Utilization of modified mRNA encoding bone morphogenetic protein-2 for periodontal regeneration: an *in vitro* study

Mr. Promphakkon Kulthanaamondhita

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การใช้ประโยชน์ของเอ็มอาร์เอ็นเอดัดแปลงที่เข้ารหัสโบนมอร์โฟเจนเนติกโปรตีน-2 ในการฟื้นฟูเนื้อเยื่อปริทันต์: การศึกษาในห้องปฏิบัติการ

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By	Mr. Promphakkon Kulthanaamondhita
Field of Study	Periodontics
Thesis Advisor	Professor RANGSINI MAHANONDA, Ph.D.
Thesis Co Advisor	Sathit Pichyangkul, Ph.D.

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

.....
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Thesis Advisor

(Professor RANGSINI MAHANONDA, Ph.D.)

.....
Thesis Co-Advisor

(Sathit Pichyangkul, Ph.D.)

.....
External Examiner

(Professor Stitaya Sirisinha, Ph.D.)

พรหมภัคกร กุลธนอมรดิษฐ์ : การใช้ประโยชน์ของเอ็มอาร์เอ็นเอดัดแปลงที่เข้ารหัสโบนมอร์โฟเจนเนติกโปรตีน-2 ในการฟื้นฟูเนื้อเยื่อปริทันต์: การศึกษาในห้องปฏิบัติการ. (Utilization of modified mRNA encoding bone morphogenetic protein-2 for periodontal regeneration: an *in vitro* study) อ.ที่ปรึกษาหลัก : ศ.ทญ. ดร.รังสินี มหานนท์, อ.ที่ปรึกษาร่วม : ดร.สาธิต พิชญางกูร

แม้ว่าปัจจุบันเทคนิคต่างๆที่นำมาใช้ในการฟื้นฟูเนื้อเยื่อปริทันต์ให้ผลการรักษาที่ดี แต่เทคนิคเหล่านี้ไม่สามารถฟื้นฟูเนื้อเยื่อให้กลับคืนได้อย่างสมบูรณ์ เมื่อไม่นานมานี้ได้มีการดัดแปลงเอ็มอาร์เอ็นเอเพื่อนำมาใช้เป็นนวัตกรรมใหม่ในการฟื้นฟูเนื้อเยื่อ การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาความสามารถของเซลล์เอ็นยึดปริทันต์ในการผลิตโบนมอร์โฟเจนเนติกโปรตีน-2 (บีเอมพี -2) ภายหลังการนำส่งด้วยเอ็มอาร์เอ็นเอที่ดัดแปลงเบสเป็นเอ็น-1 เมทิลซูโดยูริดีน (N-1 methylpseudouridine) และเข้ารหัสบีเอ็มพี-2 รวมถึงทดสอบประสิทธิภาพของบีเอมพี -2 ที่ถูกผลิตขึ้น

เซลล์เอ็นยึดปริทันต์จากเนื้อเยื่อปริทันต์ของผู้ป่วยที่มีสภาวะปริทันต์ปกติจะถูกนำส่งด้วยเอ็มอาร์เอ็นดัดแปลง จากนั้นจึงทำการเก็บเซลล์และส่วนใสที่ระยะเวลา 24 48 และ 72 ชั่วโมง เพื่อนำไปใช้วัดปริมาณของบีเอมพี -2 ด้วยอีไลซา และทำการทดสอบการมีชีวิตของเซลล์ตามช่วงเวลา ส่วนใสที่เก็บที่ 24 ชม. จะถูกนำมากระตุ้นเซลล์เอ็นยึดปริทันต์และทำการวัดผลการเจริญเติบโตด้วยอะลามาร์บลู นอกจากนี้ส่วนใสที่ระยะเวลา 72 ชม. จะถูกนำมากระต้นเซลล์บุผิวหลอดเลือดเพื่อดูความสามารถในการสร้างหลอดเลือดใหม่

ผลการศึกษาพบว่าการสร้างบีเอ็มพี-2 ภายในเซลล์สูงที่ 24 ชั่วโมง และพบบีเอ็มพี-2 ภายนอกเซลล์เพิ่มขึ้นจนถึง 72 ชั่วโมง เซลล์ยังคงมีชีวิตอยู่มากกว่าร้อยละ 90 ตลอดการทดสอบ นอกจากนี้ส่วนใสก็ส่งเสริมการเจริญเจริญเติบโตของเซลล์เอ็นยึดปริทันต์ได้ สรุปได้ว่าการนำส่งเซลล์เอ็นยึดปริทันต์ด้วยเอ็มอาร์เอ็นดัดแปลงเข้ารหัสบีเอ็มพี-2 กระตุ้นการผลิตบีเอ็มพี-2 ในปริมาณที่สูง ส่งเสริมการเจริญเติบโตของเซลล์เอ็นยึดปริทันต์ รวมถึงสามารถส่งเสริมให้เซลล์บุผิวหลอดเลือดเกิดการสร้างเส้นเลือดใหม่ ซึ่งอาจนำไปใช้ฟื้นฟูเนื้อเยื่อปริทันต์ที่ถูกทำลายจากโรคปริทันต์ได้

สาขาวิชา ปริทันตศาสตร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2562 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORD: periodontal regeneration, mRNA, periodontitis, bone morphogenetic protein-2

Promphakkon Kulthanaamondhita : Utilization of modified mRNA encoding bone morphogenetic protein-2 for periodontal regeneration: an *in vitro* study. Advisor: Prof. RANGSINI MAHANONDA, Ph.D. Co-advisor: Sathit Pichyangkul, Ph.D.

Current modalities for periodontal regeneration provide modest success, however complete periodontal regeneration is still not achievable. Recently *in vitro* synthesized nucleoside-modified messenger RNA (mRNA) has emerged as a novel platform in regenerative medicine. The aims of this study are to investigate the ability of human periodontal ligament cells (PDLCs), clinically relevant target cells, to produce bone morphogenetic protein-2 (BMP-2), a significant protein for bone formation after transfected with modified mRNA that encode this protein. We investigated the ability of translated protein for enhancing PDLC proliferation and promotes endothelial cell tube formation, marker of angiogenesis. Isolated PDLCs from healthy periodontal tissue were transfected with N1-methylpseudouridine modified mRNA encoding BMP-2 complexed with transfecting agent, Lipofectamine 2000. Cell lysates and supernatants were collected at 24, 48 and 72 hours (h) after transfection for protein production by ELISA and cell viability by AlamarBlue assay.

High levels of BMP-2 production were detected intracellulary and extracellulary. Secreted BMP-2 gradually increased up to 72 h. Cell viability was maintained above 90% throughout the observation period. The post-transfection supernatants were able to promote PDLC proliferation and endothelial cell tube formation. In conclusion, transfection of PDLCs with N1-methylpseudouridine modified mRNA encoding BMP-2 in lipofectamine 2000 led to high levels of functional BMP-2 protein. Using the *in vitro* synthesized nucleoside-modified

Field of Study:	Periodontics	Student's Signature .....
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		Co-advisor's Signature .....

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Promphakkon Kulthanaamondhita

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CHAPTER I: INTRODUCTION

Background and rationale

The Global Burden of Disease Study (2010) ranked severe periodontitis as the sixth-most prevalent disease in the world, affecting 11.2% (743 million people) worldwide. In Thailand, severe periodontitis has been recognized as the major oral health problem among adults (age 35-59) and particularly in elderly people (age 60 and over). The disease cause destruction of tooth supporting gingiva and bone and eventually lost in severe cases which can affect chewing.

So far, there has been no therapeutic approach that effectively regenerates periodontal tissue. Current treatments including guided tissue regeneration and bone grafts result in limited periodontal regeneration. New therapeutic approaches such as the use of growth factors, stem cells and gene therapy have produced mixed clinical outcomes and costly.

