Expression of Vascular Endothelial Growth Factor Protein in mRNA-Transfected Human Periodontal Ligament Cells

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Abstract

The complete regeneration of periodontal tissues following current periodontal therapy remains challenging and unpredictable. Nucleoside-modified messenger RNA (mRNA) technology can be a promising novel platform in regenerative medicine. This study aimed to evaluate whether mRNA encoding vascular endothelial growth factor (VEGF) could induce VEGF production in human periodontal ligament cells (PDLCs) and this translated protein function by promoting *in vivo* blood vessel formation using chorioallantoic membrane (CAM) assay. Isolated PDLCs from healthy periodontal tissue were transfected with pseudouridine modified mRNA encoding VEGF (VEGF mRNA) complexed with a transfecting agent, Lipofectamine 2000 (L2000) and L2000 alone (control). Supernatants collected at 24 hours (h) after transfection were evaluated for protein production by ELISA and cell viability by Alamar Blue assay. The supernatants of the VEGF mRNA-L2000, L2000 (control), and DPBS (negative control) were applied on filter papers, and individually placed these grafts onto the CAM surface through the window on day 8 of embryonic development (E8), and incubated for another three days. Angiogenesis assessment, counting numbers of blood vessels convergence to the grafts, was carried out by photographing with stereomicroscopic on E8 and E11. The result showed that PDLCs, transfected with mRNA encoding VEGF, produced a high level of VEGF protein than controls at 24 h. The transfection of mRNA encoding VEGF showed a negligible effect on PDLC viability. When supernatants were applied in CAM assay, translated protein VEGF protein was able to significantly induce blood vessel formation ($p < 0.001$). In conclusion, modified mRNA encoding VEGF promoted VEGF production and had angiogenic properties, increased blood vessel formation in the CAM. Thus, this mRNA platform technology may allow future application as a novel therapeutic platform for periodontal regeneration.

Keywords: *mRNA, vascular endothelial growth factor, angiogenesis, CAM assay, periodontal regeneration*

1. Introduction

In 2017, over 800 million global population (approximately 11%) were diagnosed with severe periodontitis (GBD Oral Disorders Collaborators, 2020). Periodontitis is a chronic inflammatory disease that results from the host's immune response to dental plaque. The disease destroys periodontium, a tooth-supporting structure including gingiva, periodontal ligaments, cementum, and alveolar bone. In severe periodontitis, such damaging inflammation could lead to tooth loss. Initial therapy for periodontal treatment consists of removal of dental plaque and calculus by scaling and root planing, allowing resolution of inflammation to occur. In the advanced form of periodontitis, common surgical procedures utilized for periodontal regeneration include guided tissue regeneration and bone grafting (Bowers et al., 1982; Nyman et al., 1982). However, these treatments provide unpredictable mixed clinical outcomes and are costly

(Avila- Ortiz et al., 2015; Kao et al., 2015). Therefore, novel periodontal treatments need to be developed to restore the damaged or lost tissues to their original form and function.

Current therapeutic strategies for periodontal regeneration are based on the key concept of tissue engineering using stem cells, scaffolds, and growth factors in the context of an adequate blood supply (Larsson et al., 2016). Growth factors are important tools to stimulate multipotent cells in periodontal tissues to proliferate and differentiate into the desired soft and hard tissues. Currently, only a few recombinant human growth factor proteins including platelet derived-growth factor-BB (GEM-21®), bone morphogenetic protein-2 (INFUSE®), and fibroblast growth factor-2 (Regroth®) are used as an adjunct to periodontal surgery in periodontal defects and dental implant ridge augmentation. Despite the attractive properties of these protein growth factors, several studies showed inconclusive clinical efficacy of recombinant growth factor application in periodontal regeneration (Donos et al., 2019). The half-life of growth factors *in vivo* is relatively short usually ranging from several hours to days (Rennel et al., 2008), therefore, supraphysiologic dose or several administrations are required (Zara et al., 2011). Such a high dose of growth factors may cause undesirable side effects and increase the cost of therapy.

