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

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Abstract

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. This study aims 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 encodes this protein. We investigated the biological activity of the translated protein for enhancing PDLC proliferation. Isolated PDLCs from healthy periodontal tissue were transfected with N1-methylpseudouridine modified mRNA encoding BMP-2 (m1Ψ-BMP-2 mRNA) complexed with a 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 intracellularly and extracellularly. Secreted BMP-2 gradually increased up to 72 hours. Cell viability was maintained above 90% throughout the observation period. In conclusion, the 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 mRNA may allow future application as a novel therapeutics platform for periodontal regeneration; however, further studies are required.

***Keywords*:** *Periodontal regeneration; mRNA; periodontitis; bone morphogenetic protein-2*

1. Introduction

Severe periodontitis is one of the major dental associated problems in adults and the elderly. The disease was ranked 6th in the most common chronic conditions in the world. An 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.

The primary treatment of periodontal disease comprises 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 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, periodontal tissues are prone to disease progression. The teeth are possibly mobile and disrupting normal occlusal function, speech, and personality that leads to alteration in the quality of life (Newman et al., 2011).

The ultimate goal of periodontal treatment is to regenerate periodontium that was 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. Guided tissue regeneration is based on the use of barrier membrane as an epithelium exclusion from the periodontal defects and helps to maintain 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 used 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 a year in order for the periodontium to regenerate. Currently, treatment outcomes by guided tissue regeneration and bone grafts still lack efficacy and effectiveness. There are still limitations that can be used only in specific types of periodontal defects. Until now, complete regeneration is not achievable and not highly predicted, and the cost of treatment is high (Avila-Ortiz et al., 2015; Kao et al., 2015).

mRNA technology is a new and highly innovative method that is safe and provides cost-effective benefits. Recently, the nucleoside-modified mRNA has emerged as a novel alternative in the non-viral gene therapy. One of the major drawbacks of mRNA in tissue regeneration is its ability to elicit innate immune response leading to an undesirable inflammatory reaction. Recently, modification of mRNA in the base region will provide the ability to evade toll-like receptors 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 the 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, will inhibit the innate immune response and enhancing protein production.

The method of mRNA delivery into target cells is greatly important for the effective production of desired proteins *in vitro* or *in vivo*. As mentioned before, mRNA can be degraded by nuclease found in most of the tissue; the need for an effective delivery system is crucial for preventing degradation and enhancing protein production. The encapsulation of mRNA increases the stability of cellular uptake and endosomal escape after entering the target cell. The most widely studied method of mRNA delivery into cells indicating good outcome is cationic lipid nanoparticles. In general, a cationic lipid which is positively charged will engage with the negatively charged mRNA forming lipid nanoparticles. These nanoparticles have been shown to successfully delivered mRNA into target cells *in vivo* (Guan & Rosenecker, 2017).

Bone morphogenetic protein (BMP) are multifunctional cytokines belonging to the TGF-β superfamily, which comprised approximately 50 genes. 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 BMP 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., 2012). Besides, bone morphogenetic protein-2 (BMP-2) also promotes the 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 condition (Vandana et al., 2016). An 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 (Wikesjo et al., 1999).

Considering that mRNA technology may be applicable in regenerating lost periodontal tissues, especially alveolar bone, our group aims to develop a new, highly innovative therapeutics platform of mRNA encoding BMP-2 that is highly efficient, safe, and cost-effective. Future development may lead to industrialization that allows public access to the treatment.

2. 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 (m1Ψ-BMP-2 mRNA) and test the biological function of produced BMP-2 as measured by cell proliferation ability.

3. Materials and Methods

3.1 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 was kindly provided by Dr. Norbert Pardi from the University of Pennsylvania (Pardi et al., 2017).

3.2 Cells isolation and culture

This study was approved by the Ethics 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 the 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 periapical granulation tissues. Then, PDL tissues were minced into a 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^oC and then filtered through a 70 µm cell strainer. The pass-through was then washed twice with culture medium. The PDLCs were cultured with a culture medium (Alpha MEM) at 37^oC in a humidified atmosphere of 5% CO_2 in the air. The culture medium was changed twice a week. After 80% confluent monolayer of cells was 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).

