1*, *GLI1*, and *HHIP1*), Notch (*HES1*, *HEY1*, and *LFNG*), TGF-β (*BMP-2*, *TMEFF1*, and *CXXC5*), and Wnt signaling (*LEF1* and *TCF7*) were analyzed by using RT-qPCR at day 1 and 3 post-osteogenic induction.

**Validation of potential signaling pathway**

Based on its target gene expression, TGF-β signaling pathway was selected as the most potent signaling pathway that was involved in OGP treatment on hPDLSCs. To validate of potential signaling pathway, a specific inhibitor was employed. The inhibitor used was 4 μM of SB-431542 to confirm TGF-β signaling pathway. Cells were collected to perform osteogenic differentiation potential at day 1, 3, 7 ,14 and 21 post-osteogenic induction.

**Statistical analysis**

To analyze the statistical analysis between control and experiment groups, obtained data was calculated by using SPSS Statistics Program Ver 22.0 (IBM, USA). The independent *t*-test was performed to analyze two independent groups. Experiments with two more than groups employed Kruskal-Wallis test and post-hoc Mann-Whitney U test. The significant level was considered at $p$-value < 0.05.

# CHAPTER IV
# RESULTS AND DISCUSSION

## RESULTS

### hPDLSCs possess MSCs criteria

The MSCs isolated from human periodontal ligament showed the characteristics of its adherence ability on a plastic surface and fibroblast-like morphology (Fig. 8A). The cells also expressed MSCs markers, including CD73, CD105, CD90, and CD44 and lowly expressed HSCs marker CD45 by flow cytometry analysis (Fig. 8B). Moreover, pluripotency (*Rex1*, *Nanog*, and *Oct4*) and proliferative (*Ki67*) markers were expressed (Fig. 8C). In Fig. 8D-E, the cells were demonstrated able to form colonies. The potential of hPDLSCs to differentiate into osteogenic, adipogenic, and chondrogenic lineages was shown through staining and specific lineage-related gene expression results in Fig. 8F-K.