The Gene therapy approach may provide better bioavailability of growth factors within the damaged tissue such as periodontal defects in periodontitis patients. This approach involves the delivery of nucleic acids, either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) encoding growth factors into the patient's cells and the cells become the growth factor protein factory. mRNA-based technology has an advantage over DNA-based technology since mRNA does not enter the nucleus (no risk of mutagenesis), and requires simple and uncomplicated methods (Warren et al., 2019; Youn et al., 2015). Until recently, the major limitations of using mRNA were its high immunogenicity (mRNA-medicated immune activation), low translatability, and limited stability (mRNA degradation by enzyme RNase) (Sahin et al., 2014). The landmark studies of Katalin Karikó and Drew Weissman demonstrated that nucleoside modification, replacing uridine with pseudouridine, in *in vitro*-transcribed (IVT) mRNA reduced immune activation and inflammation and simultaneously enhanced protein expression (Karikó et al., 2005; Karikó et al., 2008). Besides the important discovery of nucleoside-modified mRNA, the identification of lipid encapsulation as an efficient vehicle for IVT mRNA delivery (Pardi et al., 2015) has greatly advanced mRNA application in the field of medicine. Of note, the nucleoside-modified mRNA platform has proven to be a successful vaccine modality against COVID-19, demonstrating safety and high efficacy in humans (Baden et al., 2021; Polack et al., 2020). It is the first mRNA product approved by US FDA (U.S. Food and Drug Administration). The same fundamental technology platform could be applied to facilitate the development of mRNA-based regenerative therapy.

Pioneer work using mRNA encoding vascular endothelial growth factor (VEGF mRNA) demonstrated promising data on heart tissue regeneration in rat and swine models of myocardial infarction (Carlsson et al., 2018; Zangi et al., 2013). VEGF is an important angiogenic growth factor that induces new blood vessel formation, thus providing oxygen and nutrients to damaged tissue and facilitating wound healing and regeneration. At present, this VEGF mRNA (AZD8601, Moderna, and AstraZeneca) is being tested in human clinical Phase 2a trial (AstraZeneca, 2021), thus suggesting the therapeutic potential of VEGF mRNA for regenerative angiogenesis.

Our research team aims to develop nucleoside-modified mRNA encoding growth factors such as VEGF for periodontal regeneration. In this present study, we investigated protein expression after VEGF mRNA transfection in clinical relevance, human periodontal ligament cells (PDLCs), and analyze the biological activity and the angiogenic effect of the translated protein using a chorioallantoic membrane (CAM) assay.

2. Objectives

1)To evaluate VEGF protein production from the culture supernatant of pseudouridine-modified mRNA encoding VEGF-transfected PDLCs

2)To evaluate the angiogenic effect of translated VEGF protein using CAM assay

3. Materials and Methods

3.1 Preparation of nucleoside-modified mRNA encoding VEGF

The Nucleotide Sequence of modified mRNA encoding VEGF (VEGF mRNA) designed by Dr. Rangsini Mahanonda is as follows.

VEGF-A gene sequence:

ATGAACTTTCTGCTGTCTTGGGTGCATTGGAGCCTTGCCTTGCTGCTCTACCTCCACCA
TGCCAAGTGGTCCCAGGCTGCACCCATGGCAGAAGGAGGAGGGCAGAATCATCACGAAGTGG
TGAAGTTCATGGATGTCTATCAGCGCAGCTACTGCCATCCAATCGAGACCCTGGTGGACATCT
TCCAGGAGTACCCTGATGAGATCGAGTACATCTTCAAGCCATCCTGTGTGCCCCTGATGCGAT
GCGGGGGCTGCTGCAATGACGAGGGCCTGGAGTGTGTGCCCACTGAGGAGTCCAACATCACC
ATGCAGATTATGCGGATCAAACCTCACCAAGGCCAGCACATAGGAGAGATGAGCTTCCTACA
GCACAACAAATGTGAATGCAGACCAAAGAAAGATAGAGCAAGACAAGAAAATCCCTGTGGGC
CTTGCTCAGAGCGGAGAAAGCATTTGTTTGTACAAGATCCGCAGACGTGTAAATGTTCCTGCA
AAAACACAGACTCGCGTTGCAAGGCGAGGCAGCTTGAGTTAAACGAACGTACTTGCAGATGT
GACAAGCCGAGGCGGTGA

The sequence was sent to TriLink Biotechnologies (USA) for the construction of pseudouridine (Ψ)-modified mRNA encoding VEGF with cap1, DNase, and phosphatase treatment, and silica-membrane purification, which was packaged as a solution in 1 mM sodium citrate, pH 6.

