The use of modified mRNA encoding platelet-derived growth factor-BB as an innovation in periodontal regeneration

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จุฬาลงกรณ์มหาวิทยาลัย
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ธีระภัทร์ สุริแสง : การใช้เอ็มอาร์เอ็นเอดัดแปลงที่เข้ารหัสเพลตเลตดีไรฟ์โกรตแฟ็คเตอร์-บีบี เป็นนวัตกรรมในการคืนสภาพเนื้อเยื่อปริทันต์. (The use of modified mRNA encoding platelet-derived growth factor-BB as an innovation in periodontal regeneration) อ.ที่ปรึกษาหลัก : ผศ.ทญ. ดร.จันทรกร แจ่มไพบูลย์, อ.ที่ปรึกษาร่วม : ดร.สาธิต พิชญางกูร

เนื่องจากเอ็มอาร์เอ็นเอแพลตฟอร์มถูกนำมาใช้รักษาทางการแพทย์ และการคืนสภาพเนื้อเยื่อปริทันต์ในปัจจุบันไม่อาจทำนายผลความสำเร็จได้ ดังนั้นการใช้เอ็มอาร์เอ็นเอจึงเป็นวิธีที่คาดหวังว่าจะให้การคืนสภาพเนื้อเยื่อปริทันต์ได้อย่างสมบูรณ์ จุดประสงค์ของการศึกษานี้ เพื่อดูผลการใช้เอ็มอาร์เอ็นเอเข้ารหัสเพลตเลตดีไรฟ์โกรตแฟ็คเตอร์-บีบี (พีดีจีเอฟ-บีบี) ต่อการสร้างพีดีจีเอฟในเซลล์เอ็นยึดปริทันต์ และทดสอบประสิทธิภาพของพีดีจีเอฟ-บีบีที่ผลิตได้ ต่อการกระตุ้นการแบ่งตัวของเซลล์เอ็นยึดปริทันต์ และการกระตุ้นการสร้างหลอดเลือดใหม่ของเซลล์บุผิวหลอดเลือด เซลล์เอ็นยึดปริทันต์แยกมาจากฟันของผู้ป่วยที่มีอวัยวะปริทันต์ปกติ และถูกนำส่งด้วยเอ็มอาร์เอ็นเอดัดแปลง พีดีจีเอฟ ทำการเก็บสารส่วนใสที่ 24 48 และ 72 ชั่วโมง วัดระดับโปรตีนด้วยอีไลซา และทดสอบความมีชีวิตของเซลล์ และนำสารส่วนใสที่ 48 ชั่วโมงและ 72 ชั่วโมง มาทดสอบการกระตุ้นการแบ่งตัวของเซลล์ และกระตุ้นการสร้างหลอดเลือดใหม่ของเซลล์บุผิวหลอดเลือดตามลำดับ ผลการศึกษาพบว่าเซลล์เอ็นยึดปริทันต์ที่ถูกนำส่งด้วยเอ็มอาร์เอ็นเอ ผลิตพีดีจีเอฟในเซลล์สูงกว่ากลุ่มควบคุมที่ 24 ชั่วโมง มีการหลั่งพีดีจีเอฟในสารส่วนใส และหลั่งต่อเนื่องจนถึง 72 ชั่วโมง อีกทั้งการใช้เอ็มอาร์เอ็นเอไม่ส่งผลต่อความมีชีวิตของเซลล์ นอกจากนี้สารส่วนใสสามารถกระตุ้นการแบ่งตัวของเซลล์เอ็นยึดปริทันต์ได้ และสามารถกระตุ้นเซลล์บุผิวหลอดเลือดให้เกิดการสร้างของหลอดเลือดใหม่ได้ โดยสรุปเอ็มอาร์เอ็นเอดัดแปลงสามารถกระตุ้นการสร้างโปรตีนพีดีจีเอฟ-บีบีทั้งใน และนอกเซลล์ อีกทั้งพีดีจีเอฟที่สร้างขึ้นสามารถกระตุ้นการแบ่งตัวของเซลล์เอ็นยึดปริทันต์ และการสร้างหลอดเลือดใหม่ของเซลล์บุผิวหลอดเลือดได้ ดังนั้นเอ็มอาร์เอ็นเอแพลตฟอร์มอาจเป็นวิธีหนึ่งที่สามารถใช้คืนสภาพเนื้อเยื่อปริทันต์ได้

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สาขาวิชา	ปริทันตศาสตร์	ลายมือชื่อนิสิต .....
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Theeraphat Surisaeng : The use of modified mRNA encoding platelet-derived growth factor-BB as an innovation in periodontal regeneration. Advisor: Asst. Prof. CHANTRAKORN CHAMPAIBOON, Ph.D. Co-advisor: Sathit Pichyangkul, Ph.D.

Since the mRNA platform has been recently introduced to several fields in medicine and the achievement of periodontal regeneration is currently unpredictable, therefore, the use of mRNA technology is a promising approach to pursue the complete periodontal regeneration. The aims of this study are to learn if mRNA encoding platelet-derived growth factor-BB (PDGF-BB) induces PDGF production in human periodontal ligament cells (PDLCs) and to investigate the effect of secreted PDGF on PDLC proliferation and promoting endothelial cell tube formation. PDLCs were obtained from extracted teeth of healthy periodontal patients. The N1-methylpseudouridine modified mRNA encoding PDGF-BB were transfected into PDLCs. The supernatants were collected from 24-, 48- and 72 h and measured the protein production using ELISA assay. PDLCs were also tested for cell viability. In addition, the supernatants collected at 48 h and 72 h were used for proliferation assay and induction of endothelial cell tube formation, respectively. The results showed that PDLCs transfected with mRNA encoding PDGF-BB, produced higher level of intracellular PDGF-BB than the control at 24 h. PDGF-BB was detected in the supernatants started from 24 h and constantly secreted up to 72 h. The transfection of PDLCs by mRNA had no effect on the cell viability. The supernatants containing secreted PDGF-BB were able to promote PDLC proliferation and endothelial cell tube formation. In conclusion, modified mRNA encoding PDGF-BB promotes PDGF-BB production both intra- and extracellularly. Moreover, the supernatants containing PDGF-BB induce PDLC proliferation and endothelial cell tube formation. Thus, mRNA platform technology is possibly applicable for periodontal tissue regeneration.

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CHAPTER I: INTRODUCTION

Background and rationale

Periodontal disease is considered as a chronic condition which causes the destruction of the periodontium. This condition results in loss of masticatory function, esthetics, and also affects quality of life. Globally, periodontitis was ranked as the 6th most common chronic disease (Kassebaum et al., 2014). In Thailand, approximately eight million people have been affected by periodontitis (Oral Health Survey of Thailand 2017) (Bureau, 2018). Although it is not a life-threatening disease, it contributes to a large public burden on medical expenses and health care providers.