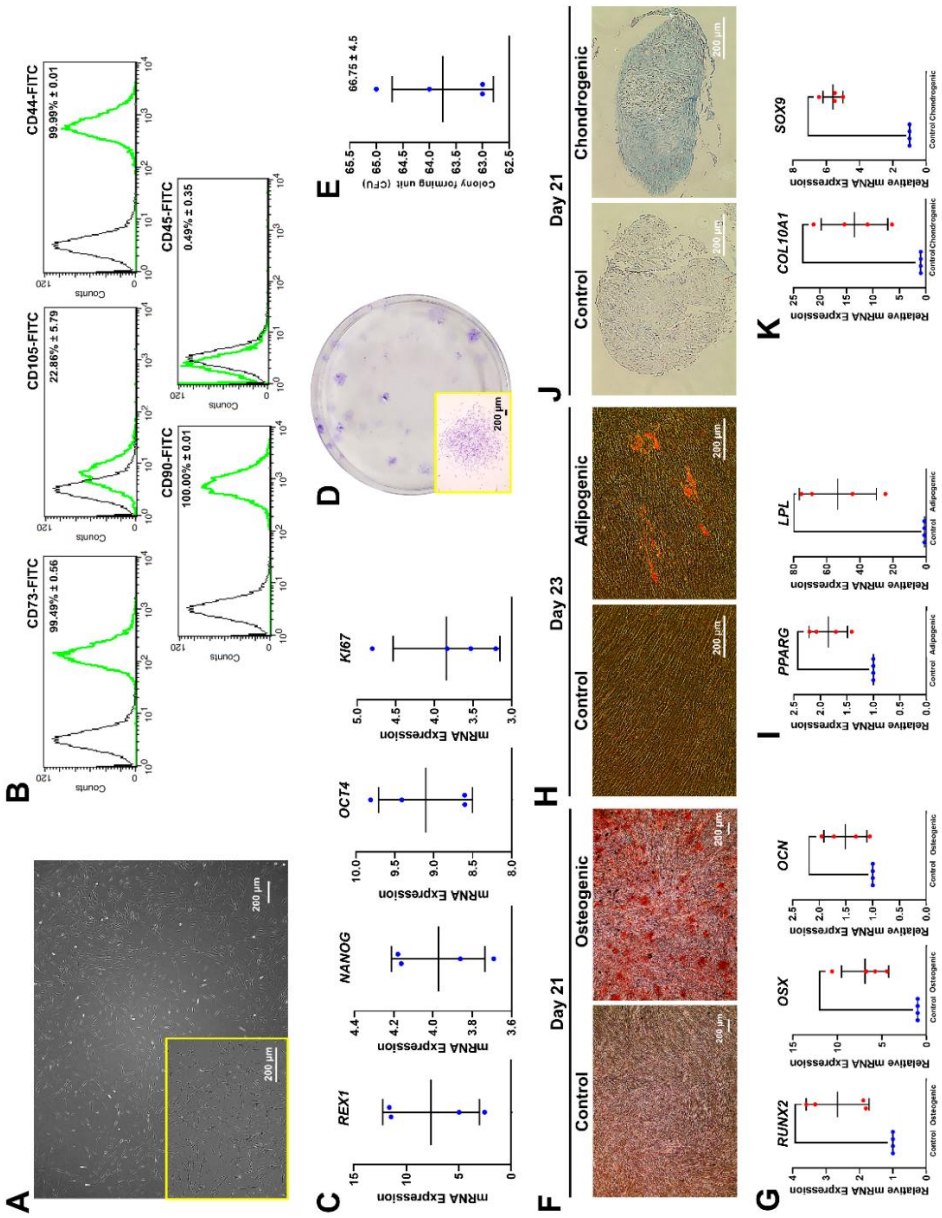


**Figure 8 Characterization features of isolated hPDLSCs.**

(A) The cell adherence on a plastic surface and morphology, (B) MSCs surface markers, (C) pluripotency (*REX1*, *NANOG*, and *OCT4*) and proliferative (*KI67*) marker expression, (D-E) functional for colony forming ability and colony count, and (F-K) multipotency with specific lineage marker expression into osteogenic, adipogenic, and chondrogenic lineages were assessed. Normalization of relative mRNA expression was done with 18S as reference gene and to control group. Bars indicate the significant difference between groups at $p < 0.05$.

**OGP at lower concentration shows higher mitogenic activity**

The experiment diagram of OGP treatment on proliferation is presented at Fig. 9A. At day 1 and 5, a significant increased proliferation was found at concentration 0.01, 0.1, and 1 nM of OGP, while 10 and 100 nM of OGP were found insignificant comparing with control. On the other hand, there were no significant results found at day 7 between OGP at any concentrations and control groups (Fig. 9B). Live/dead staining results were demonstrated and correlated with the proliferation assay results. The staining showed that dead cells were observed in only a few numbers of cells observed comparing to the viable cells after OGP treatment at any concentrations at day 1, 5, and 7 (Fig. 9C). Thus, low-dose OGP is proliferative with non-cytotoxic effect of hPDLSCs.