Recently, the development of nucleoside modified mRNA encoding growth factors as a next generation therapeutic platform for periodontitis are emerging. By stimulating periodontal fibroblast proliferation, promoting ligamentogenesis, angiogenesis and osteoblastic differentiation and bone formation, this therapeutic modality lead to regeneration of loss periodontal tissues caused by periodontal diseases. Expression of proteins by mRNA could be detected within hours following delivery and can last from several days and up to a week. The strength of modified

mRNA as a next generation therapeutic platform is in its safety, transiently, and high expression activity. In addition, unlike growth factor, stem cell and DNA-based therapy, modified mRNA is cost effective.

Objectives

To investigate the ability of human periodontal ligament cells (PDLCs) to produce or secrete bone morphogenetic protein-2 (BMP-2) after transfected with mRNA encoding BMP-2 and test the function of produced BMP-2 as measured by cell proliferation ability and the ability to induce new blood vessels using endothelial cell tube formation assay *in vitro.*

Hypothesis

1. Human PDLCs can produce and secrete BMP-2 after transfection with mRNA encoding BMP-2.

2. Secreted BMP-2 can induce PDLCs proliferation and can induce tube formation by endothelial cell.

Field of research

In vitro study of human periodontal ligament cells transfected with mRNA encoding BMP-2 using biotechnologies.

Inclusion criteria

Human periodontal ligaments around the extracted wisdom tooth from healthy periodontal patients will be obtained.

Limitation of research

Since this *in vitro* study requires samples from human, the sample size will be limited.

Application and expectation of research

This study will provide information regarding the ability of mRNA technologies which can be used to formulate an mRNA therapeutics platform. This platform could further be used in in-vivo study or eventually clinical trial in order to develop a new novel therapeutic agent for periodontal regeneration.

Keywords

Periodontal regeneration, mRNA, periodontitis, bone morphogenetic protein-2

CHAPTER II: REVIEW LITERATURE

Periodontal disease

Periodontitis and its impact on global health

Severe periodontitis is one of the major dental associated problem in adults and elderly. The disease was ranked 6th in the most common chronic conditions in the world. Estimated world population of 243 million had severe periodontitis (Kassebaum et al., 2014). The report of the 8th Thai National Dental Health Survey in 2017 showed that 78.8% of Thai adults age ranges between 35-85 had severe periodontitis. Even though severe periodontitis is not a life-threatening disease, its high prevalence and how it affects the quality of life make this disease considered to be a major public health problem.

Pathogenesis of periodontal disease

Periodontal disease is a chronic inflammatory disease affecting the periodontium which comprises of the gingiva, periodontal ligament, cementum and alveolar bone. The loss of these supporting tissues may have negative effects mastication and speech, leading to poor quality of life.

Healthy gingiva has pale pink color and firm consistency. The gingival sulcus is shallow within the range of 0.5-3 mm. The gingival margin is at or coronal to the cementoenamel junction, with no bleeding upon probing or during tooth brushing.

Periodontal disease can be divided into 2 categories depending on the severity of the disease:

- Gingivitis: The inflammation is confined to the gingiva without alveolar bone involvement. The gingiva appears erythema, edematous and easily bled during brushing or chewing.
- Periodontitis: The inflammation spread wide and deep causing destruction of periodontium. The gingival sulcus become deepened creating the periodontal pocket. The tooth is mobile and when the disease progresses, tooth loss may occur.

Although the main cause of the periodontal disease are the pathogenic bacteria in the dental plaque, the mechanism and the severity of the disease are mainly associated with the chronic immune responses to pathogenic bacteria especially gram-negative species. This process involves the immunopathogenesis of the innate and adaptive immune responses. In the diseased tissues, a large amount of cellular infiltrations of polymorphonuclear neutrophils, B lymphocytes and T lymphocytes are detected. These cells produce the high-level of inflammatory mediators and cytokines such as interleukin-1, tumor necrosis factor-α, interleukin-17, matrix metalloproteinase and receptor activator of nuclear factor kappa-beta ligand. These molecules lead to the chronic inflammation, destruction of the surface lining epithelium and connective tissue

of the periodontium consequently the destruction of the alveolar supporting bone (Figure 1). (Kornman, 2008; Mahanonda & Pichyangkul, 2007; Seymour, 1991)

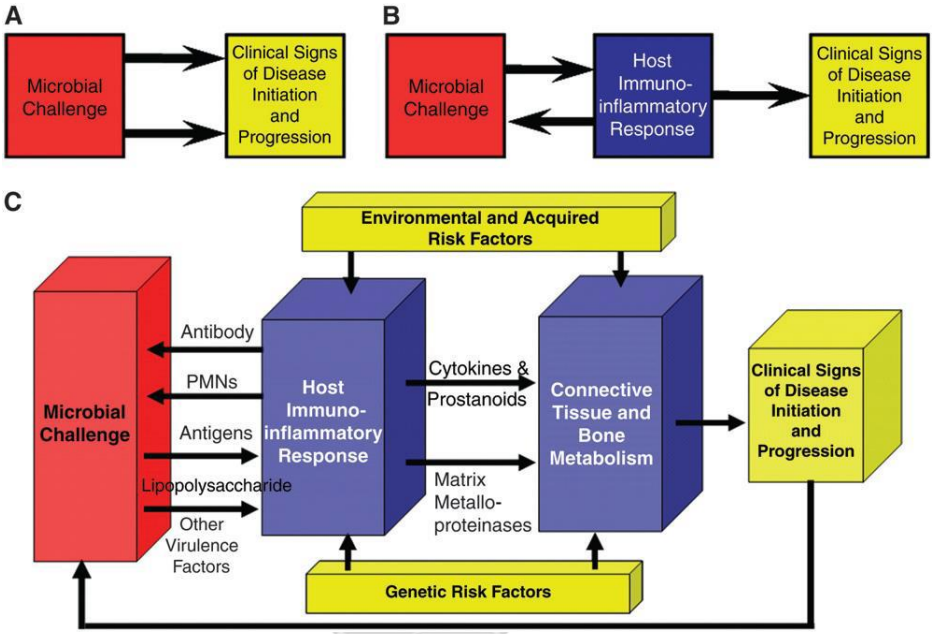

Figure 1. The pathogenesis of periodontal disease. Upon challenging with microbial plaque, various host immune-inflammatory responses occur. If the balance is maintained, the disease does not occur and remains in a homeostasis. The imbalance of host immune response to subgingival plaque biofilm leads to tooth-supporting tissue destruction and subsequently tooth loss (Kornman, 2008).

Treatment of periodontal disease

The primary treatment of periodontal disease comprises of scaling and root planing to remove bacterial plaque which is the major cause of the disease. In case of severe periodontitis, apart from scaling and root planing, periodontal surgery is required

in order to remove the bacterial plaque and calculus which located deeply within the periodontal pocket. However, these treatments can only detain the progression of the disease without the ability to regenerates the destroyed periodontium. Moreover, within the state of this reduced periodontium, the periodontal tissues are prone for disease progression. The teeth are possibly mobile and disrupting normal occlusal function, speech and personality that leads to alteration of quality of life.

Periodontal regeneration

Current treatments for periodontal regeneration

The ultimate goal of periodontal treatment is to regenerate periodontium that were destroyed by the disease. Periodontal regeneration, if achieved, will help the periodontium to function properly, improving the prognosis of the tooth and detaining the tooth to function.

Current treatment modalities for periodontal regeneration that are widely accepted comprises of guided tissue regeneration and the use of bone grafts (Figure 2). Guided tissue regeneration is based on the use of barrier membrane as an epithelium exclusion from the periodontal defects and help maintaining the space for periodontium regeneration (Nyman et al., 1982). The successful outcome of guided tissue regeneration is based on many factors and the ability and technique uses by the practitioner are one of them. Furthermore, the healing time of this technique and also

with the bone grafts are quite long as it takes several months to year in order for the periodontium to regenerates.

In conclusion, treatment outcome by guided tissue regeneration and bone grafts are still lack in efficacy and effectiveness. There are still limitations that can be used only in specific types of periodontal defects. Until now, the complete regeneration is not achievable and not highly predicted, and the cost of treatment are high (Avila-Ortiz et al., 2015; Kao et al., 2015).

