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Plant-produced recombinant Osteopontin-Fc fusion protein enhanced osteogenesis

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ABSTRACT

Osteopontin (OPN) plays an important role in the bone regeneration process. Previous investigation showed that recombinant human OPN was able to express in *Nicotiana benthamiana* leaves and induced the osteogenic related genes. Nevertheless, the purification of OPN from plant proteins with Ni affinity chromatography was still not effective enough. To improve the quality of protein expression and purification in plants, we constructed an Fc-based form of OPN. The complete OPN protein was fused to the human IgG1 Fc domain. Here, we showed that the plant-produced OPN-Fc increases the protein expression level and facilitates the purification of the recombinant protein. Our result showed that the plant-produced OPN-Fc can stimulate the expression of osteogenic related genes such as DMP1, OSX, and Wnt3a and also the calcium deposition in hPDL cells. These findings suggest that the plant-produced OPN-Fc has potential application in tissue engineering in the future.

1. Introduction

Tissue engineering is the technology aiming to restore lost tissues and impaired organs. It composed of three important factors, which are cells, scaffolds, and signaling molecules [1–3]. Various proteins play important role in tissue engineering as signaling molecule such as structural proteins, growth factors, and cytokines [1,4]. However, there are few limitations that slow down the application of these proteins in tissue engineering such as cost, availability, stability, and batch-to-batch variability. These limitations have been overcome by the development of recombinant protein technology.

Among various recombinant protein production platform, plants have advantages over others such as low cost, production speed, post-translational modification, and lack of human pathogen. Plants have been used to produce vaccines and therapeutic proteins previously [5–7]. For tissue engineering application, plant-produced osteopontin protein was shown to induce osteogenic related genes [8]. However, the yield and purity of osteopontin produced in plants need to be improved. Novel approach to improve is using the Fc domain of immunoglobulin. Fc-fusion proteins have been investigated for their effectiveness with several successes [9–11].

In Fc-based fusion protein, the Fc-domain of immunoglobulin is fused with a protein of interest, which often increases its biological and pharmacological characteristics [12,13]. The fusion of the protein to Fc domain can increase its plasma half-life [14,15] because of the slow clearance due to higher molecular weight [16]. Besides, the Fc fusion proteins can be easily purified through a one-step protein A affinity chromatography. Furthermore, the safety of use of Fc in fusion proteins in human has been clearly established from the development of Fc-based protein drugs and therapeutic monoclonal antibodies previously [17–19].

For all these reasons, we believe that the Fc fusion platform is a promising approach to improve the expression, purification and function of plant-produced OPN protein. In this study, we transiently expressed recombinant OPN fused with the Fc domain of human IgG1 in *N. benthamiana*. We found that the fusion of Fc did not affect the biological function of OPN protein. However, the expression level and the purity of OPN-Fc fusion protein produced in plants were improved, comparing to the production of OPN alone. Out study suggested that the OPN-Fc

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fusion protein is a potential candidate for tissue engineering applications in the future.

2. Materials and methods

2.1. Construction of recombinant OPN fused with human IgG1 Fc

The sequence of OPN-Fc fusion gene was generated by overlap PCR. First amplification of OPN [8] and Fc [20] genes using specific primers NcoI-SP-OPN forward primer (5'-CCATGGAAC TTGGACTTTCTTGGATTTTCCTCTTGGCTATCCTTAAGGGTG-3') & linker-OPN reverse primer (5'-CCACCTCCAGAACCTCCACCTCCT-GAACCTCCACCTCCATTGACCTCA GAAGATGCAC-3') and linker-Fc forward primer (5'-GGAGGTGGAGGTTCAGG-3') & SacI-KD-Fc reverse primer (5'-GAGCTCTTAAAGCTCATCCTTCTCAGACTTGC-CAGG GGACAAAG-3'), respectively. The PCR products of OPN and Fc were mixed and amplified again using NcoI-SP-OPN forward primer and SacI-KD-Fc reverse primer. For Fc construct as negative control, Fc gene [20] was amplified using specific primer XhoI-ATG-L forward primer (5'-CTCGAGATGGGAGGTGGAGGTT-CAGGAG-3') and SacI-KD-Fc reverse primer. The PCR product of Fc and OPN-Fc fusion genes and were purified and cloned into pGem-T Easy (Promega, USA) for sequencing. The correct sequence was cut and inserted into geminiviral vector pBY030.2R with *Nco*I and *Sac*I site. The pBY-Fc and pBYOPN-Fc was transformed to *E. coli* strain DH10B by heat shock method. The plasmid DNA was confirmed by colony PCR using the gene specific primers and enzyme restriction. The plasmid was transformed into *Agrobacterium tumefaciens* stain GV3101 for expression in plant.