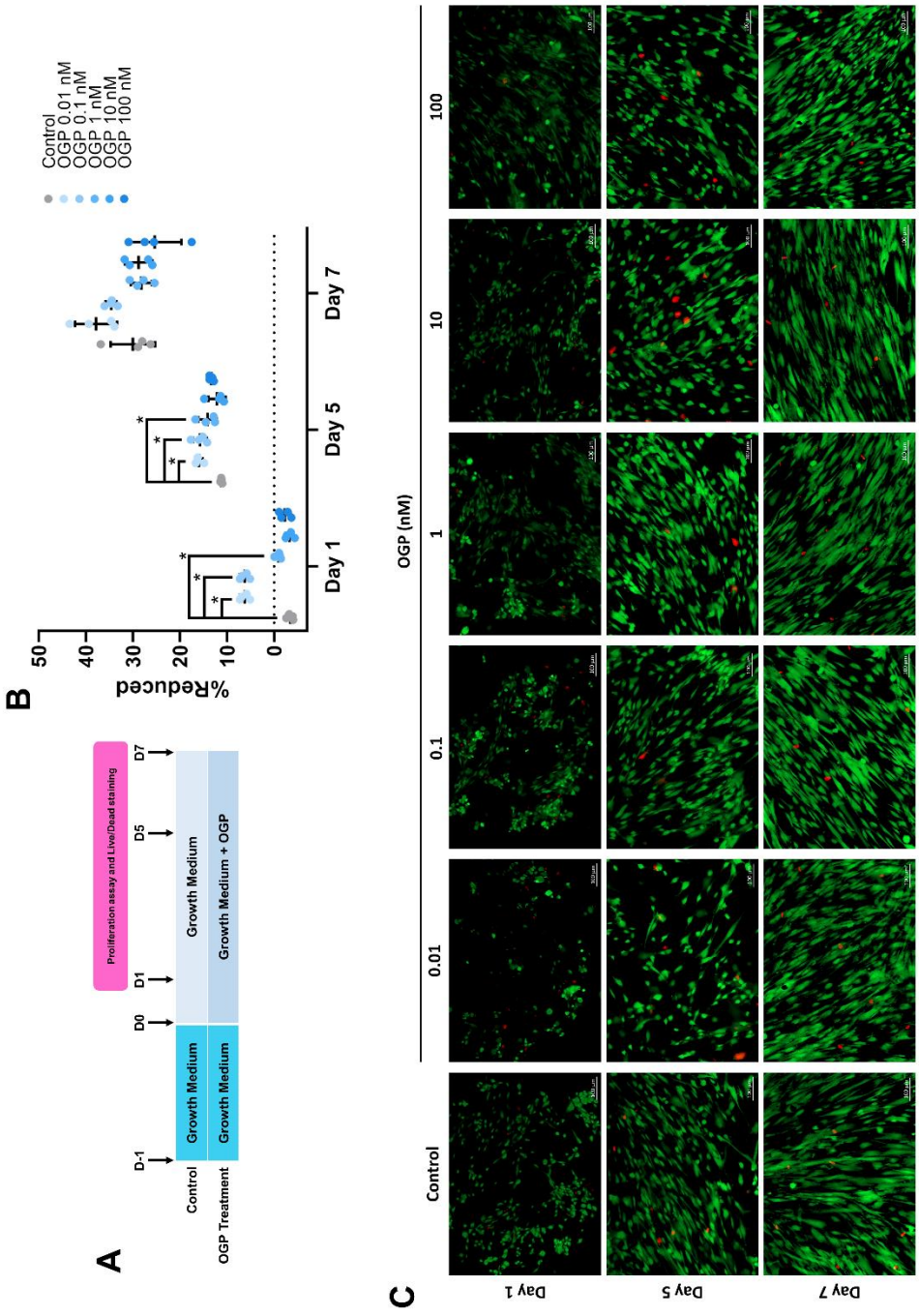


**Figure 9 OGP effects on proliferation and cytotoxicity of hPDLSCs.**

(A) The experiment workflow and (B) proliferation assay of OGP treatment were demonstrated. (C) Cytotoxicity assay was visualized with dead cells stained by propidium iodide (PI) in red-colored and viable cells stained by calcein-AM in green-colored. Bars on proliferation assay indicate the significant difference between control and OGP groups at $p < 0.05$.

**OGP at lower concentration enhances osteogenic differentiation of hPDLSCs**

Further experiment to evaluate osteogenic differentiation potential in OGP treatment was conducted. The experiment diagram of OGP treatment toward osteogenic lineage is presented at Fig. 10A. Illustration of the ALP activity result shows that undifferentiated control group had higher ALP activity than other groups at day 7, despite low concentration (0.01 and 0.1 nM) of OGP showed higher result trend than high concentration (1, 10, and 100 nM) of OGP. In addition, 0.01 and 0.1 nM of OGP were also illustrated having higher ALP activity than other experimental groups (Fig. 10B). The mRNA expression of osteogenic markers revealed that 0.01 and 0.1 nM of OGP upregulated *ALP*, *OPN*, and *OCN*. Moreover, higher expression of *RUNX2*, *OSX*, and *COL1A1* was found at 1 nM comparing with 0.01 and 0.1 nM of OGP (Fig. 10C). Mineral deposition result illustrated 0.01 and 0.1 of OGP had more mineral deposits than higher 1, 10, and 100 nM of OGP (Fig. 10D). According to the results, low-dose OGP has the potential in enhancing osteogenic differentiation of hPDLSCs.