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In general, the treatments of periodontal disease include scaling and root planing to reduce the causes of the disease. If tissue defects or diseases remain, surgical procedures such as guided tissue regeneration and bone grafting are necessary in order to reconstruct the diseased periodontium. Unfortunately, to date, there is limited treatment which can achieve true regeneration in humans.

Several novel biological therapeutic approaches have been provided to promote tissue regeneration including growth factors, cell- based therapy and gene therapy. Nevertheless, these approaches are laborious, complex and require expensive equipment. Moreover, some approaches, especially gene therapy, can develop carcinogenesis or risk mutation.

Promisingly, researchers recently developed nucleosides modified mRNA encoding growth factors to establish a therapeutic platform for periodontal regeneration. Comparing to the use of recombinant human growth factors, this platform is capable of producing a larger amount of protein. With advanced innovation in mRNA technology, this study presented an *in vitro* design of the mRNA biometrics by selecting an mRNA that was encoded PDGF and had modified bases which effectively enhances the expression in human periodontal ligament cells (PDLCs). This study also explored appropriate bioactivity such as PDLC proliferation and the ability to induce tube formation by endothelial cells.

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Objectives

1. To learn if mRNA encoding PDGF-BB induces PDGF-BB production in human periodontal ligament cells.

2. To investigate the effect of PDGF-BB induced by mRNA encoding PDGF-BB transfection on PDLC proliferation and tube formation by endothelial cells.

Hypothesis

1. mRNA encoding PDGF-BB induces PDGF-BB production in human periodontal ligament cells.
2. PDGF-BB induced by mRNA encoding PDGF-BB transfection enhances PDLC proliferation and tube formation by endothelial cells.

Field of research

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An *in vitro* study of human periodontal cells transfected with mRNA encoding PDGF-BB using biotechnologies.

Inclusion criteria

Human periodontal ligament cells were obtained from healthy periodontal patients undergoing tooth extraction due to orthodontic reasons or wisdom teeth.

Limitation of research

Since this study required a construction of the mRNA, the expenses for this process was high.

Application and expectation of research

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This study will provide the foundation knowledge regarding the feasibility of mRNA technologies in periodontal regeneration which can be formulated as an mRNA therapeutic platform. This platform could further be used in an *in vivo* study and eventually a clinical trial in order to develop a novel therapeutic agent for periodontal regeneration.

Keywords

Periodontal regeneration, mRNA, periodontitis, Platelet-derived growth factor-BB

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CHAPTER II: LITERATURE REVIEW

Periodontal disease

Periodontal disease is known as an immune condition which responds to oral pathogens. It results in the destruction of the tooth supporting structures such as gingiva, periodontal ligament, cementum and alveolar bone. The disease progression contributes to tooth loss and subsequently affects an individual's functions including eating, speaking, esthetics, and the quality of life. In general, periodontal disease can be roughly divided into 2 major groups (Figure 1):

- **Gingivitis** is found at any age. The inflammation is confined within the gingiva and does not spread to the alveolar bone. The clinical features of gingivitis are red-colored, edema and easily bleeds when brushing or chewing food.
- **Periodontitis** commonly affects middle-aged and elderly people. The inflammation spreads down to periodontal structures, deepens periodontal pockets and loosens teeth. The latter leads to the loss of dentition in severe cases.

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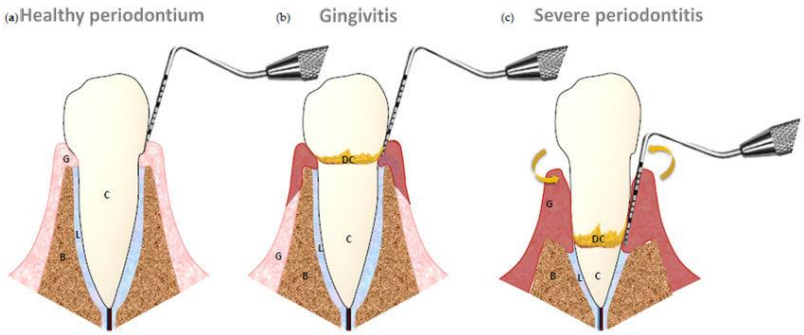

Figure 1. (a) Healthy periodontium, (b) Gingivitis: presence of calculus (DC) and reversible inflammation, and (c) Severe form of chronic periodontitis: gingival inflammation, periodontal pocket, subgingival calculus, and tooth mobility. (G = gingiva, L= periodontal ligament, C= root cementum, and B= alveolar bone (Román-Malo & Bullon, 2017)).

Severe forms of periodontitis are considered a major oral health problem, ranked at 6th among the most common chronic diseases worldwide. Approximately 243 million people suffered from this disease (Kassebaum et al., 2014), while the Oral Health Survey of Thailand 2017 (Bureau, 2018) reported that eight million Thais have experienced severe periodontitis. Although periodontitis is not a life-threatening disease, it is considered as a huge public health burden which requires global attention.

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Treatment of periodontal diseases

The treatment of periodontitis is to remove dental plaque biofilm, the cause of the disease and the elimination of other factors that contribute to the disease, such as dental calculus. Current treatments include scaling and root planing, along with oral hygiene instruction. In addition, deep plaque removal and bony defect correction will be performed with periodontal surgeries. However, the outcomes of these treatments are mainly to halt spreading of the disease, control inflammation, and promote healing, especially by repair. Unfortunately, since the periodontium cannot be restored and reconstructed by repair, it results in being unable to return to its normal condition and also lacks efficiency in chewing. Moreover, recurrence of the disease may occur ((Hirschfeld & Wasserman, 1978);(Kaldahl et al., 1996)).

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The ultimate goal of periodontal treatment is to regenerate the destroyed periodontium due to periodontitis to improve functions; for example, eating, speaking, esthetics, and the quality of life. To the best of our knowledge, guided tissue regeneration (GTR) and bone grafting (Figure 2) are acceptable approaches commonly applied for periodontal regeneration.

Interestingly, bone grafting is one of the treatment options used for the correction of periodontal osseous defects. It provides clinical improvement superior to surgical debridement alone (Reynolds et al., 2003). However, the success varies mainly depending on the number of residual wall defects. While the concept of GTR is to exclude gingival connective tissues and prevent apical migration of the gingival epithelium by placing a barrier membrane between the flap and the root surface, this promotes the repopulation of the periodontal ligament and bone cells into the periodontal defect (Nyman et al., 1982). A systematic review indicated that GTR was more effective than a surgical debridement in terms of clinical attachment gain and probing depth reduction in intrabony and furcation defects (Murphy & Gunsolley, 2003).