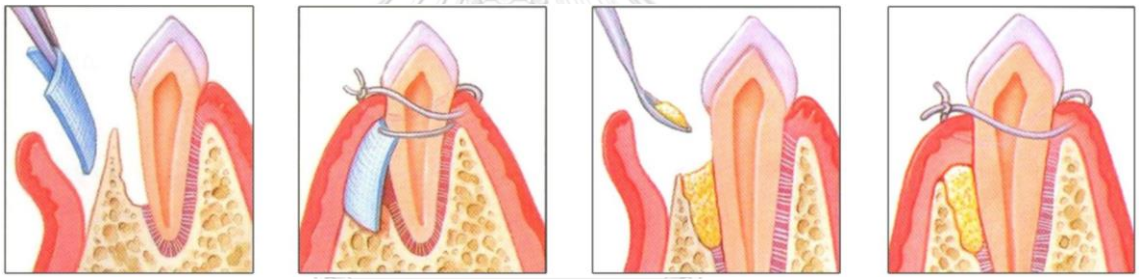

Figure 2. Schematic illustration of guided tissue generation and bone grafting. In guided tissue regeneration, after the inflammation are under control, collagen or synthetic membrane is used to control cells population during the healing process. While in bone grafting, various types of bone replacement grafts such as autogenous bone graft are used to facilitate the healing process (Newman et al., 2011).

Emerging approaches for periodontal regeneration

During the past decades, many attempts were made to find new approaches for periodontal regeneration, and these includes the use of stem cells and cell sheet, growth factor proteins and gene therapy.

Cell-based periodontal regeneration

Advancement in medical tissue engineering have allowed the use of mesenchymal stem cells (MSCs) in periodontal regeneration (Bartold et al., 2000). One of the major sources of MSCs for treating periodontal defects is periodontal ligament.

So far, most study combined the stem cells with a scaffold for periodontal regeneration, for example, collagen, fibrin, hydrogel, gelatin. An alternative, non-scaffold tissue engineering has been of increasing interest in tissue regeneration. Cell sheet engineering has been developed as a novel, scaffold-free method and has shown positive results. Transplantation of periodontal ligament cell (PDLCs) sheets in an athymic rat mesial dehiscence model led to identifiable periodontal ligament-like tissues that included an acellular cementum-like layer and fibrils anchoring into this layer (Akizuki et al., 2005).

Despite the significant advances of stem cell technology in periodontal regeneration, several challenges have to be overcome including: 1) the optimal source of MSCs for periodontal regeneration, 2) The low number of isolated mesenchymal stem

cells especially from elderly people, 3) *in vitro* growing large number of cells and risk of accumulating genetic and epigenetic abnormalities leading to cell transformation.

Protein growth factors

Growth factors have been receiving a lot of attention as a tool to stimulate multi-potent cells within periodontium to proliferate and differentiate into desired tissue. These groups of proteins include platelet-derived growth factors (PDGFs), transforming growth factor-B (TGF-b), insulin growth factor (IGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs) (Kao et al., 2009).

PDGFs are well known for their ability to promote tissue regeneration and wound healing (Dereka et al., 2006). Three different subtypes of PDGFs have been identified including, PDGF-AA, PDGF-BB and PDGF-AB. Among these, PDGF-BB is the only growth factor that has been approved by the Food and Drug administration (FDA) for use in periodontal therapy (intrabony defects, furcation lesions and gingival recession) (Suarez-Lopez Del Amo et al., 2015).

FGFs are a group of proteins that have been studied in tissue regeneration. They consist of 23 subtypes (FGF-1 to FGF-23) and among these, FGF-2 is the most well study in periodontal regeneration. Recent data suggest that FGF-2 has potent angiogenic, osteogenic activities and stimulates MSC proliferation and differentiation (Kao et al., 2009; Murakami, 2017). Despite its attractive properties for tissue

regeneration, several studies of FGF-2 showed inconclusive clinical efficacy in periodontal regeneration (Li et al., 2017). In addition, the use of FGF-2 in tissue regeneration may need several administrations to maintain therapeutic levels, therefore treatment could be costly.

Bone morphogenetic protein-2 (BMP-2)

The use of demineralized bone matrix to heal bone defects was reported at the end of the nineteenth century, but the osteogenic proteins involved in this process was introduced in the 1960s, when an osteoinductive role for bone morphogenetic proteins (BMPs) was postulated. However, isolation of a first BMP from bovine bone extract, occurred in the 1980s. Subsequently, the cloning of BMP-2 and 4 were made (Wozney, 1998), followed by the publication of human BMP-2 and OP-1 crystal structure in the 1990s.

BMPs are multifunctional cytokines belonging to the TGF-β superfamily which comprised of approximately 50 genes (Chen et al., 2012). The TGF-β superfamily proteins are classified according to protein sequence similarity in humans and other species. BMPs can be divided into 4 distinct subfamilies (Figure 3), according to their sequences and functions: BMP- 2 and 4; BMPs 5, 6, 7, 8a, and 8b; BMPs 9, 10; and BMPs 12, 13, 14. BMP-2, BMP-4, and BMP-7 are expressed in dental epithelium and they display osteogenic potential. Nevertheless, some BMP show distinct actions

compared to others, such as cartilage formation, trabecular formation in the embryo heart, and oocyte development and follicular formation (Miyazono et al., 2010).

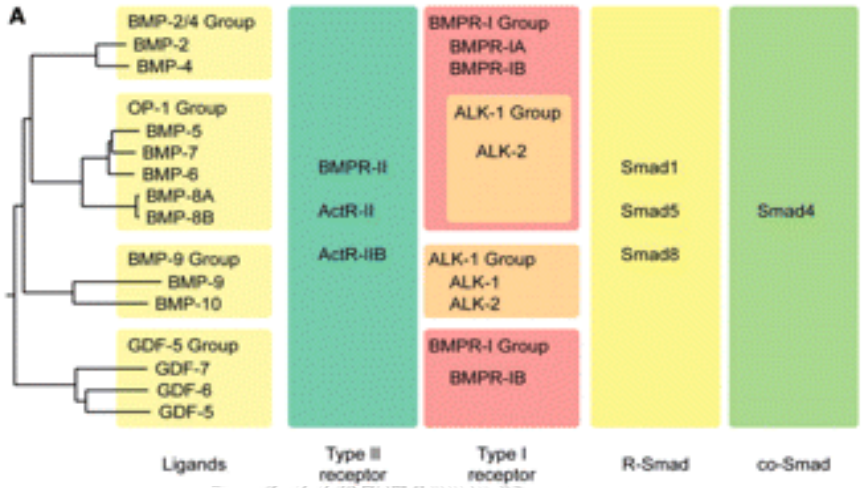

Figure 3. Bone morphogenetic proteins are divided into 4 subfamilies: BMP-2/4 group, OP-1 group, BMP-9 group and GDF-5 group. Signal transduction of BMP involves 2 main types of surface receptors for BMP; type I and type II and intracellular Smad proteins; Smad1/5/8 (R-Smad) and Smad4 (Co-Smad). (Miyazono et al., 2010)

Osteoprogenitor cells, osteoblasts, chondrocytes, platelets, and endothelial cells produce BMP in the bone tissues. Upon secretion, the extracellular matrix temporarily stores BMP. The proteins then released during bone repair and remodeling. The role of BMP in mesoderm formation, heart development, cartilage development, and post-natal bone formation and metabolism are further understood through the advanced in the gene knockout technology (Chen et al., 2004).

The action of BMPs is time-dependent, triggering a sequential cascade of events, which leads to chondrogenesis, osteogenesis, angiogenesis, and controlled synthesis of extracellular matrix by modulation of several genes (Bustos-Valenzuela et al., 2011). Depending on the arrangement of cell-surface receptors, different signaling pathways can be activated.