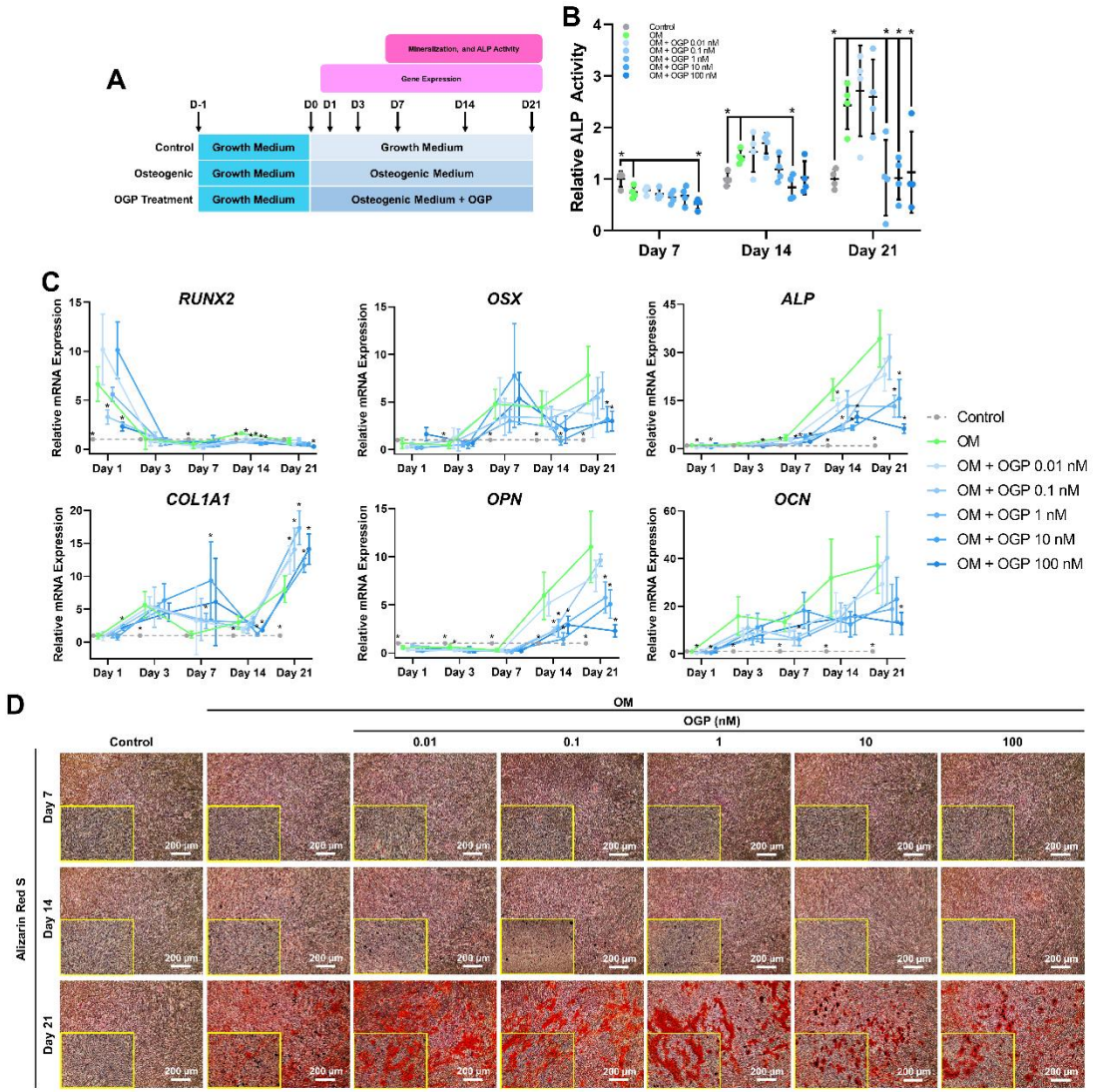


**Figure 10 OGP effect on osteogenic differentiation of hPDLSCs.**

(A) The experiment workflow of OGP treatment workflow on hPDLSCs in an osteogenic condition. (B) The ALP activity, (C) osteogenic-related marker expressions, and (D) mineral deposition were demonstrated. Bars and asterisks on ALP activity and relative mRNA expression, respectively, indicate the significant difference between control OM and other groups at $p < 0.05$.

**OGP dependently enhances osteogenic differentiation of hPDLSCs via TGF-β pathway**

Dissemination of signaling pathways involved in OGP treatment on hPDLSCs was conducted by assessing target gene expression of several signaling pathways, including bone morphogenetic protein (BMP), Hedgehog, Notch, transforming growth factor-beta (TGF-β), and Wnt at day 1 and 3 after osteogenic induction. The experiment diagram of osteogenic-regulated signaling screening during OGP treatment in an osteogenic condition is illustrated at Fig. 11A. This experiment used OGP at concentration 0.1 nM as it was found the best concentration to induce osteogenic differentiation on hPDLSCs from previous experiment.

The expressions of BMP signaling target genes was found upregulated, only *DLX5* was significant difference to OM group, both at day 1 and 3 (Fig. 11B). Meanwhile, at day 1 and 3, *PTCH1* and *GLI1* of Hedgehog signaling were also found upregulated but not significant different comparing with OM group. For *HHIP*, the expression in OGP group was extremely downregulated and resulted in OM group had higher expression at day 3 (Fig. 11C). Interestingly, OGP upregulated *HES1* at day 1, yet downregulated at day 3 comparing with OM. Also, *HEY1* and *LFNG* of Notch signaling were expressed lower in OGP than OM at day 3 (Fig. 11D). A distinguished result of TGF-β signaling pathway comparing with the other signaling pathways was illustrated that *BMP2*, *TMEFF1*, and *CXXC5* were significantly upregulated both at day 1 and 3 (Fig. 11E). For Wnt signaling pathway, it was shown that *LEF1* and *TCF7* were upregulated but not significant difference at both observed days (Fig. 11 F). Altogether, TGF-β signaling is the most potent signaling pathway regulated during OGP treatment toward osteogenic lineage on hPDLSCs.