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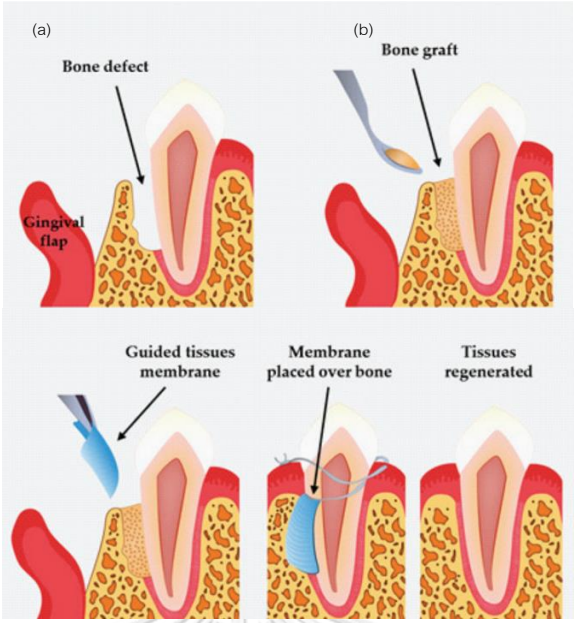

Figure 2. Schematic illustration of (a) Bone grafting procedure and (b) Guided tissue regeneration (Sheikh et al., 2014).

Unfortunately, it is difficult to achieve complete periodontal regeneration by GTR and bone grafting approaches due to several limitations such as patients' systemic conditions and behaviors, types of periodontal defects, previous restorations, and surgical techniques (Bashutski et al., 2011). In addition, both treatments were also considered to be highly expensive procedures ((Avila-Ortiz et al., 2015); (Kao et al., 2015)).

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Periodontal regeneration

During the past decade, tissue engineering has been introduced as an innovation to regenerate the destructed periodontium. The three essential constituents of tissue engineering are comprised of stem cells, scaffolds and signaling molecules. Firstly, stem cells reproduce themselves and differentiate into various types of cells. Secondly, a scaffold is a construction or framework that provides target cells to attach, proliferate and differentiate into target tissue organs. Lastly, signaling molecules or growth factors are proteins that induce stem cells to proliferate and differentiate via their receptors (Figure 3) ((Smith, 2006); (Taba et al., 2005)).

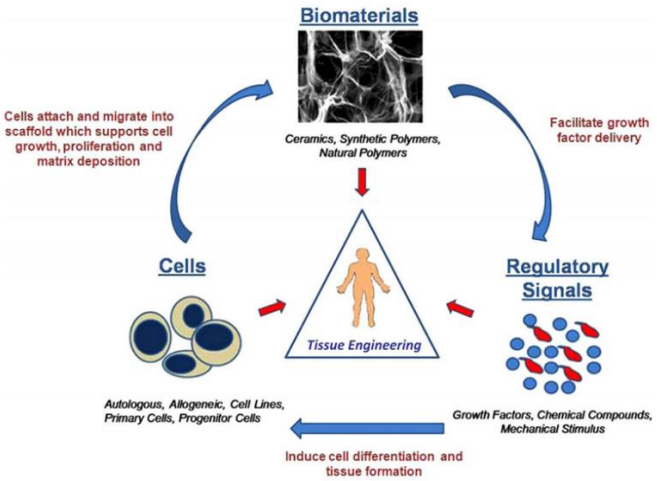
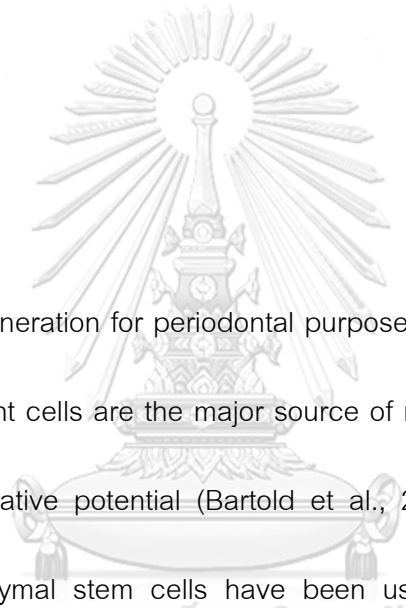


Figure 3. The three essential components of a tissue engineering triad (Murphy et al., 2013).

Several attempts to develop innovations for periodontal regeneration have continued to emerge. These innovations during the past decade have included

- cell-based regeneration,
- recombinant growth factors, and
- gene therapy.



Cell-based regeneration

Cell-based regeneration for periodontal purposes use mesenchymal stem cells. The periodontal ligament cells are the major source of mesenchymal stem cells, which have shown a regenerative potential (Bartold et al., 2000). To enhance success in regeneration, mesenchymal stem cells have been used in combination with either scaffolds such as collagen, fibrin, hydrogel, gelatin etc. or non-scaffold materials such as cell sheets (Akizuki et al., 2005). However, the use of mesenchymal stem cells and cell sheets are still limited due to the lack of cell sources and being a time-consuming and complicated procedure.

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Recombinant growth factors

The use of growth factors has been considered for decades as one of the developed therapies for periodontal regeneration by stimulating mesenchymal stem cell proliferation and differentiation. Growth factors used in regeneration include fibroblast growth factor (FGF), platelet derived growth factor (PDGF), insulin growth factor (IGF), vascular endothelial cell growth factor (VEGF), epidermal growth factor (EGF), and bone morphogenetic proteins (BMPs) (Kao et al., 2015).

Platelet-derived growth factor (PDGF)

Platelet-derived growth factors (PDGFs) are members of a multifunctional polypeptide family. PDGFs are produced by many cell types comprising platelets, fibroblasts, keratinocytes, myoblasts, epithelial cells, and macrophages. PDGFs have been classified into five isomeric forms, namely, PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD (Figure 3). They bind to cell-membrane tyrosine kinase receptors, resulting in autophosphorylation of the cytoplasmic domain and subsequently exerting biological effects. The biological activities of PDGFs include the promotion of wound healing by upregulating the stem cell population, bone regeneration by upregulating the

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osteoblastic population, capillary ingrowth by enhancing endothelial cell proliferation, and connective tissue regeneration by replicating and stimulating fibroblasts (Shin et al., 2015).