3.2 Isolation and culture of periodontal ligament cells (PDLCs)

The study protocol was approved by the Ethical Committee (No.071/2020) and Institutional Biosafety Committee (No.0621.06/2935) from the Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand. All participants were provided written informed consent. PDLCs were harvested from extracted teeth due to orthodontic purposes or therapeutic reasons at the Faculty of Dentistry, Chulalongkorn University. PDLCs were extracted from the tooth by the enzyme-digestion method. The extracted teeth were washed twice with Dulbecco's Phosphate-Buffered Saline (DPBS) (Gibco®, Thermo Fisher Scientific, USA) and the PDL tissues were scraped out from the middle third of the root under a sterile condition. Care was exercised to avoid contamination from gingival or periapical granulation tissues. PDL tissues were minced into a fragment of 1-2 mm^2 and immediately placed into a solution of 2 mg/ml collagenase and dispase for 60 min at 37°C for digestion and then filtered through a 70-µm cell strainer. Subsequently, the pass-through was washed twice with the culture medium. Then, PDLCs were cultured with the medium (Dulbecco's Modified Eagle Medium (DMEM), Gibco®, Thermo Fisher Scientific, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco®, Thermo Fisher Scientific, USA) at 37°C in a humidified atmosphere of 5% CO_2 in the air. The medium was changed twice weekly. After a confluent monolayer of cells was reached, PDLCs were trypsinized, washed, and then sub-cultured to a new tissue culture flask. The cell from the 3rd to 8th passages of three different donors were used in this study (Iwata et al., 2010; Surisaeng et al., 2020).

3.3 In vitro PDLC transfection and production of VEGF protein

In this study, pseudouridine (Ψ)-modified mRNA encoding VEGF (VEGF mRNA) were complexed with Lipofectamine® 2000 (L2000, Invitrogen) (VEGF mRNA-L2000) according to the manufacturer's instructions. The PDLCs (100,000 cells/well, 24 well tissue culture plate) were transfected with VEGF mRNA-L2000 (1 µg VEGF mRNA, 1.5 µl L2000) or with L2000 only (1.5 µl) (L2000 control) in a final volume of 50 µl. Our previous kinetic study of green fluorescent protein (GFP) mRNA-L2000 showed that peak protein expression occurs at 24 hours (h) transfection in PDLCs (Ratreprasatsuk et al., 2019). Therefore, the culture supernatants in our experiment were collected after 24 h incubation, and VEGF protein production was measured by ELISA kit (Quantikine®, R&D System, Minneapolis, MN, USA).

ELISA procedure is briefly explained as follows. 200 µl of diluted culture supernatants (test/control) were incubated in an individual well of ELISA plate at room temperature for 2 hours and washed 5 times with washing solution. Subsequently, the detection antibody (200 µl) was added to each well, incubated at room temperature for 2 hours, and washed 5 times. Then, a substrate solution (200 µl) was added and incubated at room temperature for 30 minutes. When the color was developed, the stop solution was added, and analyzed by a microplate reader (at 450 nm).

3.4 Cell viability

Besides protein production, cell viability was assessed using Alamar Blue assay after 24-hour PDLC transfection. 10% Alamar Blue solution was added to the transfected cells (alamarBlue™, BIO-RAD, CA, USA), then incubate at 37°C in a humidified atmosphere of 5% CO_2 for 4 hours. After incubation, culture supernatants were measured at an absorbance of 570 nm using a microplate reader (Epoch™, Biotek™, VT, USA).