3.3 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 m1Ψ-BMP-2 mRNA complexed with Lipofectamine® 2000 (Invitrogen) according to the manufacturer's instructions. To analyze the BMP-2 protein expression and secretion levels, the transfected cells and control cells (Lipofectamine only) were cultured for 24-72 hrs. Supernatants and cells were harvested at 24, 48, and 72 hours 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).

3.4 Cell viability analysis

To analyze cell viability, PDLCs transfected with either m1Ψ-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^oC in a humidified atmosphere of 5% CO_2. After 1-4 hours, cell culture supernatants were measured at an absorbance of 570 nm using a microplate reader.

3.5 Cell proliferation ability

For the ability of secreted BMP-2 to enhance PDLCs proliferation, PDLCs were plated in 96 wells plate (3000 cells per well) and either control medium or supernatant of PDLCs that has been transfected with m1Ψ-BMP-2 mRNA complexed with Lipofectamine 2000. 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 an absorbance of 570 nm.

4. Results and Discussion

4.1 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. At each time-point of 24, 48, and 72 hours, supernatants and cell lysate were collected.

After 24 hours of transfection, high levels of BMP-2 were observed intracellularly in the experimental group, with the mean concentration of 22,188 picograms 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. As shown in Figure 1A, PDLCs from the experimental group produced a higher amount of BMP-2 than cells from the control group.

A high extracellular concentration of BMP-2 was observed in supernatants collected from the transfected cells. The BMP-2 concentration gradually increased from each time-point with the mean concentration of 12,285, 23,964, and 36,162 pg/ml, respectively. Whereas in control, the concentration was low and was hardly detected by an ELISA. As shown in Figure 1B, extracellular concentrations of BMP-2 were high and gradually increased up to 72 hours in the experimental group compared to the control group.