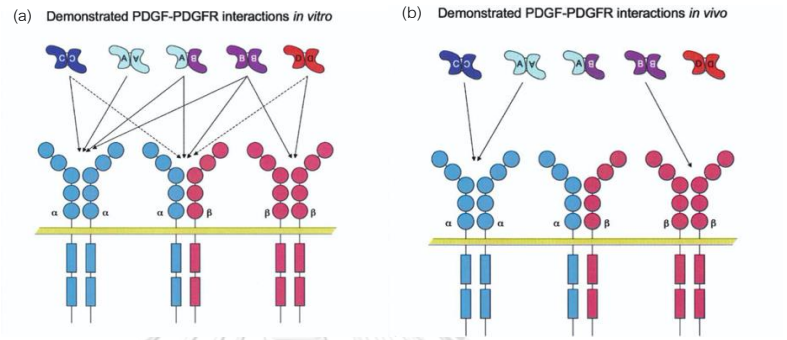

Figure 4. PDGF–PDGFR interactions. Each chain of the PDGF dimer interacts with one receptor subunit. The active receptor configuration is therefore determined by the ligand dimer configuration. Figure (a) shows the interactions that have been demonstrated in cell culture. Hatched arrows indicate weak interactions or conflicting results. Figure (b) shows the interactions proven to be of importance in vivo during mammalian development (Andrae et al., 2008).

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In regenerative medicine, recombinant human PDGF-BB (rhPDGF-BB) (Regranex® or Becaplermin gel) has been introduced for clinical use as a topical therapy for chronic neuropathic diabetic ulcers (Robson et al., 1992). Furthermore,

PDGF-BB has also been used for pressure wounds and accelerating wound healing in various surgical procedures. The tissue repair mechanisms induced by PDGF-BB appear to involve fibroblast proliferation, collagen production and vessel formation (Pierce et al., 1994). Augment® Bone Graft comprises an osteoconductive scaffold of β-tricalcium phosphate (β-TCP) and rhPDGF-BB. It is intended as an alternative to an autologous bone graft in the hindfoot or ankle joint fusion sparing the patient the morbidity of autograft harvest (Solchaga et al., 2012).

PDGFs are known as a group of growth factors promoting periodontal tissue regeneration (Dereka et al., 2006). In the late 1980's, Lynch and co-workers first revealed that PDGF promoted the regeneration of periodontal tissues including bone, cementum and periodontal ligaments following the application of PDGF to periodontitis defects in beagle dogs. From histological findings, a significant amount of new bone and cementum formation was exhibited in experimental sites. These preliminary results indicated that *in vivo* application of PDGF may enhance periodontal regeneration (Lynch et al., 1989). According to the meta-analysis, the use of 0.3 mg/ml rhPDGF-BB positively impacted on defect bone fill, bone height and clinical attachment gain in periodontal defects (Li et al., 2017). A clinical study has also shown that the use of rhPDGF provided comparable outcomes in periodontal regeneration to GTR or bone grafting (Darby &

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Morris, 2013). After rhPDGF-BB was used for the first time as Regranex® to promote soft tissue healing, GEM 21S® was later introduced for use in promoting periodontal bone regeneration (Solchaga et al., 2012). It was approved by the Food and Drug Administration (FDA) to use 0.03% PDGF-BB in combination with beta-tricalcium phosphate to treat intrabony defects, furcation defects and gingival recession (Suárez-López Del Amo et al., 2015). Even though PDGF-BB has potential in stimulating PDL stem cells to proliferate and differentiate (Mihaylova et al., 2018), the leakage of this protein from treated areas and the expensive cost are its limitations.

Gene therapy

Besides using recombinant growth factors, the use of DNA in gene therapy has been introduced in treating several diseases. Gene therapy can be performed by processing plasmid DNA or viral vectors. Foreign DNA is delivered to the nucleus by passing through the cell and nuclear membranes. Foreign DNA is integrated into the host genome and sustained transgene expression even after host cells replicate (Kim & Eberwine, 2010). Adenoviruses have been used as gene delivery vectors because of high transduction efficiency in both dividing and non-dividing cells and non-inducing phenotypic changes in transduced cells (Gu et al., 2004). Compared with recombinant

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growth factors, adenovirus encoding PDGF gene sequences can effectively transduce cells, prolong growth factor expression and induce downstream signaling pathways (Chen & Giannobile, 2002).

For example, an adenovirus encoding PDGF-AA induced PDGF-AA protein production in gingival fibroblasts (Chen & Giannobile, 2002). In an animal study, adenovirus encoding the PDGF-B gene promoted nearly complete alveolar bone bridging in the adenovirus PDGF-B-treated group, whereas limited bridging was observed in the control group (Chang et al., 2009). Nonetheless, gene therapy should be used with caution due to safety issues including mutation and tumorigenesis (Kim & Eberwine, 2010).

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The drawback of the current strategies used for tissue regeneration includes the unexpected treatment outcomes due to inappropriate defect selection and increased risk of mutation in cases using DNA therapy. Some methods required large amounts of substances while its presence in the tissue remained only for a short period of time. Moreover, the cost of treatment was considered as another limitation because most materials used for regeneration were expensive and required importing. In addition,

current techniques such as bone grafting and guided tissue regeneration were unable to provide complete regeneration of the periodontium.

Messenger RNA (mRNA) technology

A novel technique called "mRNA therapy" is the use of central dogma from molecular biology concepts, where specific mRNA is delivered by a carrier into the cytoplasm of the patient's cell to achieve the translation process for the desired protein (Figure 5). These proteins have properties as signaling molecules or growth factors that induce cell proliferation and differentiation in periodontal tissue regeneration.

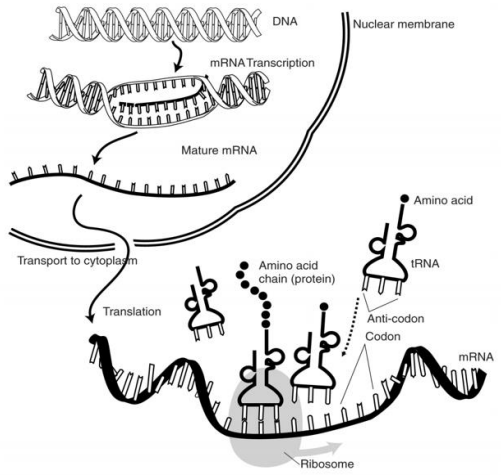

Figure 5. Overview of the central dogma of molecular biology with gene expression in eukaryotes. DNA is first transcribed to the messenger RNA in the nucleus.

After transporting out of the nucleus, protein synthesis begins with the help of a ribosome and transports RNA by translating nucleotide triplets to amino acids (Mesuere et al., 2015).