3.5 CAM assay

Chorioallantoic membrane (CAM) assay has been recognized as a well-established model for studying angiogenesis due to its simplicity, rapid development, and cost-effectiveness. The CAM is an extraembryonic membrane that is formed by the fusion of chorion and allantois which serves as a gas exchange surface supported by a dense capillary network. Its growth starts on day 3 of embryonic development (E3) as a small vesicle and it enlarges very rapidly from days 3-10 of development (Nowak-Sliwinska et al., 2014). The immune system of CAM is not fully developed and is naturally immunocompetent until day 18, allowing this model to be used for testing various test substances (Kundeková et al., 2021).

All animal procedures were conducted under the approval of the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Science, Chulalongkorn University, Thailand (approval no.2031021). In this study, fertilized White Leghorn chicken eggs were obtained from Kasetsart University, Thailand. They were sterilized with 75% ethanol and incubated in an incubator at 37.5° with 60% humidity for the duration of their development

After 3 days of incubation (E3), the eggs were windowed on the eggshell by first aspirating 3 ml of albumen from the blunt end by using a 25G syringe to prevent the shell membrane from adhesion. After removing the albumen, the eggshell was cut like a window (size 10 x 20 mm) by using small dissecting scissors. The window was sealed with transparent tape and returned the egg to the incubator. On day 8 of incubation (E8), filter papers (5 mm diameter, Whatman filter, Grade 41, 220 µm thick with pore size 20-25 µm) with substances of interest (test/control) were individually placed on to the CAM through the window and incubated for three days (Figure 1). The eggs were randomly divided into 3 groups (n = 5) as follows:

Group 1 (DPBS control, a negative control): Whatman filters with DPBS (10 µl)
Group 2 (L2000 control): Whatman filters with supernatant from L2000 control transfected PDLCs (10 µl)
Group 3 (mRNA): Whatman filters with supernatant from VEGF mRNA-L2000 transfected PDLCs (10 µl)

After placement of the filter papers with substances of interest, the window was again covered with transparent tape and returned to the incubator. The evaluation was carried out by photographing with stereomicroscopic on E8 and E11. On day 11 of incubation (E11), all the eggs were euthanized by rapid decapitation (Figure 1) (Ribatti et al., 2006).

3.6 Stereomicroscopic evaluation

After the implantation of the material, the angiogenic response can be evaluated by counting the total number of the microvessels (3-10 µm) that convergence toward the *grafts (filter plus substance of*

interest) under a stereomicroscope (Olympus Stereomicroscope (SZ61), Rozzano, Italy) at 10x magnification on E8 and E11 (Ribatti et al., 2006). Angiogenic response was characterized by an increased number of blood vessels. All the images were generated in Otsu grey threshold using ImageJ software (National Institutes Health, Bethesda, MD, USA). The counting was done by three independent observers in a blind manner (Ribatti et al., 2006)

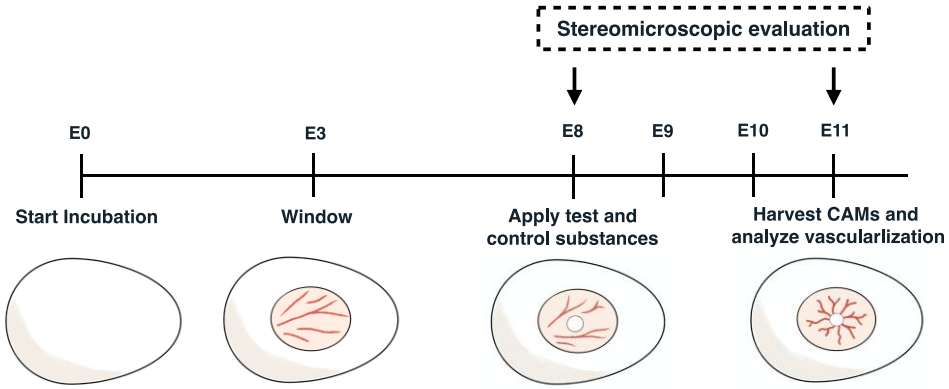

Figure 1 Diagram showing the timeline of the experiment in CAM assay. E — Embryonic day

4. Results and Discussion

4.1 Results

4.1.1 VEGF protein production in pseudouridine (Ψ)-modified mRNA encoding VEGF complexed with Lipofectamine 2000 transfected PDL cells

PDLCs were transfected with Ψ-modified mRNA encoding VEGF complexed with Lipofectamine 2000, while Lipofectamine alone was used as control. After collecting supernatants from the cultures at 24 h, quantification of secreted VEGF protein using ELISA revealed the mean concentration of VEGF in the mRNA group was 25,105 ± 1,326.84 pg/ml and 545.93 ± 44.49 pg/ml in L2000 control group (Figure 2). Levels of VEGF protein in the mRNA group were significantly higher than L2000 control group ($p < 0.001$, $n = 3$).