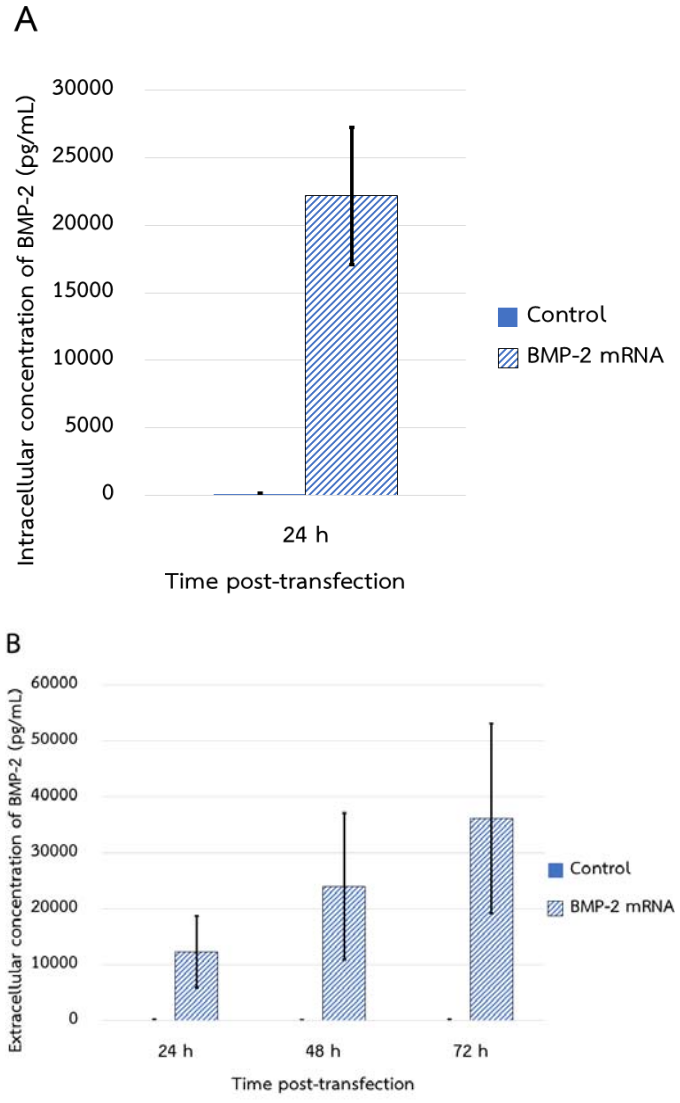

Figure 1 *In vitro* production of BMP-2 protein from PDLCs in experimental comparing with control groups after transfection with m1Ψ-BMP-2 mRNA. (A) Intracellular and (B) Extracellular concentrations after 24-72 hours of transfection. Data shown are mean ± SE (n=3).

4.2 Cell viability after transfection with m1Ψ-BMP-2 mRNA

At each time-point of observation, the AlamarBlue solution was added into the transfected cell culture. After four hours, the cell viability was analyzed using a microplate reader. As shown in Figure 2, m1Ψ-BMP-2 mRNA and Lipofectamine 2000 had modest effects on PDLC viability. The overall percentage of viability was maintained above 90% throughout the observational period.

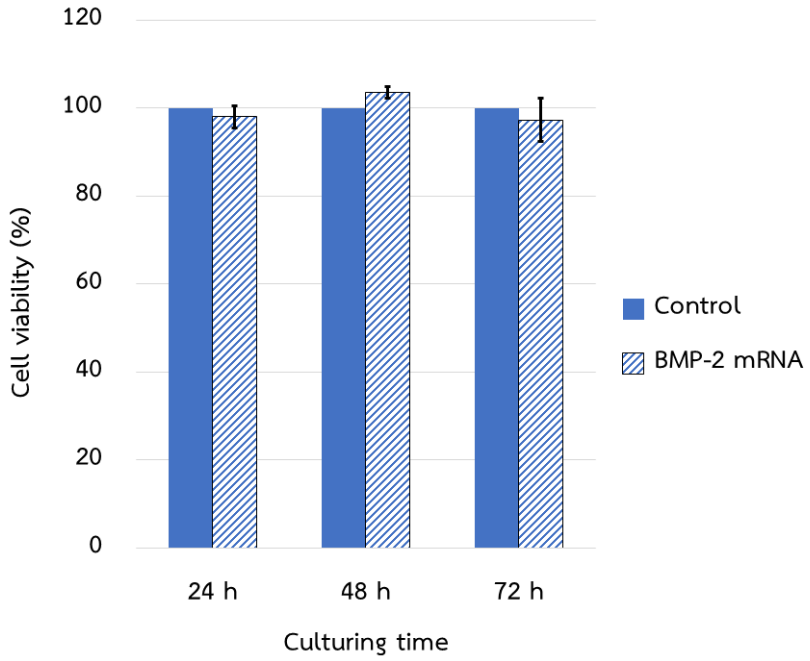

Figure 2 Cell viability after transfection with m1Ψ-BMP-2 mRNA. Mean and SE of cell viability of transfected PDLCs comparing between experimental and control group after 24-72 hours of transfection (n=3).

4.3 Biological activity of translated BMP-2 protein

Supernatants collected at 24 hours from the experimental group and control group were added to a fresh PDLC culture. After 48 hours of incubation, the media were removed, and a 10% alamarBlue solution was added. Cell proliferation ability was measured after four hours using a microplate reader. As shown in Figure 3, the supernatants from the experiment group were able to enhance cell proliferation but not markedly significant from control.