BMPs signaling pathways comprises mainly of 2 pathways: Smad dependent and Non-Smad pathway (Figure 4) (Gomez-Puerto et al., 2019). BMPs ligands can bind either cooperatively to pre-formed receptor complexes or first to a BMP type I receptor (BMPRI) that later recruits BMP type II receptor (BMPRII). BMP can bind to activin type II and type IIB receptors (Mueller & Nickel, 2012). In this pre-formed complex-type signaling, active BMPRII receptors are already linked to BMPRI upon BMP binding. BMPRII then catalyzes phosphorylation of BMPRI, which, in turn, catalyzes phosphorylation of Smads 1, 5, and 8, that are associated with co-Smad-4, forming a protein complex. This protein complex is directed to the nucleus and activate Runx2, Dlx5, and Osterix (Osx) genes (Chen et al., 2012).

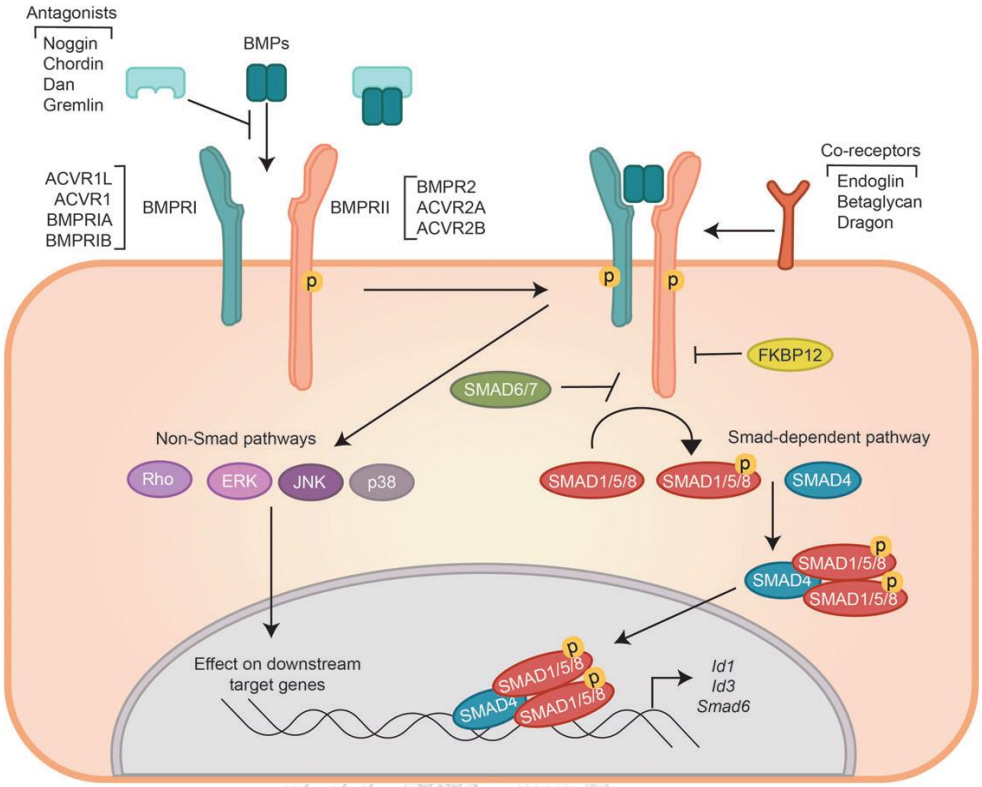

Figure 4. BMPs signaling pathways. BMPs signal via complexes of type I (BMPRIs) and type II (BMPRIIs) transmembrane kinase receptors. The type II receptor, a constitutively active kinase phosphorylates and activates the type I receptor, upon which intracellular canonical signaling is initiated by phosphorylation of SMAD1, 5, and 8 (R-SMAD). Activated R-SMADs then coupled with SMAD4 to regulate expression of specific target genes. BMP receptor activation can also induce non-SMAD signaling pathways , by activating p38 and JNK MAP kinases and small GTPases such as Rho and Rac and subsequently induces target genes expression in the nucleus (Gomez-Puerto et al., 2019).

The roles of BMPs have been extensively studied in the areas of embryonic development and their effects on cellular functions such as growth, differentiation and apoptosis. Recent studies have revealed that BMPs signals the proliferation and differentiation of chondrocytes, differentiation of mesenchymal stem cells into osteoblasts and control the bone quality (Carreira et al., 2015; Chen et al., 2004). In addition, BMP-2 also promotes formation of new blood vessels or angiogenesis through the production of vascular endothelial growth factor A (Deckers et al., 2002).

The therapeutic abilities of BMP-2 have been evaluated in various clinical settings such as calvarial, mandibular, and cleft palate reconstruction; alveolar augmentation; dental implant fixation; and for endodontic and periodontal conditions (Lindholm TC, 1996; Wikesjo et al., 1999). Early study reported the effect of applying recombinant BMP-2 in a polylactic acid-polyglycolic acid copolymer carrier into dog intrabony periodontal defects promoted significantly greater regeneration of alveolar bone and cementum.

Nevertheless, following the application of BMP-2 into the periodontal defects, ankylosis was encountered and compromised the healing process. *In vivo* study in beagle dogs with created periodontal alveolar defects showed that application of recombinant BMP-2 rapidly stimulate new bone formation within 4 weeks. Areas exhibiting characteristics of periodontal ligament with a fine layer of acellular cementum

and inserting Sharpey's fiber were found but ankylosis was more frequent (Selvig et al., 2002). Therefore, the application of BMP-2 for periodontal tissue regeneration may need to be combined with other growth factors.

The osteoconductive capacity of BMP-2 has also been demonstrated in clinical trials. From a randomized double-blind control trial have showed that the application of recombinant human BMP-2 following periodontal flap surgery in vertical defects significantly enhanced probing pocket depth reduction, clinical attachment gain, and radiographic bone fill at 9 months postoperatively (Vandana et al., 2016).

Gene therapy

The use of DNA in gene therapy enhances the bioavailability of growth factor proteins within damaged tissue leading to better regeneration ability. Several studies have been using DNA for promoting periodontal tissue regeneration. Gene therapy are carried out using plasmid DNA or viral vector such as adenovirus. The use of adenovirus encoding PDGF-AA can transduced the gingival fibroblast, enhancing the ability of proliferation and migration (Chen & Giannobile, 2002). Furthermore, plasmid DNA encoding BMP-4 in conjunction with scaffold delivery system have shown the ability to induce new bone formation. However, the major drawbacks of DNA in gene therapy are the safety in terms of mutation and tumorigenesis. In addition, the amount of growth factor proteins produced are still rather low.

Messenger RNA (mRNA) technology

Background of mRNA technology

mRNA technology is a new and highly innovated method that is safe and provide cost-effective benefits. Recently, the nucleoside-modified mRNA has been emerged as a novel alternative in the non-viral gene therapy. During 1990s, researchers used mRNA as the gene therapy by intramuscular injecting mRNA encoding reporter gene in mice, the expression of proteins were observed (Wolff et al., 1990). However, during that time, the mRNA technology was still overlooked due to the fragility and instability. Until recently, these limitations have been overcome by

1. The use of mRNA in combination with cationic lipid or polymers resulting in mRNA complex that preventing the degradation of mRNA by RNase enzyme and also promote cell uptake.

2. The translation efficiency of mRNA has been increased by the nucleoside modification and the incorporation of 5'cap, 5', 3' UTR and increasing the length of poly A tail. The nucleoside modification also reduces the ability of mRNA to induce inflammation.

3. The ability to synthesize a larger amount of mRNA and becoming a good manufacturing practice *in vitro* making it possible for the clinical application.

One of the major drawbacks of mRNA in tissue regeneration is its ability to elicit innate immune response leading to an undesirable inflammatory reaction. *In vitro*,

resident cells can recognize mRNA through various receptors such as Toll-like receptors (TLR), RIG-I receptors, MDA 5, NOD2 and IFIT-T (Goubau et al., 2013). These receptors recognized mRNA as a foreign antigen or virus causing the interaction between the receptors and mRNA. This interaction will activate the NF-**K**B and interferon and resulting in inhibition of proteins production (Pollard et al., 2013).