2.2. Expression and purification of OPN-Fc fusion protein

The OPN-Fc fusion protein was expressed by using pBYOPN-Fc co-infiltrating with p19 vector (Fig. 1). *A. tumefaciens* cells were infiltrated into 6–8 weeks-old *Nicotiana benthamiana* plants. Plants were incubated in growth chamber for 4 days after infiltration. Leaves were harvest for determination of protein expression and purification. For purification of OPN-Fc fusion protein, the infiltrated tobacco leaves were extracted with tris buffer (20 mM Tris-HCl pH 7.4, 50 mM NaCl). Crude extract was centrifuged at 26,000 *g* at 4 °C for 30 min. The supernatant was filtered with 0.45-micron filter and loaded into proteinA sepharose (GE Healthcare, Sweden) column. After washing with tris buffer, the purified protein was eluted with 0.1 M Glycine pH2.7 and neutralized with 1.5 M Tris-HCl pH8. The purified protein was analyzed by SDS-PAGE and Western blot. The Fc protein was expressed and purified using the same protocol as OPN-Fc fusion.

2.3. SDS-PAGE and Western blot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% acrylamide gels. Generally, gels were run for 1 h at 160 V. The proteins were visualized by Coomassie brilliant blue staining or electrophoretically transferred to nitrocellulose membrane (Biorad, USA). Membranes were blocked in 5% non-fat milk (dissolved in TBST buffer containing 25 mM Tris, 250 mM NaCl, 0.1% Tween-20, pH 7.4) for 1 h at room temperature. To detect OPN, the membrane was incubated with mouse anti-OPN antibody (Abcam, UK) diluted 1:5000 in 1% skim milk in TBST at 4 °C overnight and goat anti-mouse IgG-HRP conjugated (Promega, USA) diluted 1:20,000 in 1% skim milk in TBST for 1 h at room temperature. Moreover, OPN-Fc was confirmed using anti human IgG gamma chain. The membrane was probed with goat anti-human gamma-HRP conjugated (The Binding site, UK) diluted 1:5000 and incubated with membrane at 4 °C overnight. The membranes were developed by chemiluminescence using ECL plus detection reagent (GE Healthcare, UK).

2.4. Cell culture

Human periodontal ligament (hPDL) cells were obtained from healthy periodontal ligament tissue of non-carious, freshly extracted third molars removed for orthodontic reasons at Faculty of Dentistry, Chulalongkorn University. The protocol was approved by the Ethical Committee, Faculty of Dentistry, Chulalongkorn University and informed consent was obtained from each patient. Briefly, each tooth was rinsed with sterile PBS, pH 7.4 and hPDL was removed from the middle third of the root. The explants were harvested on 35-mm tissue culture dishes to allow cell migration in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 nM L-glutamine, 100 units/mL of penicillin, 100 μg/ml of streptomycin and 5 μg/ml of amphotericin B. Media and supplements were supplied from Gibco BRL (Thermo Fisher Scientific, USA). The cells were cultivated at 37 °C in a humidified atmosphere 5% CO_2. After cells were confluence, they were detached with 0.25% trypsin-EDTA and sub-cultured at ratio of 1:3 on 60-mm tissue culture