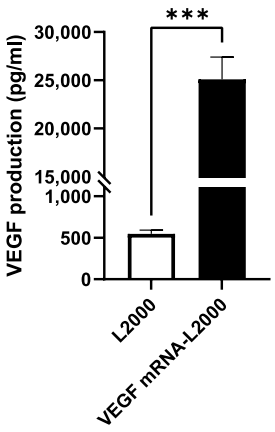

Figure 2 *In vitro* production of VEGF protein from PDLCs in VEGF mRNA-L2000 group vs L2000 control group. Data are shown as mean ± SE ($n = 3$). *** $p < 0.001$ indicates a significant difference between the mRNA and the control groups.

4.1.2 Cell viability following the VEGF mRNA transfection complexed with L2000

The cell viability after VEGF mRNA transfection in PDLCs was assessed after 24 hr incubation by Alamar Blue assay. It was found that cell viability greater of than 85 percent was observed amongst the mRNA and control groups (Figure 3).

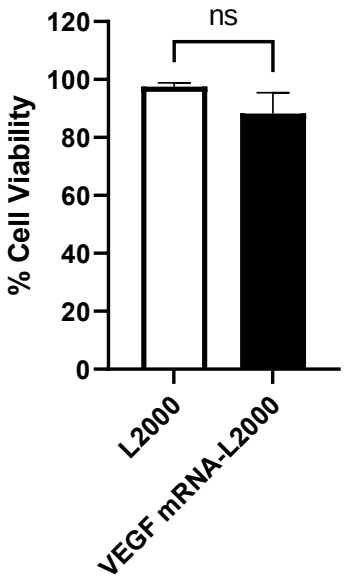

Figure 3 Cell viability after transfection with VEGF mRNA-L2000. Data are shown as mean ± SE of cell viability of transfected PDLCs compared between experimental and control groups after 24 h incubation (n = 3). NS = not significant (p = 0.094).

4.1.3 Effect of VEGF mRNA-L2000 transfected PDLCs upon angiogenesis in the chorioallantoic membrane assay

Following measuring the *in vitro* protein expression, the biological function of translated protein in the culture supernatants of the mRNA and L2000 control groups was tested *in vivo* for its ability to induce blood vessel formation using CAM assay. At E8, 10 µl of culture supernatant group (at a predetermined dilution of 1:40, unpublished data) was loaded onto Whatman filters placed on CAM. The numbers of blood vessels were counted at E8 and E11 under the stereomicroscope. Figure 4a shows a few microvessels in the membrane were detected on E8 in both groups (Figure 4a). The mean numbers of blood vessels were 21.8 ± 1.8 in the mRNA group, 25.73 ± 3.07 in the L2000 control group, and 20.33 ± 2.95 in the DPBS control group. On E11, more abundant microvascular networks as a spoked-wheel pattern were observed in the mRNA group than in the L2000 group while the negative controls did not show this pattern (Figure 4b). The numbers of blood vessels markedly increased to 50.27 ± 1.24 in mRNA group, 31.73 ± 1.65 in L2000 control group (p *<0.001)* and 30.33 ± 3.39 in DPBS control group (p *<0.01)* (n = 5) (Figure 4c).