Modifications of mRNA

Several methods have been used to overcome this problem for example, modification of mRNA in the base region provides the ability to evade TLR recognition that leads to inhibition of type 1 interferon production. The modification of the base region to pseudouridine or N-1 methylpseudouridine are also effective in inhibiting innate immune response and enhances the production of proteins (Andries et al., 2015; Kariko et al., 2008). Furthermore, the decontamination of double stranded RNA, which was generated during *in vitro* synthesis, with liquid chromatography inhibit the innate immune response and enhancing the proteins production (Foster et al., 2018; Weissman et al., 2013).

Methods of mRNA delivery

Method of mRNA delivery into target cells are greatly importance for effective production of desired proteins *in vitro* or *in vivo*. As mentioned before, mRNA can be degraded by nuclease found in most of the tissues, the need for effective delivery system is crucial for preventing degradation and enhancing the proteins production. The

encapsulation of mRNA increases the stability of cellular uptake and endosomal escape after entering the target cell. Several materials can be used to encapsulate mRNA such as lipids (Mintzer & Simanek, 2009), polymers (Pack et al., 2005) and peptides (Martin & Rice, 2007).

The most widely studied method of mRNA delivery into cells indicating good outcome is cationic lipid nanoparticles. In general, cationic lipid which is positively charged will engage with the negatively charged mRNA forming lipid nanoparticles (Figure 5). These nanoparticles have been shown to successfully delivered mRNA into target cells *in vivo*. For example, lipid nanoparticles were used to deliver mRNA encoding for erythropoietin and factor IX into the cells and promoted the effective production of proteins in rats and monkeys (DeRosa et al., 2016; Kariko et al., 2012).

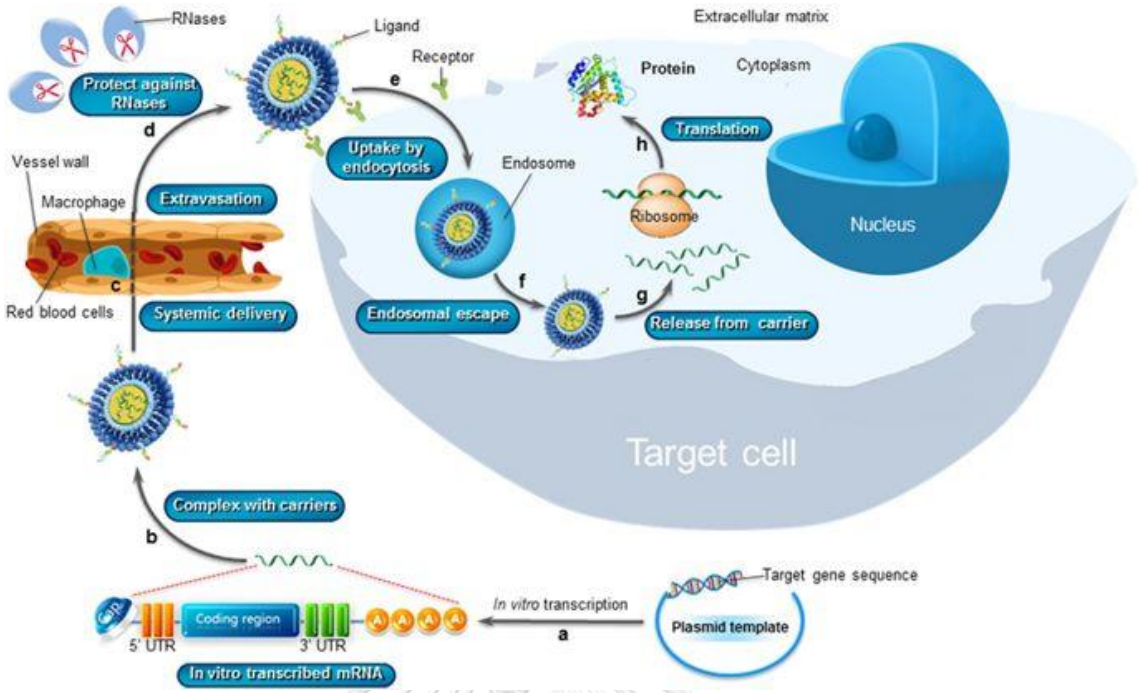

Figure 5. Schematic illustration of the production and delivery of mRNA using nonviral approach. Chemically modified mRNA is complex with a cationic lipids carrier and delivered to the patient using various routes. Upon arriving at the target cell, mRNA complex is uptake into the cell via endocytosis and then undergone endosomal escape to the cytoplasm where translation of desired proteins take place. (Guan & Rosenecker, 2017)

Medical tissue engineering using mRNA technology

In the recent year, the development of modified-mRNA has been used in medical tissue engineering as a novel alternative to stem cells, growth factor proteins and gene therapy with DNA or virus as a vector. Interesting results has been provided and showing the efficacy and effectiveness of modified-mRNA encoding VEGF-A in

promoting heart tissue regeneration that were destroyed from myocardial infraction (Zangi et al., 2013). *In vivo* results in mice after receiving modified-mRNA encoding VEGF-A in lipid indicated the increase in capillary density, reduction of the infraction size and increase the survival time when compared to plasmid DNA vector or controls. The result achieved might be associated with the ability of VEGF-A that can recruit epicardial progenitor cells into the diseased area and promoting its differentiation into cardiovascular cell type. Recent publication from the same group has reported the results of the study conducted in pigs. They injected the modified mRNA encoding VEGF-A formulated with biocompatible citrate buffer into the heart of the pigs that were experiencing failure and after 1 week, they found improvement in cardiac function (Carlsson et al., 2018).

With the advances in technologies, researches are driven into human clinical trials in a short period of time. The randomized, double-blind, placebo-controlled, phase 1 study of chemically modified mRNA encoding VEGF-A was first conducted with the aims to evaluate safety and potential therapeutic effects. Men with type 2 diabetes mellitus received intradermal injection of this mRNA on the forearm showed elevated VEGF-A protein at the treated sites and enhancement in basal skin blood flow as compared to the control sites (Gan et al., 2019). These investigators currently carried out Phase 2 clinical trial using VEGF mRNA in cardiovascular patients. In addition, more supporting safety data of chemically modified mRNA were from phase 1 clinical trial of

mRNA vaccines against H10N8 and H7N9 influenza viruses. The patients who received mRNA vaccine were well tolerated and elicited robust humoral immune responses (Feldman et al., 2019). Therefore, these recent data favor the safe and efficient uses of modified mRNA in human and may have therapeutic potential for periodontal tissue regeneration.

This may be concluded that mRNA has several advantages over stem cells and DNA/viral vector in tissue regeneration as follows:

1. mRNA technology is safe due to the property of temporary carriage of genetic information that can be degraded naturally without the risk of DNA integration.

2. mRNA enters multiple types of cells regardless replicating or non-replicating cells. After entering the cell, mRNA is rapidly translated into protein for sustained period of time.

3. When compared to a viral vector, the use of mRNA is not restricted by the preexisting anti-vector immunity.

4. mRNA can be synthesized in a large amount with a low cost and can be stored in room temperature of a long period of time.

CHAPTER III: MATERIALS AND METHODS

Production of mRNA encoding BMP-2

Nucleotide sequences of human BMP-2 were designed by Prof. Rangsini Mahanonda and her team. In collaboration, the synthesis of N1-methylpseudouridine modified mRNA encoding BMP-2 (m1Ψ-BMP-2 mRNA) was kindly provided by Dr. Norbert Pardi from University of Pennsylvania (Pardi, Hogan, et al., 2018; Pardi, Parkhouse, et al., 2018; Pardi et al., 2017).

Medium and reagents

Minimum Essential Medium with Alpha modification (Alpha MEM) supplemented with 10% heat-inactivated fetal calf serum, 2 mM GlutaMax-I, 100 U/ml penicillin, 100 µg/ml streptomycin and 5 µg/ml amphotericin B (Life Technologies) were used throughout the study. Opti-MEM I, Lipofectamine 2000 was purchased from Invitrogen. Human recombinant BMP-2 was purchased form R&D Systems. Primary human umbilical vein endothelial cells, endothelial cell growth basal medium and Matrigel were purchased from Lonza Bioscience.