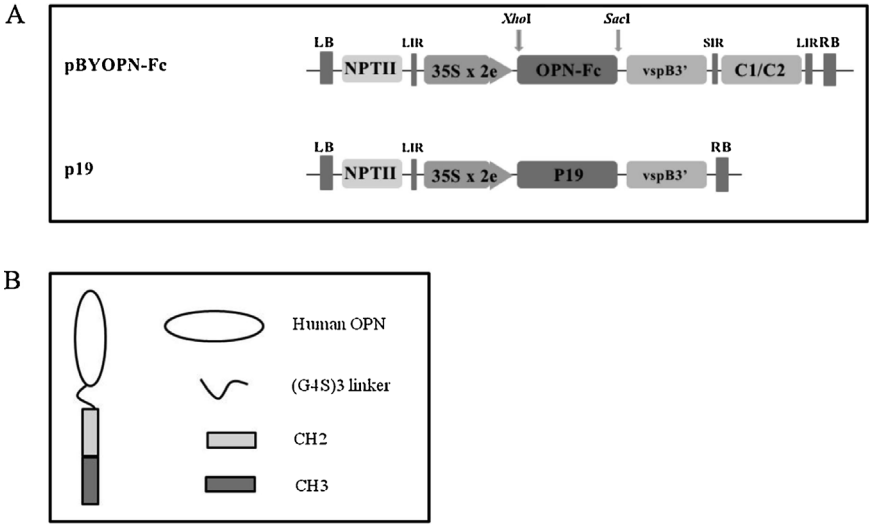

Fig. 1. Diagram of Geminiviral vector using to express OPN-Fc protein (A) and the diagram of OPN-Fc protein (B).

dishes, considering as passage one. The experiments were conducted using cells from passage 3–7. The cells from at least three different donors were utilized in experiments.

2.5. MTT assay

The tetrazolium-based colorimetric assay (MTT test) (USB Corporation, USA) is a tetrazolium compound which will be reduced to a formazan product by mitochondrial dehydrogenase and measures living cells and in *vitro*. Furthermore, MTT was used to confirm the *in vitro* proliferation assays because formazan product represents the metabolic activity of viable cells at a particular time point.

Cell proliferation can be indirectly determined by the changes in the amount of formazan. The hPDL cells were seeded in 24- well microtiter plate pre-coated by protein G and OPN-Fc, respectively at a density of 5×10^4 cells/cm^2/well for 24, 48, or 72 h. The cells were treated with 300 μl of 0.5 mg/ml MTT solution and incubated for 20 min at 37 °C in a humidified atmosphere 5% CO_2. After that, the MTT solution was aspirated. The wells were washed with PBS and 500 μl of Glycine:DMSO (1:9) was added to each well. After the formazan crystals had dissolved, measurements of the optical density of formazan solutions on the spectrophotometrically at 570 nm using a reference wavelength of 630 nm on an ELX800UV universal microplate reader (Bio-Tek Instruments Inc., USA). The experiments were done in triplicate.

2.6. Enzyme-linked immunosorbent assay (ELISA)

The protein concentration of plant-produced OPN-Fc fusion protein was determined using ELISA assay according to the manuals of Human Osteopontin (OPN) ELISA Kit (Sigma-Aldrich, USA). The final product was evaluated the absorbance at 450 nm using microplate reader (BioTek, ELx800, USA).

2.7. Preparation of OPN coated plate

The 24-well microtiter plate was pre-coated on the surface using 50 μg/ml Protein G (Invitrogen, Thermo Fisher Scientific, USA) 250 μl per well and incubated overnight at room temperature. The plate was blocked with 10 mg/ml BSA for 2 h at room temperature before washing with PBS 3 times. The plate was coated with 50, 100, 200 and 300 ng of plant-produced OPN-Fc fusion protein and incubated 2 h at room temperature. The hPDL cells were seeded in 24- well microtiter plate at a density of 8×10^4 cells per well. After 24 h treatment, RNA was extracted for real-time polymerase chain reaction (RT-PCR) analysis.

Table 1
Primer sequence for gene expression analysis using RT-PCR.