The process of mRNA technology is an advanced innovation that will transform the medical treatment base with biotechnology for safe and affordable treatment. There are many advantages such as high delivery efficiency and no risk of integration into the host genome, so is superior to DNA therapy (Kim & Eberwine, 2010).

Currently, the use of mRNA has been developed by various methods to prevent mRNA degradation and increase the effectiveness in protein production. The encapsulation of mRNA enhances its stability and helps in uptake of mRNA into the cell (Figure 6). mRNA encapsulation or transfection can be performed in several forms such as lipids encapsulation (Mintzer & Simanek, 2009), polymers (Pack et al., 2005) and peptides (Martin & Rice, 2007). The most reliable system for delivering mRNA to the cell is cationic lipid nanoparticles. These nanoparticles are easy to synthesize and import into target cells with specific ligands. A positive charge of a cationic lipid is subjected to attach to the negative charge of mRNA, then it aggregates into lipid nanoparticles ((DeRosa et al., 2016); (Karikó et al., 2012)). Furthermore, increasing the stability of

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mRNA by gene sequence coding modification such as 5′cap, 5 ′ and 3 ′ UTR and the length of poly A, especially chemical modification of nucleosides, leads to enhance the effectiveness of protein production (Zhang et al., 2019).

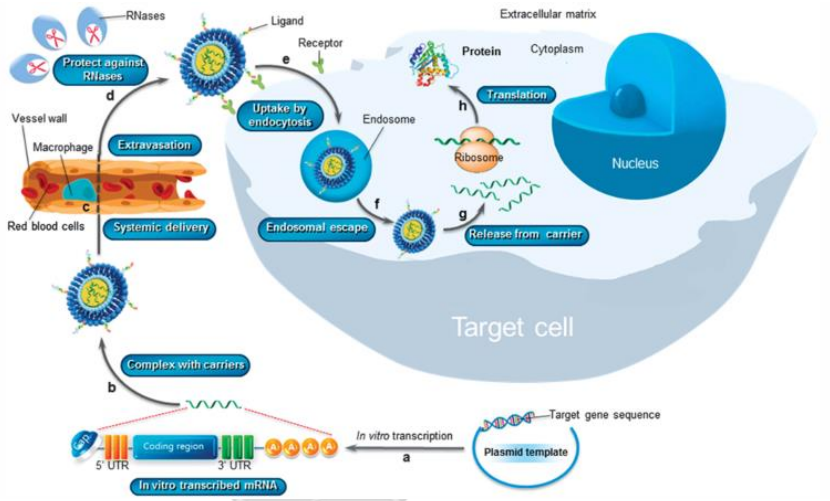

Figure 6. mRNA technology using a non-viral vector approach. At (a), a linearized DNA template with a target gene sequence is used for the production of IVT-mRNA. At (b), IVT-mRNA spontaneously forms nanosized complexes with cationic materials. At (c), accumulation occurs to target organ or tissues without nonspecific interactions. At (d), the delivery system protects IVT-mRNA against ubiquitous endonucleases within the extracellular matrix, and at (e), it possibly promotes target cell entry efficiency. At (f), endocytosed formulations locate in the endosome, and the carrier of IVT-mRNA facilitates endosomal escape via endosome disruption. At (g) IVT-mRNA is released in cytoplasm and transported to ribosome. Finally, at (h), a protein of interest is

translated from IVT-mRNA by using protein synthesis machinery of the target cell (Guan & Rosenecker, 2017).

The limitation of the use of mRNA in tissue regeneration is that mRNA stimulates the immune system. The induction of innate immune response to mRNA is an undesired effect (Goubau et al., 2013). Since mRNA can be seen as foreign bodies or viruses and can be recognized by various receptors, the binding between receptors and mRNA possibly leads to cell activation resulting in the inhibition of protein formation (Pollard et al., 2013).

Thus, an attempt to inhibit the immune activation by modifying the base of the mRNA was suggested. Particularly, base adjustment in either pseudouridine or N1-methylpseudouridine inhibits the innate immune response via type 1 interferon and promotes protein production ((Andries et al., 2015); (Karikó et al., 2008)).

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Currently, the development of modified mRNA is used as an innovation in biotechnology platforms for medical tissue regeneration instead of stem cells, protein growth factors and gene therapy (DNA / viral vector). For example, an experiment of

mRNA specific to the VEGF-A growth factor in mice with ischemic heart disease demonstrated a successful outcome for using mRNA in tissue regeneration. After receiving the VEGF-A encoded mRNA injection, an increase of new blood vessels was reported. Smaller muscle lesions became smaller and mice lived longer than mice injected with plasmid DNA or those in the controls (Zangi et al., 2013).

In conclusion, mRNA technology has several advantages compared with stem cell and plasmid DNA /viral vector technologies in periodontal regeneration as follows: mRNA technology is safe because mRNA is a transient genetic transporter that degrades naturally. It also has no risk to integrate into host genomes. mRNA can also uptake into different cell types and produce high protein levels. Unlike the use of a viral vector, mRNA technology can be used without inducing the immune response. Therefore, the ability to produce large amounts of mRNA and good manufacturing practice (GMP) grades *in vitro* can be used in future clinical settings.

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CHAPTER III: MATERIALS AND METHODS

Construction of modified mRNA encoding PDGF-BB

Nucleotide sequences of human PDGF-BB was designed by the immunology laboratory at the Faculty of Dentistry, Chulalongkorn University. In collaboration, the production of N1-methylpseudouridine mRNA encoding PDGF-BB was provided by Dr. Norbert Pardi from the University of Pennsylvania, United States ((Pardi et al., 2017); (Pardi, Parkhouse, et al., 2018); (Pardi, Hogan, et al., 2018)).

Medium and reagents

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Minimum Essential Medium with Alpha modification (Alpha MEM, ThermoFisher Scientific, Waltham, MA, USA) supplemented with GlutaMax-I (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (5 µg/ml), and 10% heat-inactivated fetal calf serum (Gibco Laboratory, Grand Island, NY, USA) was prepared and used throughout the study. Opti-MEM I and Lipofectamine® 2000 were obtained from Invitrogen, Waltham, MA, USA.

Cell isolation and culture

All participants were provided written informed consent forms. The study protocol was approved by the medical ethical committee at the Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand.

Human periodontal ligament tissues were obtained from healthy periodontal patients (age 15-35 years) undergoing wisdom tooth extraction or tooth extraction for orthodontic reasons at the Faculty of Dentistry, Chulalongkorn University, Thailand. The extracted tooth was immediately placed in a sterile tube containing Alpha MEM, kept on ice and transferred to the laboratory within a few hours for cell isolation.