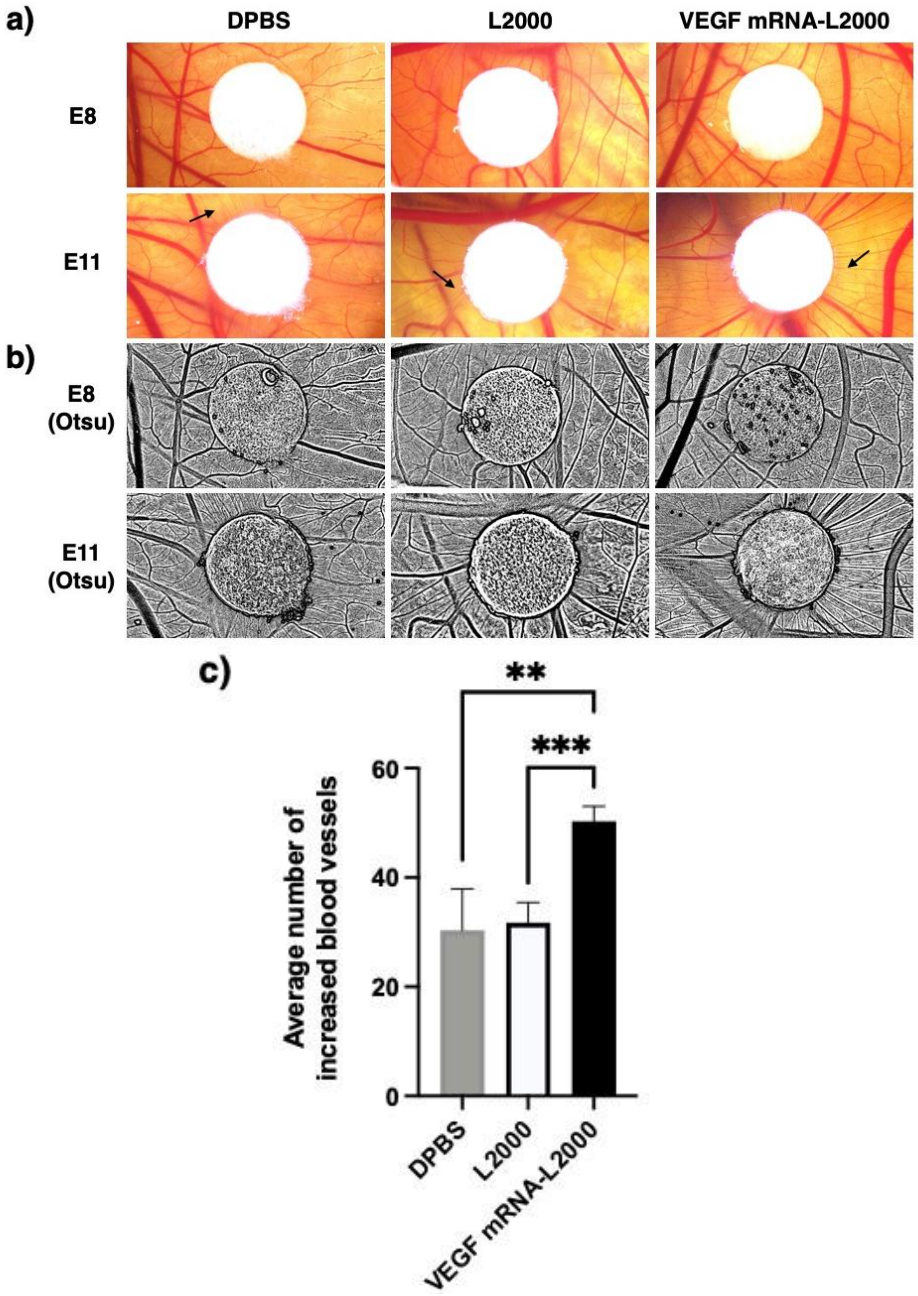

Figure 4 Chick embryo chorioallantoic membrane (CAM) assay. (a) A representative of stereomicroscope image of CAM at E8 and E11 after placing Whatman filters with DPBS (negative control), supernatant from L2000 control and supernatant from VEGF mRNA-L2000. On E11, more abundant microvascular networks as a spoked-wheel pattern (arrows) were observed in the mRNA group. (Original magnification, 10x) (b) Image analysis of CAM at E8 and E11 of individual control/test was generated by using the Otsu technique (c) The mean number of increased blood vessels from E8 to E11 in DPBS control, L2000 control, and mRNA groups. mRNA group significantly increased the number of blood vessels as compared to the controls. Data are shown as mean ± SE (n = 5). ** $p < 0.01$, *** $p < 0.001$ indicate a significant difference between the mRNA and the control groups.