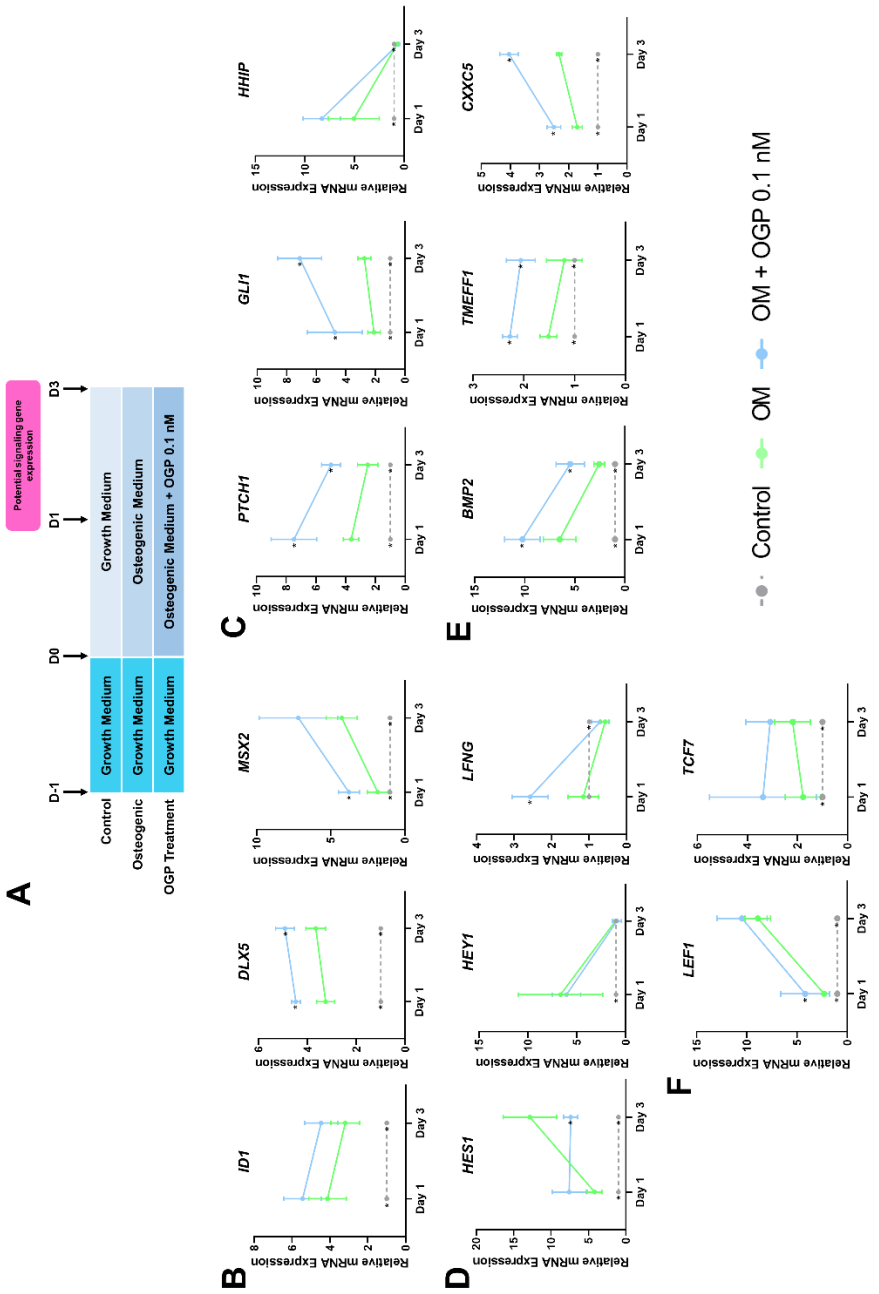


**Figure 11 Osteogenic-regulated signaling specific target gene expressions toward osteogenic lineage by OGP treatment on hPDLSCs.**

(A) Signaling pathway screening workflow of OGP treatment in an osteogenic condition. Bone morphogenetic protein (BMP), (C) Hedgehog, (D) Notch, (E) transforming growth factor-beta (TGF-β), and (F) Wnt signaling pathway target gene expression observation was presented. Asterisks on gene expression result indicate the significant difference between OM and other groups at $p < 0.05$.

Validation of previous experiment was then performed for the most potent signaling pathway, TGF-β signaling, by employing SB-431542 with OGP at 0.1 nM toward osteogenic lineage for 21 d. The experiment diagram of OGP treatment validation on TGF-β signaling toward osteogenic lineage is illustrated at Fig. 12A. The results indicate that SB-431542 suppressed unremarkably the osteogenic differentiation effect of OGP on the activity of ALP (Fig. 12B). In contrast, osteogenic gene expression was attenuated in those treated with SB-43152 (Fig. 12C). In addition, at day 14 and 21, the addition of SB-431542 in OGP treatment showed mineral deposition of hPDLSCs, yet slightly lesser than cells treated with OGP alone (Fig. 12D). In short, TGF-β signaling pathway is getting involved in OGP treatment on hPDLSCs toward osteogenic lineage.