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Human periodontal ligament cells (PDLCs) were separated from the tooth by the enzyme-digestion method (Tanaka et al. 2011). Briefly, the tooth was washed twice with culture medium or Dulbecco's phosphate-buffered saline (DPBS), to remove blood clots and adherent erythrocytes. Then PDL tissues were scratched out from the middle third of the root under sterile conditions to avoid contamination from gingival or periapical granulation tissues. Then PDL tissues were minced into a fragment of 1-2 mm^2 and digested with a solution of collagenase (2 mg/ml) and dispase (2 mg/ml) for 60 minutes

at 37°C and then filtered through a 70-μm cell strainer. The pass-through was washed twice with culture medium. The PDLCs were cultured with the medium at 37°C in a humidified atmosphere of 5% CO_2 in air. Culture medium was changed twice a week. After a confluent monolayer of cells was reached, PDLCs were trypsinized, washed twice and then transferred into new tissue culture dishes. The cells from the 3rd to 8th passages from 3 different subjects were used throughout the study (Iwata et al., 2010).

In vitro cell transfection and production/secretion of PDGF-BB protein

For *in vitro* transfection of periodontal ligament cells, modified N1-methylpseudouridine mRNA encoding PDGF-BB was complexed with Lipofectamine® 2000 (PDGF-BB mRNA-L2000) and transfected into PDLCs according to the manufacturer's instructions. To analyze the amount of PDGF-BB protein production and secretion, the transfected cells were cultured for 24-72 h. The cells were lysed using RIPA buffer (Pierce® RIPA buffer, ThermoFisher Scientific, Waltham, MA, USA). The cell lysates were collected at the 24 h time-point for determining the amount of intracellular protein production, whereas the supernatants were collected at the 24, 48 and 72 h time-points for determining the amount of extracellular protein secretion. PDGF-BB protein production and secretion were measured by a commercially available ELISA kit

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(Quantikine®, R&D System, Minneapolis, MN, USA). The assay was performed according to the manufacturer's protocol.

Cell viability

To investigate cell viability, PDLCs transfected with either PDGF-BB mRNA-L2000 or L2000 alone were harvested at the 24, 48 and 72 h time-points. Next, the cells were incubated with 10% AlamarBlue solution (AlamarBlue®, BIO-RAD, CA, USA) then incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After 4 h, cell culture supernatants were collected and measured for optical density at an absorbance of 570 nm using a microplate reader (Epoch®, Biotek®, VT, USA).

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The stimulation of PDLCs proliferation

To determine the effect of secreted PDGF-BB proteins on PDLC proliferation, fresh PDLCs were plated in a 96-well plate ($3x10^3$ cells per well) and either the control medium, supernatants of PDGF-BB mRNA-L2000 or L2000 alone transfected PDLCs were added. After a 48 h incubation, 10% AlamarBlue solution was added. After 4 h

incubation, the cell proliferation ability was measured via the optical density at the absorbance of 570 nm. Capillary- like tube formation of endothelial cells.

Capillary-like tube formation of endothelial cells

To determine the biological activity of secreted PDGF-BB protein from PDLCs on capillary-like tube formation, an endothelial cell tube formation assay was performed. Briefly, primary human umbilical vein endothelial cells (HUVECs) were cultured for 24 h and plated in a 96-well plate (2 x 10^4 cells per well) coated with Matrigel (Matrigel® Matrix, Corning®, NY, USA). The supernatants of PDLCs transfected PDGF-BB mRNA-L2000 or L2000 alone as a control were added. After 5 h incubation, each plate was determined under a light microscope (Olympus® life science, Waltham, MA, USA) and photographs were taken for analyzing capillary-like tube formation.

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Statistical analysis

Values were presented in means with standard error. The data were analyzed using computer program JMP 15.0 (SAS Institute Inc. NC, USA). Student's t test was used to determine the difference between the modified mRNA encoded PDGF-BB and

the control groups. One-way ANOVA analysis was performed to determine the differences among different time-points. *P* values less than 0.05 were regarded as statistically significant.

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CHAPTER IV: RESULTS

Analysis of PDGF-BB protein production and secretion

PDLCs were transfected with N1-methylpseudouridine mRNA encoding PDGF-BB complexed with Lipofectamine 2000 or Lipofectamine alone. The cell lysates were collected at 24 h for determining the amount of intracellular PDGF-BB protein production. While the supernatants were collected at 24, 48 and 72 h for investigating the amount of extracellular PDGF-BB secretion.

After transfection for 24 h, the PDGF-BB mRNA-L2000 transfected PDLCs were capable of producing a significantly higher intracellular protein level than the control ($p < 0.05$), with a mean of 155,540.3 picograms per milliliter. The amount of intracellular proteins produced from the control was limited (Fig. 7A).

The supernatants were collected at 24, 48 and 72 h and subjected to measure the amount of extracellular PDGF-BB protein secretion. The secreted PDGF-BB was detected at 24 h and continuously increased up to 72 h. The mean concentrations of

PDGF-BB in the PDGF-BB mRNA-L2000 transfected group were 26,815, 44,985 and 57,938 picograms per milliliter at 24, 48 and 72 h, respectively (Figure 7B). At all time-points, the protein levels detected in the PDGF-BB mRNA transfected group were significantly higher than the controls ($p< 0.05$). However, the difference of PDGF-BB levels among time-points in mRNA transfected groups was not significant (Fig. 7B).

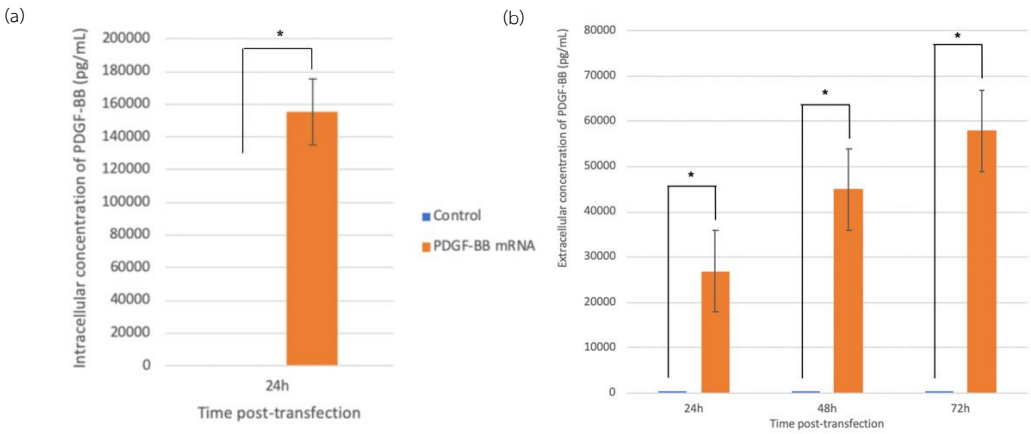

Figure 7. The production of the PDFG-BB protein. Human PDLCs at $1x10^5$ cells/well were transfected with 2µg of PDGF-BB mRNA-L2000. Culture supernatants were analyzed by ELISA. At (a) was the mean concentration of intracellular PDGF-BB at 24 h. At (b) was the mean concentration of extracellular PDF-BB at 24, 48, and 72 h. Data were shown as mean ± SE (n=3). *, = a significant difference between the mRNA and the control groups ($p<0.05$).