Cells isolation and culture

This study was approved by Ethic Committee (No.042/2019) and Institutional Biosafety Committee (No.011/2019) of Faculty of Dentistry, Chulalongkorn University. Human periodontal ligament cells (PDLCs) were obtained from healthy periodontal patients (age 15-35 years) undergoing extraction of third molars for orthodontic or

therapeutic reasons at Chulalongkorn Dental School. PDLCs were obtained from the tooth by enzyme-digestion method. Briefly, the teeth were extensively washed twice with Dulbecco's phosphate-buffered saline (DPBS) and the PDL tissues were scraped out from the middle third of the root under sterile condition. Care was exercised to avoid contamination from gingival or pericapical granulation tissues. Then, PDL tissues were minced into fragment of 1-2 mm^2 and digested with a solution of 2 mg/ml collagenase and 2 mg/ml dispase for 60 minutes at 37^{o}C and then filter through a 70 μm cell strainer. The pass-through was then washed twice with culture medium. The PDLCs were cultured with culture medium (Alpha MEM) at 37^{o}C in humidified atmosphere of 5% CO_2 in air. Culture medium was changed twice a week. After 80% confluent monolayer of cells were reached, PDLCs were trypsinized, washed and then sub-cultured to new tissue culture flasks. The cells from 3rd to 8th passages from 3 different donors were used in this study (Iwata et al., 2010).

***In vitro* cell transfection and production/secretion of BMP-2 protein**

Human periodontal ligament cells (PDLCs) were plated in 12 wells plate (100,000 cells per well). These cells were then transfected with 2 μg of m1Ψ-BMP-2 mRNA complexed with 1.5 μl of Lipofectamine 2000. After 4 h of transfection, the medium in each well were removed and new medium were added.

To analyze the BMP-2 protein expression and secretion levels, the transfected cells and control cells (Lipofectamine 2000 only) were cultured for 24-72 hrs.

Supernatants and cells were harvested at 24, 48 and 72 h time-point. and were used for analysis. Harvested cells were lysed using RIPA buffer solution (Pierce® RIPA buffer, ThermoFisher Scientific) and the lysate was collected for further analysis. Monoclonal antibodies specific to BMP-2 were used to determine the protein production and secretion by using ELISA (Quantikine®, R&D Systems).

Cell toxicity analysis

To analyze cell toxicity, PDLCs transfected with either BMP-2 mRNA complexed with Lipofectamine 2000, Lipofectamine 2000 alone or control medium were cultured with 10% Alamar Blue solution (alamarBlue®, BIO-RAD) then incubated at 37^{o}C in humidified atmosphere of 5% CO_2. After 1-4 hours, cell culture supernatants were measured at absorbance of 570 nm using microplate reader.

Cell proliferation ability

For the ability of secreted BMP-2 to enhance PDLC proliferation, PDLCs were plated in 96 wells plate (3000 cells per well) and either control medium, supernatant of PDLCs that has been transfected with BMP-2 mRNA complexed with Lipofectamine 2000 was added. After 48 hours of incubation, 10% Alamar Blue solution (alamarBlue®, BIO-RAD) was added. After 2-4 hours, cell proliferation ability was measured by a microplate reader at absorbance of 570 nm.

Ability to induce endothelial cell tube formation

The ability of secreted BMP-2 to induce tube formation by endothelial cells was determined using endothelial cell tube formation assay. Primary human umbilical vein endothelial cells (HUVECs) were plated in 96 well plate (20,000 cells per well) coated with Matrigel. When the cell confluent was reached, HUVECs were then transferred and cultured in the serum deprived medium for 24 hours. Supernatants at 72 h from the previous experiment of each sample were diluted 10 folds to reduce the concentration of FCS to 0.5%, mixed with serum free culture medium and added to the designated well. Supernatants at 72h from the Lipofectamine 2000 alone group were used as control. After 5 hours of incubation, culture plate was examined under the microscope and tube formation ability were compared by taking photographs (Buesch et al., 2017).

Statistical analysis

Transfections in PDLCs were performed by using n=3 individual donors. For each donor, the experiments were performed in triplicate. Data are reported as mean ± standard error of the mean (SE). Statistical analysis was performed by using SPSS 20.0 statistical software. Normal distribution of data was analyzed by Shapiro-Wilk test. The Mann-Whitney test was utilized for the comparison between groups. $P \leq 0.05$ indicates a statistical significance.

CHAPTER IV: RESULTS

Analysis of BMP-2 production after transfection with m1Ψ-BMP-2 mRNA

PDLCs were transfected with m1Ψ-BMP-2 mRNA complexed with Lipofectamine 2000. PDLCs transfected with Lipofectamine 2000 alone were used as controls. Intracellular BMP-2 protein of cell lysates were determined at 24 h culture and extracellular protein from culture supernatants were determined at 24, 48 and 72 h culture.

After 24 h of transfection, significantly higher levels of BMP-2 were observed intracellularly in the experimental group compared to control. The mean concentration was 22,188 picogram per milliliter (pg/ml). Whereas in control, the amount of BMP-2 production was rather low and was unable to be detected by an ELISA (mean minimum detectable dose = 11 pg/ml). As shown in figure 6A, PDLCs from the experimental group produced significantly higher amount of intracellular BMP-2 than those from the control group ($P < 0.05$).

High extracellular concentration of BMP-2 were observed in supernatants collected from the transfected cells in the experimental group. The BMP-2 concentrations were gradually increase from each time-point with the mean concentrations of 12,285, 23,964 and 36,162 pg/ml respectively. Whereas in control, the concentrations were significantly lower and were negligible by an ELISA measurement.

As shown in figure 6B, extracellular concentrations of BMP-2 were significantly higher and gradually increased up to 72 h in the experimental group compared to control group ($P < 0.05$).