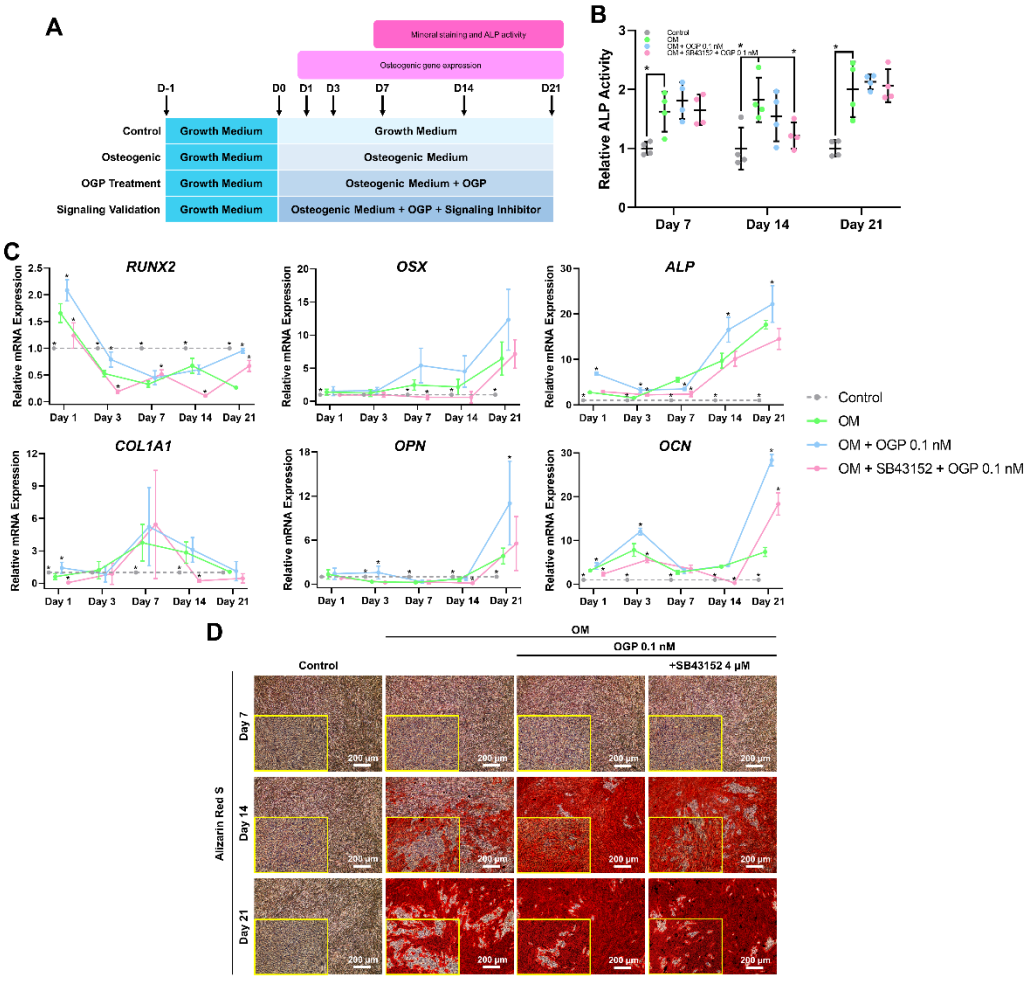


**Figure 12 Validation of TGF-β signaling pathway toward osteogenic lineage on hPDLSCs during OGP treatment.**

(A) Experiment workflow of OGP treatment on TGF-β signaling pathway on hPDLSCs in an osteogenic condition. (B) The ALP activity, (C) osteogenic-related marker expressions, and (D) mineral deposition were demonstrated. Bars and asterisks on ALP activity and relative mRNA expression, respectively, indicate the significant difference between OM and other groups at $p < 0.05$.

**DISCUSSION**

Investigation of OGP toward osteogenic lineage was performed in this study on hPDLSCs. This present study showed that OGP, specifically at low-dose, is able to enhance osteogenic differentiation potential in hPDLSCs on ALP activity, osteogenic marker gene expression, and mineral deposition.

The isolated hPDLSCs used in this study showed that the cells were characterized as MSCs following the criteria of the International Society for Cellular Therapy (ISCT) (Dominici et al., 2006) by verifying the plastic adherence, fibroblast-like shaped morphology, MSCs surface marker expression, and multipotency to osteogenic, adipogenic, and chondrogenic lineages. The cells were found highly expressing CD73, CD105, CD90, and CD44, but CD45, but relatively lower expression in CD105. This similar finding was also found in previous study (Kuncorojakti et al., 2020). This result is supported by the other parameters to define the cells as MSCs. Furthermore, hPDLSCs used in this present study demonstrated one of many abilities of human MSCs in forming colonies (Lin et al., 2008). This result is supported by the expression of pluripotency and proliferative markers our cells presented. In another word, the cells have the abilities in self-renewal and proliferation (Aponte and Caicedo, 2017). Previous published reports have also been reported similar result in different species origin-MSCs derived from bone marrow and dental pulp (Nantavisai et al., 2019; Nantavisai et al., 2020; Purwaningrum et al., 2021). Similar to this present study findings, hPDLSCs have also been reported to differentiate into osteogenic, adipogenic, and chondrogenic lineages (Gay et al., 2007; Sawangmake et al., 2014a; Sawangmake et al., 2014b).