4.2 Discussion

This study is the first study to explore the potential use of mRNA encoding VEGF in periodontal tissue regeneration. *In vitro* transfection of PDLCs with pseudouridine-modified mRNA in L2000 demonstrated high production of VEGF protein. This translated protein functioned, showing the ability to induce significant blood vessel formation using an *in vivo* CAM assay.

In this study, PDLCs were used due to their stemness potential that can differentiate into a variety of cells including osteoblasts and cementoblasts, important cells for periodontal regeneration (Seo et al., 2004). Our transfection results support previous data in our laboratory (the Excellent Center for Periodontal Disease and Dental Implant) that used other growth factor mRNAs. With similar culture conditions, all reported high protein expression after 24 h PDLC transfection with corresponding growth factor modified mRNA complexed with transfecting agent L2000. The mean VEGF protein production in our experiment was approximately 25,105 ± 1,326.84 pg/ml with 85% cell viability, while Surisaeng et al (2020) showed PDGF-BB protein expression of 26,815 ± 7,343.31 pg/ml and with 90% cell viability in PDGF-BB mRNA-transfected PDLCs and Kulthanaamondhita et al (2020) revealed BMP-2 protein expression of 12,285 ± 6,321.95 pg/ml with 90% cell viability in BMP-2 mRNA-transfected PDLCs (Kulthanaamondhita et al., 2020; Surisaeng et al., 2020). The mRNA technology is recognized as a simple plug-and-play technology that allows us to change the coding sequence of different genes of interest. It would be a simple but powerful tool to generate different growth factors to treat periodontal tissue regeneration.

Vascular endothelial growth factor (VEGF) is an important signaling protein involved in promoting the growth of new blood vessel formation that provides oxygen and nutrients to survive. Moreover, VEGF was shown to be the key factor coupling osteogenesis and angiogenesis since the inactivation of VEGF concomitantly subdued blood vessel invasion and bone formation (Clarkin et al., 2013; Gerber et al., 1999). In periodontal regeneration, angiogenesis plays an important role in the success of soft and hard regeneration. However, without proper vascularization, major problems could occur, leading to a low regeneration rate in tissue engineering. Therefore, VEGF could be a key factor for periodontal regeneration.

In vivo study, CAM assay was conducted to evaluate the biological activity, angiogenesis, of translated VEGF production in PDLC culture supernatants. The mRNA group shows a significant increase in the blood vessel network, with a radial arrangement of blood vessels directed toward the graft like a spoked-wheel pattern. We agree that this assay is relatively simple and rapid. Under a stereomicroscope, the highly vascularized with thin and transparent tissue CAM offers easy monitoring of the change in the vascular network through the window. Thus, CAM assay can provide important additional information and bridge the gap between *in vitro* cell culture and larger level preclinical animal models.

The mRNA technology platform has recently emerged in the field of medicine and the mRNA COVID-19 vaccine is the first product of its kind. There have been limited studies of mRNA-based therapeutics using CAM assay for mRNA product evaluation. In our study, the translated VEGF protein from mRNA transfected PDLCs showed the ability to induce significant numbers of blood vessels in CAM. Our results agreed with a previous study, where they used VEGF mRNA transfected in osteoblast-like MG-63 cells seeded on polycaprolactone scaffold and placed on CAM (Rumney et al., 2019). The combined mRNA technology with cell therapy and scaffold could lead to a markedly increased angiogenesis.

Overall, these data can be concluded that VEGF mRNA-L2000 has the angiogenic activity to promote blood vessel formation in the CAM. The next step in the development of this mRNA therapeutic platform is to further evaluate the ability of VEGF mRNA to promote soft and bone tissue regeneration in animal models.

5. Conclusion

In this study, we demonstrated that modified mRNA encoding VEGF can promote VEGF production after being transfected in PDLCs with negligible effect on cell viability. This translated protein had angiogenic property to promote the formation of blood vessels in the CAM. Thus, this mRNA could be beneficial in the treatment of periodontal regeneration.

6. Acknowledgements

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