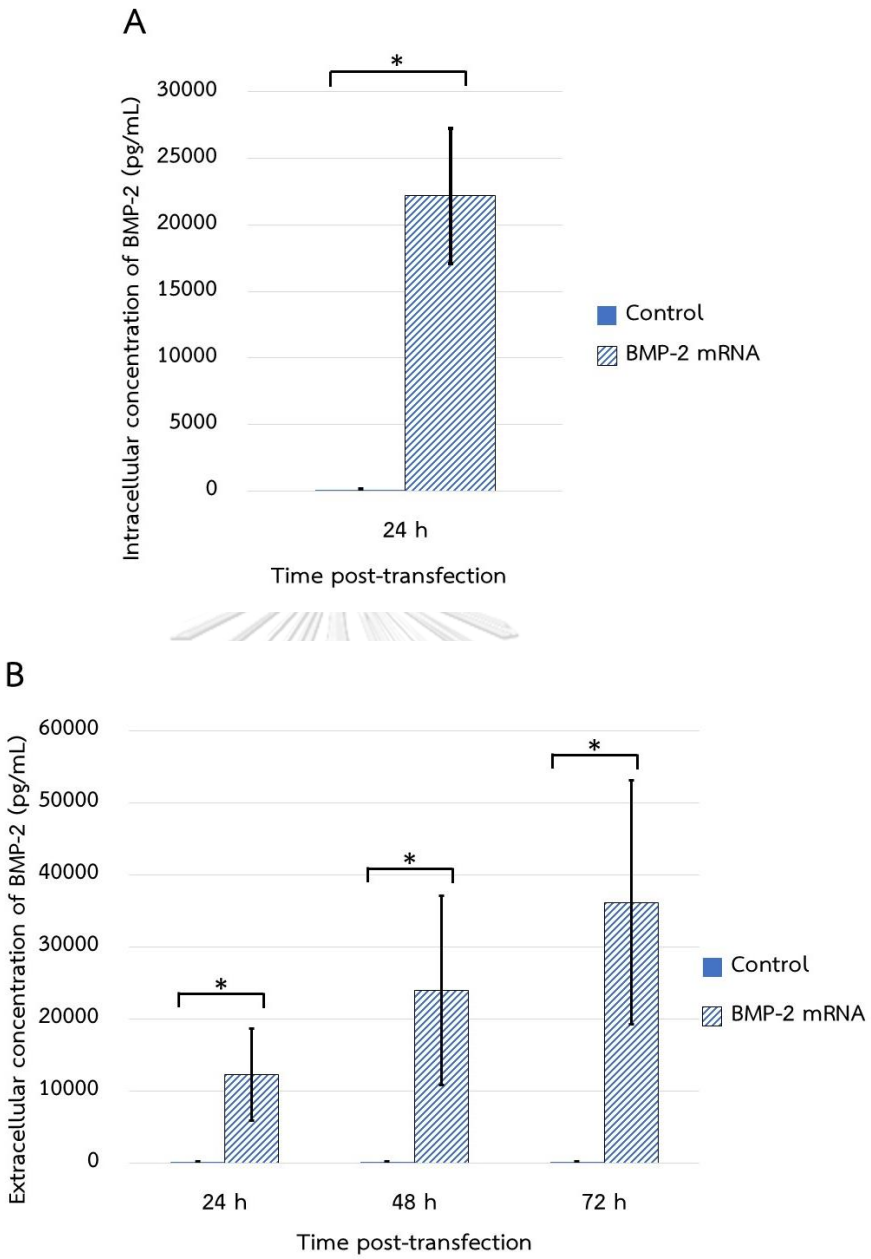

Figure 6. *In vitro* production of BMP-2 protein in clinically relevant target cells after transfection with m1Ψ-BMP-2 mRNA. Human PDLCs at 100,000 cells/well were transfected with 2 µg of BMP-2 mRNA in Lipofectamine 2000. (A) After 24 h of cell transfection, cells were harvested and measured for BMP-2 by ELISA. (B) After 24, 48 and 72 h of cell transfection, culture supernatants were harvested and analyzed by ELISA. Data shown are mean ± SE (n=3). *$P < 0.05$.

Cell viability after transfection with m1Ψ-BMP-2 mRNA

At each time-point of observation, AlamarBlue solution were added into the transfected cells culture. After 4 h, the cell viability was analyzed using a microplate reader. As shown in figure 7, m1Ψ-BMP-2 mRNA and Lipofectamine 2000 had a very minimal effect on PDLC viability. The overall percentage of viability were maintained above 90% throughout the observational period without significance different from controls.

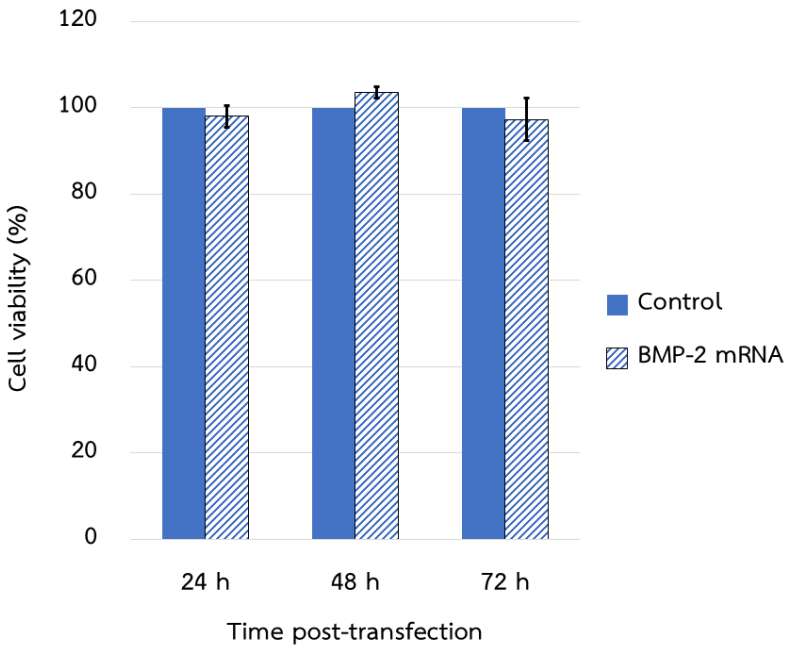

Figure 7. Cell viability after transfection with m1Ψ-BMP-2 mRNA. Human PDLCs at 100,000 cells/well were transfected with 2 μg of BMP-2 mRNA in Lipofectamine 2000. At each time-point of observation, cell viability of PDLCs was assessed by AlamarBlue assay. Data shown are mean ± SE (n=3).

Biological activity of translated BMP-2 protein

Cell proliferation ability

Supernatants collected at 48 h from the experimental group and control group were added to a fresh PDLC culture. After 48 h of incubation, the media were removed and 10% alamarBlue solution were added. Cell proliferation ability was measured after 4h using a microplate reader. As shown in figure 8A, the supernatants from the experiment group were able to enhance cell proliferation but not markedly significant from controls.

Endothelial cell tube formation

To study the effect of produced BMP-2 on angiogenesis, endothelial cell tube formation assay was used. As shown in figure 8C (representative images), BMP-2 translated from mRNA enhanced endothelial cell tube formation seen as a honey comb appearances with increase in branch points. Whereas in control (Figure 8B), incomplete tube formation was mainly observed.