Exploration of OGP effects on hPDLSCs was firstly tested the peptide by evaluating the proliferation and cytotoxicity assays. The peptide was previously mentioned has the effect on mitogenic activity (Bab et al., 1992; Bab and Chorev, 2002). Therefore, alamarBlue™ with active compound resazurin was employed for proliferation assay in this study. We found that OGP increased the proliferation of

hPDLSCs at lower concentration. This suggested that the proliferative hPDLSCs by OGP treatment escalates the reductases number that results in increased metabolic activity. By this means, alamarBlue™ was able to be reduced greater in a greater number of viable cells (Aslantürk, 2018). The assay is known to be able to evaluate metabolic activity through redox reaction by changing oxidized blue-colored resazurin into reduced pink-colored resofurin (Rampersad, 2012). It was reported that OGP promotes the proliferation on hPDLSCs as well (Xu et al., 2009). However, their result is contrary with ours, where OGP at concentration 1 nM had the most proliferation effect on hPDLSCs. However, lower concentration, 0.01 to 1 nM, of OGP has been mentioned to have higher effect on proliferation in mice-origin bone marrow-derived MSCs (BM-MSCs), osteoblastic MC3T3 E1, and fibroblastic NIH 3T3 cells (Greenberg et al., 1993; Fei et al., 2010). Moreover, OGP was also found to have the mitogenic activity in several types of cells, including osteogenic ROS 17/2.8 (Bab et al., 1992), rabbit- (Robinson et al., 1995), and human-origin BM-MSCs (Robinson et al., 1995).

On day 7, there was an insignificant result that may be stationary phase had been reached in OGP treatment groups. This result implies treatment groups were in a stable cell growth and control group was in a log phase. Previous study mentioned that hPDLSCs are able to reach log growth phase in 3 to 8 days of culture, while stationary growth after 8 days of culture (Yu et al., 2019). This also means that reductases and cells number in control group was comparably similar to treatment groups.

The increased proliferation phenomenon can be occurred due to proteolysis of OGP into OGP(10-14) binds with the unidentified $G_i$ protein-coupled receptor (GPCR) and subsequently initiates the activation of mitogen-activated protein kinase (MAPK pathways) (Gabarin et al., 2001). It has been reported that a Ras-like domain of $G_i$ protein contributes to this matter (McCudden et al., 2005). The Ras stimulation then leads to the activation of MAPK pathways (Goldsmith and Dhanasekaran, 2007).

Thus, MAPK pathways can control the cell proliferation through the GPCR activation. It has also been reported that CDK2/CyclinA pathways is getting involved in promoting proliferation by OGP (Fei et al., 2010).

The observation of OGP treatment on hPDLSCs was found increasing the osteogenic differentiation. MAPK pathways are mentioned not only regulate proliferation, but also osteogenic differentiation (Zhang and Liu, 2002). Therefore, this supports the phenomenon of increased osteogenic differentiation by OGP through MAPK pathways.

The expression of *RUNX2*, one of major osteogenic transcription factors (TFs), was reported to be promoted by the activation of Ras (Wang et al., 2001). Moreover, MAPK regulates *RUNX2* phosphorylation and transcriptional activity (Xiao et al., 2000). Also, MAPK mediates the expression of *OSX*, another important osteogenic TF (Celil and Campbell, 2005).

MAPK pathways have also been reported able to regulate *COL1*, *ALP*, and *OPN* expressions (Kim et al., 2014). Another study also found that *COL1* is regulated by the activation of MAPK (Wang et al., 2001). Moreover, p38 MAPK takes a role in the expression of *ALP* (Suzuki et al., 2002). The activation of ERK has also been reported to increase ALP activity (Takeuchi et al., 1997).



In addition, several studies also reported the involvement of other molecules and signaling pathways to enhance osteogenic differentiation by OGP treatment. OGP was found able to form stress fibers and involving RhoA/ROCK pathway (Chen et al., 2011). In that study, OGP could enhance osteogenesis and inhibit adipogenesis. To support this evident, $G_i$ protein activation can mediate RhoA/ROCK pathway (Huang et al., 2001). Heme oxygenase-1 (HO-1), an inducible molecule, was found to be upregulated and resulting in enhanced osteogenesis and decreased adipogenesis by OGP treatment (Vanella et al., 2010). OGP also increased the expression of long non-coding RNA (lncRNA) *AK141205* and *CXCL13* via acetylation modification H4 histone

(Li et al., 2015). The increased osteogenesis from mentioned studies above was proved with their findings in the enhanced osteogenic marker expression by OGP treatment.