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Analysis of cell viability after transfection with PDGF-BB mRNA-L2000

The PDCLs were transfected with PDGF-BB mRNA-L2000, and the cells were then harvested at 24, 48 and 72 h for analyzing cell viability using an AlamarBlue assay. The results showed that the PDGF-BB mRNA-L2000 did not affect the viability of PDCLs. At each time-point, the percentages of cell viability were comparable between these two groups. In addition, it showed that the cell viability was still greater than 90 percent after 72 h incubation and similar to the controls (Figure 8).

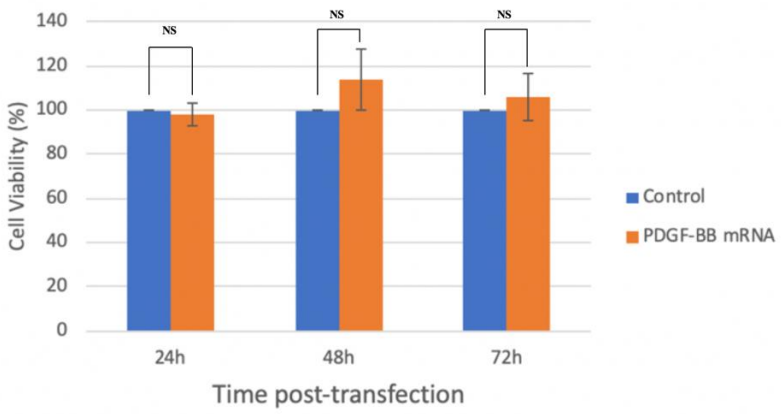

Figure 8. The mean percentages of periodontal cell viability at 24, 48 and 72 h. Human PDLCs ($1x10^5$ cells/well) were transfected with 2 µg of PDGF-BB mRNA-L2000. At each time-point, the cell viability of PDLCs was assessed by an AlamarBlue assay. Data were shown as mean ± SE (n=3). NS = not significant.

Analysis of biological activity of translated PDGF-BB protein

The stimulation of PDLCs proliferation

Fresh PDLCs were incubated with the supernatants of the PDLCs transfected with PDGF-BB mRNA-L2000 or L2000 alone for 48 h. After 48 h of incubation, AlamarBlue solution was added and cultured for another 4 h. The cell proliferation ability measured the optical density at an absorbance of 570 nm. The results showed that the supernatants from PDLCs transfected with a PDGF-BB mRNA-L2000 stimulated the periodontal ligament cell proliferation. The percentage of cell proliferation in the transfected cell group was greater than the control at 48 h ($p < 0.05$) (figure 9).

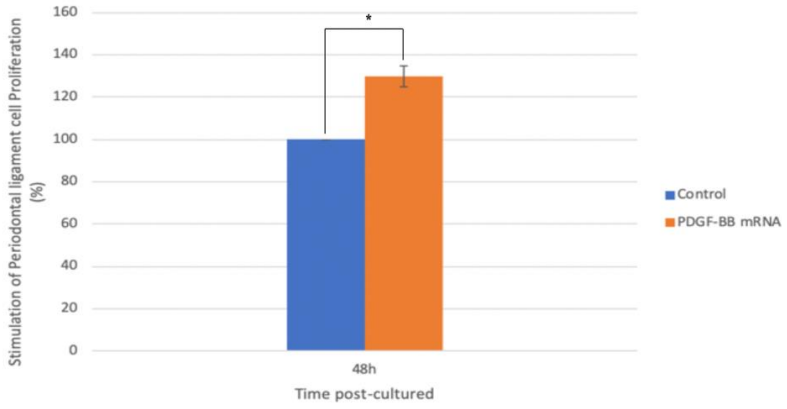

Figure 9. The biological function of PDGF-BB protein translated from mRNA *in vitro*. PDGF-BB produced from mRNA transfection was assessed for cell proliferation.

Data were shown as mean ± SE (n=3). *, = a significant difference between the mRNA and the control groups ($p<0.05$).

Capillary-like tube formation of endothelial cells

The endothelial cell tube formation assay was used to determine the biological function of the secreted PDGF-BB protein. HUVECs were cultured for 24 h and plated in a 96-well plate coated with Matrigel. The supernatants of PDLCs transfected PDGF-BB mRNA-L2000 or L2000 alone (a control) were added. Each plate was determined for capillary-like tube formation at every hour of incubation. Tube formation was initially detected in the PDGF-BB mRNA-L2000 at 2 h. The cell interconnecting cord was longer compared with the controls at every time-point starting from 2 to 4 h. As shown in Figure 10, a capillary-like structure was completely formed after 5-h incubation in the mRNA transfected supernatant group.

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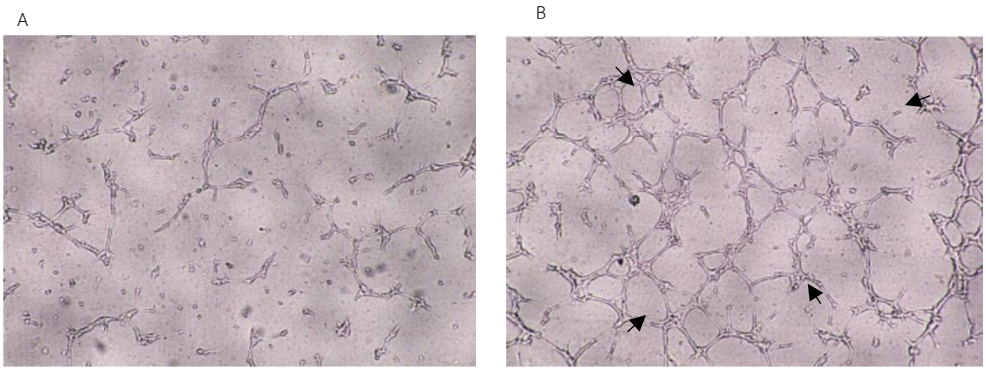

Figure 10. An *in vitro* biological function of PDGF-BB protein translated from mRNA. PDGF-BB produced from mRNA transfection were assessed for endothelial cell tube formation ability. In (A), HUVECs 2 x 10^4 cells/well were cultured with the supernatants of the control. In (B), HUVECs 2 x 10^4 cells/well were cultured with? the supernatants of PDLCs transfected PDGF-BB mRNA-L2000. Black arrows indicate completely formed a capillary-like structure after culture for 5 h. The images are the representatives of three separate experiments.