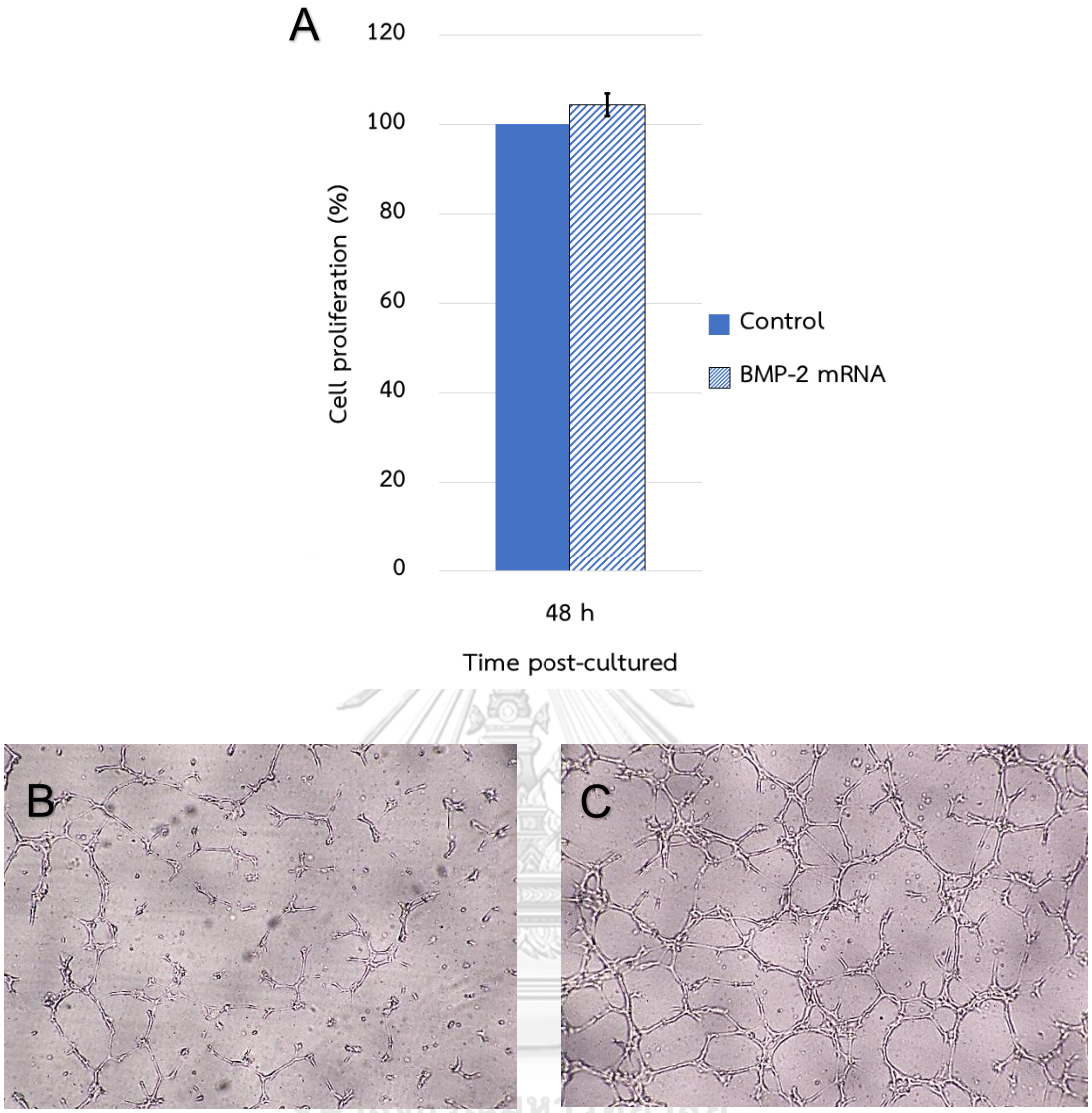

Figure 8. Biological function of BMP-2 protein translated from mRNA *in vitro.* BMP-2 produced from mRNA were assessed for cell proliferation (A), and endothelial cells tube formation assays. (B) HUVECs at 20,000 cells/well were stimulated with PDLC supernatants from controls. (C) HUVECs at 20,000 cells/well were stimulated with PDLC supernatants from 72 h experimental group. Data shown in (A) are mean ± SE and (B and C) are representative images (n=3).

CHAPTER V: DISCUSSION AND CONCLUSION

This study is a pilot study to explore the potential use of mRNA platform in periodontal tissue regeneration. Here, we investigated *in vitro* the transfection efficiency, cytotoxicity, proliferative and angiogenic potential of chemically modified mRNA encoding BMP-2 complexed with cationic lipid. For the first time, our results demonstrated the transfection ability of PDLCs with m1Ψ-BMP-2 mRNA in Lipofectamine 2000 that leads to high levels of BMP-2 production with negligible effect on cell viability. These translated proteins functioned since they were able to induce PDLC proliferation and promote endothelial cell tube formation, a marker of angiogenesis.

High intracellular and extracellular production of BMP-2 were observed in this study. The protein production was sustained until 72 h and exceeds the half-life of recombinant BMP-2 which are usually 1-6 h *in vivo* (Carreira et al., 2015; Issa et al., 2008). This may be thanks to the novel and superior biotechnology of mRNA platform. The construction of mRNA with chemically modified N1-methylpseudouridine nucleotide and optimizing coding sequences with a stringent purification through high performance liquid chromatography (HPLC), hence allowing stability, non-immunogenicity and high protein with transient expression. A few studies used chemically modified BMP-2 mRNA with the aims of aiding bone regeneration. Elangovan et al. (2015) reported the superiority of chemically modified mRNA for *in vitro and in vivo* non-viral gene therapy

application. Their results demonstrated significantly higher expression of BMP-2 from transfected human bone marrow stromal cells after 48 h of transfection compared to conventional plasmid DNA. Recent study by Zhang et al. (2019), rat muscle-derived stem cells were transfected with chemically modified mRNA encoding BMP-2. Their results demonstrated significantly higher expression of BMP-2 from transfected stem cells in every time point compared to controls and peak expression was observed at 24h with gradually declined until day 6. Even though, our results agree with both studies on the transfection ability of modified BMP-2 mRNA, it is quite difficult to compare between studies in terms of translated protein concentrations and kinetics. This may be due to the differences in cell types, differences in mRNA modification and concentrations used.

Cell viability and enhancement of cell proliferation is one of an important factor that are used to demonstrate the safety and efficacy of the biomaterials especially the one that will be used in human. In this study, the m1Ψ-BMP-2 mRNA and Lipofectamine 2000 had negligible effects on the cell viability. Furthermore, the supernatants from the transfected cells could induced PDLC proliferation. Although the differences between cell proliferation could be observed between experimental wells and those in control wells, statistically differences were not found.

Angiogenesis is one of the vital components in tissue regeneration. New blood vessel formations are important for the survival of regenerated tissue because it

provides crucial components for tissue development and maturation. Endothelial cells are the key element of angiogenesis. Proliferating endothelial cells are capable of forming three-dimensional structures as tubes and loops, the structural fundament of a functioning circulation (DeCicco-Skinner et al., 2014). In this study supernatants from the experimental group were able to clearly increase in HUVEC sprouting and increase in the number of branch point as compared to supernatants from the controls. Our translated BMP-2 protein showed a similar result to human recombinant BMP-2 protein-driven endothelial cell formation (Finkenzeller et al., 2012).

In the past, recombinant BMP-2 have been use mainly in the oral surgery field. Attempts were made for the application in periodontal regeneration but there were some drawbacks. Animal studies demonstrated high amount of bone regeneration in horizontal and furcal defects in beagle dogs (Ishikawa I, 1994). However, root resorption and ankylosis at some area of the root surface were encountered (Selvig et al., 2002; Wikesjo et al., 1999). These side effects has been hypothesized that the application of high concentration of BMP-2 protein at once, leading to extensive bone formation that exceeds the rate of cementum or PDL formation. The advantage point of mRNA is a transient expression. In addition, different types of carrier in mRNA technology platform to further sustain proteins expression have been intensively investigated. Hence, optimizing delivery system of our modified BMP-2 mRNA will be needed and further research should be carried out. Furthermore, combination of multiple mRNA encoding

different types of appropriate growth factor proteins such as PDGF that promotes soft and hard tissues formation simultaneously may be used to reduce or prevent the unwanted side effects.

During 2007, US FDA approved recombinant BMP-2 as an alternative to autogenous bone graft for maxillary sinus augmentation and alveolar ridge augmentation after tooth extraction. Previous studies had shown positive results mostly adequate augmentation for implant placement, histologically indifferent form host bone and had no effects on implant survival (Fiorellini et al., 2005; Triplett et al., 2009). Nevertheless, these products are recombinant human proteins with high production costs that leads to high treatment cost and limited the access for general population. In this case, the use of mRNA therapeutic platform may allow access for patients with reduction in cost but remain high in treatment efficacy.

The next step in development of this mRNA therapeutic platform are animal studies. Application of this mRNA technology in animal models can provide crucial data such as the development of the most appropriate delivery systems which differ form *in vitro.* The duration of action and the extent of proteins production could also be assessed. For the future application in human, safeness of this therapeutic platform is one of the important criteria that need to be evaluated. Apart from cell toxicity assessment, the ability to provoke the inflammatory responses also needs to be taken into consideration. Studies in past have shown that mRNA can elicit the innate immune

response that resulted in undesirable inflammation and poor proteins formation; however, this problem had been overcome by the modification of the mRNA. Future researches on the inflammatory response after the use of the modified mRNA are required in order to achieve highest treatment efficacy coupled with maximum safeness.

In conclusion, this study demonstrated the ability of PDLCs after transfection with m1Ψ-BMP-2 mRNA to produce high and transient amount of functional proteins. The translated BMP-2 protein were able to enhance PDLC proliferation and endothelial cell tube formation, the marker of angiogenesis. Using the *in vitro* synthesized nucleoside-modified mRNA may allow future application as novel therapeutics platform for periodontal regeneration, however further researches are required.

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VITA

NAME	พรหมภัคกร กุลธนอมรดิษฐ์
DATE OF BIRTH	29 เมษายน พ.ศ.2534
PLACE OF BIRTH	กรุงเทพมหานคร
INSTITUTIONS ATTENDED	ทันตแพทยศาสตร์บัณฑิต (เกียรตินิยมอันดับ 1) มหาวิทยาลัยรังสิต
HOME ADDRESS	77/402 ซ.พหลโยธิน 54/1 แยก 4-45 (ม.ชลลดา) สายไหม กรุงเทพมหานคร 10220