According to the result of Fig. 4, the expression of most of evaluated target genes was increased. This implies that OGP is able to activate of the most evaluated signaling pathways. The phenomenon can be suggested by the activation of MAPK pathways by OGP. Several studies reported that TGF-β (Brown et al., 1999; Mori et al., 2004; Tan et al., 2014), BMP (Sapkota et al., 2007), Hedgehog (Riobo et al., 2006; Atkinson et al., 2009; Liu et al., 2014), Notch (Yamashita et al., 2013), and Wnt signaling pathways (Liao et al., 2006; Bikkavilli et al., 2008) can be regulated by MAPK pathways. In addition, a crosstalk between pathways is suggested to be occurred as well. The increased expression of *BMP2* in TGF-β signaling can activate BMP signaling. As for Notch signaling, BMP signaling can regulate the osteogenic differentiation of Notch signaling (Manokawinchoke et al., 2021). Interestingly, this study found that Notch is not mainly governed during OGP treatment in hPDLSCs up to day 3, but not day 1 toward osteogenic lineage. Moreover, Smad3 of TGF-β was found able to mediate β-catenin of Wnt canonical to translocate into the nucleus and activate the transcription (Jian et al., 2006). This activity results in increased MSC proliferation, corroborates with this study.



Thus, this study suggested that OGP facilitates the MAPK-induced increased osteogenic differentiation of hPDSLCs via many osteogenic-related signaling pathways.

Of previous result, validation was performed by using a specific inhibitor of TGF-β signaling pathway, SB-431542. It is known that TGF-β signaling is activated through its dual receptor system of transmembrane serine/threonine kinases – type I and type II receptors (Janssens et al., 2005). The major type I receptor of this signaling is activin receptor-like kinase 5 (ALK5) or TβRI (Larsson et al., 2001). In bone

cells, ALK5 was mentioned to be the sole type I receptor for TGF-β signaling (Janssens et al., 2005). Therefore, as ALK5 is important in bone cells, SB-431542 was used to inhibit the mentioned receptor to suppress the phosphorylation of Smad2 (Inman et al., 2002).

As expected, SB-431542 suppressed the osteogenic differentiation of hPDLSCs on ALP activity, osteogenic-related gene expression, and mineralization. However, the finding was not remarkably difference. Failure in decreasing the Jagged1-osteogenic induced in human dental pulp stem cells (hPDLSCs) was previously found by the supplementation of SB-431542. This phenomenon may be the two mechanisms of TGF-β signaling. The inhibition of osteogenic differentiation is occurred because of TGF-β has positive feedback in osteoblast proliferation (Matsunobu et al., 2009). In contrast, the phases of matrix maturation and mineralization can be inhibited by the activation of TGF-β signaling with the regulation of *COL1A1*, *OCN*, *OPN*, *ALP*, and ALP activity (Breen et al., 1994; Harris et al., 1994). Thus, blocking that activity with SB-431542 in later phase, as this study performed, resulted in not remarkably difference on osteogenic differentiation. However, it is suggested SB-431542 mineralizes later than OGP. According to the changes by SB-431542 treatment, it implies that OGP partially regulate the osteogenic differentiation of hPDLSCs via TGF-β signaling pathway.

# CHAPTER V

# CONCLUSION

OGP has mitogenic activity and is not cytotoxic on hPDLSCs. The increased osteogenic differentiation of hPDLSCs were orchestrated by OGP through the activation of BMP, Hedgehog, TGF-β, and Wnt signaling pathways. With SB-431542 treatment, this study found that OGP is partially involving TGF-β signaling pathway during osteogenesis of hPDLSCs. In final, OGP is suggested to be a prospective candidate agent that can be used to treat osteoporosis and/or periodontitis.

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# VITA

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|---|---|
| **NAME** | Steven Dwi Purbantoro |
| **DATE OF BIRTH** | 6 February 1997 |
| **PLACE OF BIRTH** | Jakarta |
| **INSTITUTIONS ATTENDED** | Udayana University, Bali, Indonesia |
| **HOME ADDRESS** | Jl. Prima Dalam Blok R5 No.1 RT 04/005, Tegal Alur, Kalideres, Jakarta Barat, DKI Jakarta, Indonesia |
| **PUBLICATION** | 1. Steven Dwi Purbantoro, Erika, Kadek Yudha Ariawan, Endah Rahmawati, Zefanya Christiani, Sri Kayati Widyastuti. 2021. Schirmer Tear Test 1 as a method to measure tear production value in normal Kintamani dogs. The Thai Journal of Veterinary Medicine 51(3): 597-599.<br><br>2. Medania Purwaningrum, Nabila Syarifah Jamilah, Steven Dwi Purbantoro, Chenphop Sawangmake, Sirirat Nantavisai. 2021. Comparative characteristic study from bone marrow-derived mesenchymal stem cells. J Vet Sci 22(6):e74. |
| **AWARD RECEIVED** | 1. Scholarship awardee of Lembaga Karya Pokphand from PT. Charoend Pokphand 2017/2018 Period<br>2. 1st Runner Up for Quiz Competition Veterinary Scientific's Week 2017 held by Indonesia's Veterinary Student Association as a team (National)<br>3. 2nd Runner Up for Essay Competition Veterinary Scientific's Week 2017 held by Indonesia's Veterinary Student Association as a team (National) |