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CHAPTER V: DISCUSSION AND CONCLUSION

This study is the first study using the mRNA technology for periodontal regeneration. The cells used in the study are periodontal ligament cells since they have high stemness potential. Periodontal ligament cells differentiate into a variety of cell linages that resembles periodontal ligaments and cementum, which is an important target organ for periodontal regeneration. However, the limitation is that use of periodontal ligament cells require tooth extraction (Chen et al., 2006); (Yang et al., 2013)).

The benefits of transfecting mRNA outweighed other techniques. These benefits included no risk of gene integration into the host genome, no requirement for immune inducible vectors, high cell cycle- independent transfection efficiency, and adjustable and rapid expression (Yamamoto et al., 2009). Using mRNA transfection can introduce mRNAs into a cell, thereby, overcoming the overexpression of the gene. This is because mRNA does not need to be in a nucleus to be expressed. Moreover, other advantages of mRNA transfection are that mRNAs can be expressed within minutes after transfection because it skips several processes such as the translocation process to the nucleus and the transcription process (Kim & Eberwine, 2010).

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The success of cell transfection with mRNA was implied from our previous experiments that used the same transfecting agent (Lipofectamine 2000). In the preliminary experiment, periodontal ligament cells were transfected with mRNA encoding green fluorescence protein (GFP), and the results revealed that the transfection efficacy of mRNA encoding GFP complexed with Lipofectamine 2000 was over 90% (Unpublished data).

According to our finding, it indicated that mRNA specific to PDGB-BB stimulated target cells (periodontal ligament cells) and also effectively induced a large amount of PDGF-BB production intracellularly. The PDGF-BB was released extracellularly at 24 h and continuously released until 72 h. Similar to the use of rh PDGF-BB, the amount of PDGF-BB secretion was detected after 24 h and continuously released up to 7 days (Zaman et al., 1999). From the last decade, there were no scientific reports about using mRNA specific to PDGF-BB, there was only a study using modified mRNA encoding BMP-2 to transfect into muscle-derived mesenchymal stem cells was reported (Zhang et al., 2019). The study showed that BMP-2 production was released first at 24 h and continuously declined. In addition, BMP-2 was still detectable intracellularly and

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extracellularly until day 6. Our study showed mRNA specific to PDGF-BB stimulated PDLCs to produce PDGF-BB in relatively large amounts, moreover, the production lasted up to 72 h. mRNA induced PDGF-BB production stayed longer than rhPDGF-BB application which is quickly clear due to its short half-life (approximately 4 h) (Lynch et al., 1991).

Typically, rh PDGF-BB induced PDLC proliferation ((Mailhot et al., 1996); (Zaman et al., 1999)). Similar to using recombinant protein, our study demonstrated that secreted PDGF-BB after stimulating with mRNA was capable of inducing PDLC proliferation superior to the control. Our unpublished data demonstrated that PDGF-BB encoding mRNA transfected cells also induced VEGF protein production in PDLCs.

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Since this *in vitro* study used a mRNA platform as a delivery system, not only did it stimulate high amounts of protein over a long period of time, but also the platform had no effect on cell viability of PDLCs. Similarly, using plasmid DNA specific to a pdgf-b gene transfected into PDLCs showed no significant cell viability compared to the controls (Plonka et al., 2017).

An adequate blood supply is critical for periodontal tissue regeneration. Several biological factors were involved in the angiogenic regulation (Duran et al., 2017). However, the vascular endothelial growth factor (VEGF) was commonly considered as the most effective agent participating in modulation of vascular endothelium (Ng et al., 2006). Interestingly, a study had previously shown that Krüppel-like factor 4 (KLF4) promotes angiogenesis by activating VEGF signaling in endothelial cells while PDGF-BB induces VEGF expression through KLF4 during endothelial cell angiogenesis (Liang et al., 2017). Angiogenesis involves migration and proliferation of endothelial cells, capillary tube formation and MSC stabilization of newly formed tubes ((Carmeliet & Jain, 2011); (Chen et al., 2013)). Also, rhPDGF-BB contributes to endothelial cell angiogenesis *in vitro* ((Battegay et al., 1994); (Castellon et al., 2002); (Gehmert et al., 2011)). Our *in vitro* study found that a secreted PDGF-BB protein of the PDLCs transfected with PDGF-BB mRNA also had the ability in promoting capillary-like tube formation by endothelial cells, which may imply the initiation of angiogenesis.

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According to this study, the results suggested that *in vitro* transfection of human periodontal ligament cells leads to high protein expression and effective biological activities. For the next step, animal studies are required to prove the effectiveness of the mRNA therapeutic platform. Since animal models may provide crucial data such as the

development of the more effective delivery systems which may differ from *in vitro* studies. The results of this study suggested an optimal delivery system for an *in vitro* transfection, but further investigation in its efficacy in an *in vivo* study need to be clarified. Furthermore, the immune activation of N1-methylpseudouridine modified mRNA encoding PDGF-BB transfection in animal models should be assessed prior to performing clinical trials. The effect on aggravating the inflammatory responses need to be accounted for in further investigation.

Given the delivery of PDGF-BB transfection, more research is required to acquire a proper scaffold or carrier to introduce and/ or maintain the mRNA solution at a defect site. These additional scaffolds could comprise of controlled bio-absorbable collagen and cell sheets for promising materials ((Larsson et al., 2016); (Onizuka & Iwata, 2019)). Additionally, the combination of modified mRNA encoding PDGF-BB with other growth factors such as BMP-2, VEGF and FGF-2 is another approach which enhanced the outcomes of tissue regeneration. Altogether, these strategies may accelerate the development of mRNA-based therapy in periodontal regeneration.

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In conclusion, this study shows that the use of mRNA encoding PDGF-BB can be delivered to the periodontal ligament cells and induce cells to produce PDGF-BB without affecting the cell viability. Moreover, PDGF-BB stimulates the periodontal ligament cell proliferation and induces capillary like tube formation in endothelial cells. Thus, the result of this study will be beneficial for further *in vivo* studies and clinical trials to acquire the foundation of mRNA platform technology to restore affected periodontium and possibly apply to other regenerative fields.

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