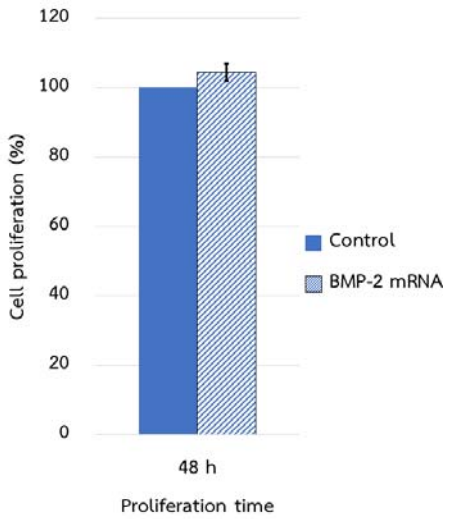

Figure 3 Biological function of BMP-2 protein translated from m1Ψ-BMP-2 mRNA *in vitro*. Effect of 24 hours supernatant from transfected cells on 48 hours of PDLCs proliferation compared with the control group. Data shown are mean ± SE (n=3).

This study, for the first time, demonstrated the transfection ability of PDLCs with m1Ψ-BMP-2 mRNA and Lipofectamine 2000 that leads to high levels of functional BMP-2 production without an effect on cell viability. Furthermore, these proteins were able to induce PDLC proliferation.

High intracellular and extracellular productions of BMP-2 were observed in this study. From the previous study, it was found that transfect rat muscle-derived stem cells with mRNA encoding BMP-2 demonstrated a much lower concentration of BMP-2 compared to our study (Zhang et al., 2019). The peak concentration was observed at 24 hours and gradually decreased, but in this study, the concentration increases gradually with the highest concentration at 72 hours. Also, protein production in the previous study was observed on day 6. When compared to gradual protein production in the present study, it may suggest a similar period of protein production.

Cell viability and enhancement of cell proliferation is an important factor for the demonstration of the safety and efficacy of the biomaterials, especially the one that will be used for tissue regeneration in human. AlamarBlue assay was used because of the non-toxic ability of the solution that permitted time-course experiments or post-measurement functional assay without interfering with the normal cell functions. In this study, the transfection of PDLCs with the nucleosides modified m1Ψ-BMP-2 mRNA and Lipofectamine 2000 had modest effects on the cell viability. Furthermore, the supernatants from the transfected cells can induce new PDLC proliferation. Similar results were observed in the previous study that transfected PDLCs with recombinant BMP-2 gene (Jian et al., 2017). Significantly higher proliferation ability was observed in the transfected cells compared to controls. Whereas, in our study, the proliferation rate in the experimental group was not significantly different from the control group, which may due to the differences in methods of transfection between DNA and mRNA and the differences evaluation method that has been used.

In the past, recombinant BMP-2 has been used mainly in the oral surgery field. Attempts were made for the application in periodontal regeneration, but there were some drawbacks. Animal studies demonstrated a high amount of bone regeneration in horizontal and furcal defects in beagle dogs (Ishikawa et al., 1994). However, root resorption and ankylosis in some areas of the root surface were encountered (Selvig et al., 2002; Wikesjo et al., 1999). These side effects may due to the application of a high concentration of BMP-2 proteins at once, leading to the extensive bone formation that exceeds the rate of cementum or PDL formation. On the contrary, mRNA technology allows the slow release of mRNA from the carrier that results in gradual protein production and release. Also, mRNA can be degraded over time, so protein production will not

prolonged. Furthermore, a combination of multiple mRNA encoding different type of proteins that promotes soft and hard tissues formation simultaneously may be used to reduce or prevent the unwanted side effects.

During 2007, the 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 proteins with high production costs that lead to the high treatment cost and limited access for the general population to receive treatment. In this case, the use of mRNA therapeutic platforms may allow access for patients with a reduction in cost but remain high in treatment efficacy.

The next step in the development of this mRNA therapeutic platform is 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 protein production could also be assessed. For the future application in humans, the 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 the past have shown that mRNA can elicit the innate immune response that resulted in undesirable inflammation and poor protein 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 the highest treatment efficacy coupled with maximum safeness.

5. Conclusion

This study demonstrated the ability of PDLCs after transfection with m1Ψ-BMP-2 mRNA to produce a high amount of functional proteins. The translated BMP-2 proteins were able to enhance PDL cell proliferation without affecting normal cell functions. Using the *in vitro* synthesized nucleoside-modified mRNA may allow future application as a novel therapeutics platform for periodontal regeneration; however, further studies are required.

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