Gene	Primer sequences
GAPDH	F: 5' CAC TGC CAA CGT GTC AGT GGT G 3' R: 5' GTA GCC CAG GAT GCC CTT GAG 3'
qALP	F: 5' CGA GAT ACA AGC ACT CCC ACT TC 3' R: 5' CTG TTC AGC TCG TAC TGC ATG TC 3'
qBMP2	F: 5' GCG TGA AAA GAG AGA CTG C 3' R: 5' CCA TTG AAA GAG CGT CCA C 3'
qDMP1	F: 5' ATG CCT ATC ACA ACA AAC C 3' R: 5'CTC CTT TAT GTG ACA ACT GC 3'
qOPN	F: 5' AGG AGG AGG CAG AGC ACA 3' R: 5'CTG GTA TGG CAC AGG TGA TG 3'
qOSX	F: 5' GCC AGA AGC TGT GAA ACC TC 3' R: 5' GCT GCA AGC TCT CCA TAA CC 3'
qWNT3a	F: 5' CTG TTG GGC CAC AGT ATT CC 3' R: 5'GGG CAT GAT CTC CAC GTA GT 3'

2.8. Quantitative Real-time polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted from each experiment with TRIsol-RNA Lysis reagent (5Prime, Gaithersburg, USA) following to the manufacturer's instruction. RNA was quantified using nano-drop2000 spectromonometer (Thermo Fisher Scientific, USA). The RNA samples were converted to cDNA using a reverse transcriptase kit (Promega, USA) according to the manufacturer's instruction.

The gene expression was detected by RT-PCR using a Light cycler Nano real-time polymerase chain reaction machine (Roche Applied Science, USA) and Fast Start Essential DNA Green Master (Roche Applied Science). The PCR protocol was set as; denaturation at 94 °C for 10seconds, annealing at 60 °C for 10seconds, and extension at 72 °C for 10 s for 45 cycles. The reaction product of GAPDH was used as a reference gene for the internal control. The primer sequences were shown in Table 1.

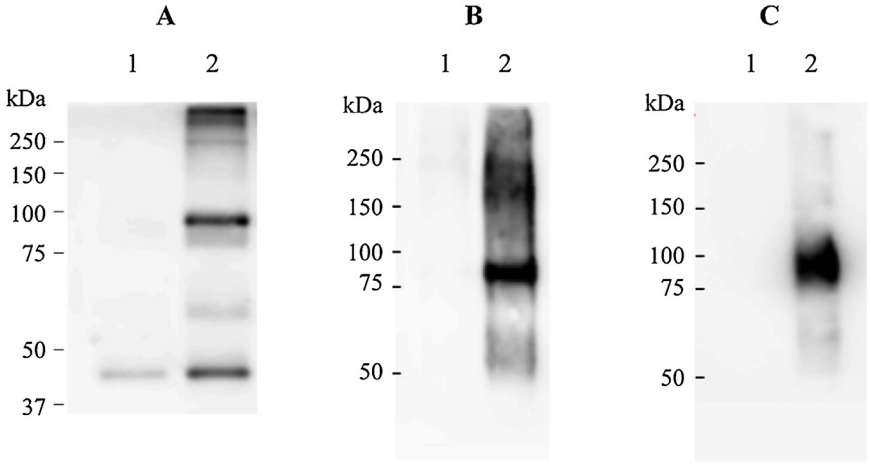

Fig. 2. The expression of OPN-Fc fusion protein in *Nicotiana benthamiana* increased comparing to OPN protein (A) and the purified protein contained both OPN (B) and human Fc (C) portions. Western blot to compare the expression level of OPN and OPN-Fc fusion proteins in crude extracts (A). Crude extracts from *N. benthamiana* leaves 4 days after agroinfiltrated with p19 and pBYOPN [8] (lane 1) or pBYOPN-Fc (lane 2) were loaded on the SDS-PAGE gel under reducing condition. Western blot was probed with mouse anti human OPN antiserum and detected with goat anti mouse IgG antiserum. Western blot to detect OPN (B) and Fc (C) portions in OPN-Fc fusion protein. Crude extract from *N. benthamiana* leaf 4 days after agroinfiltrated with p19 and pBYOPN-Fc was loaded on the SDS-PAGE gel under reducing condition. Western blot was probed with mouse anti human OPN antiserum (B) or anti human Fc antiserum (C) to detect the OPN and Fc portion in the OPN-Fc fusion protein, respectively. Lane 1: wildtype plant, Lane 2: plants infiltrated with pBYOPN-Fc and p19.

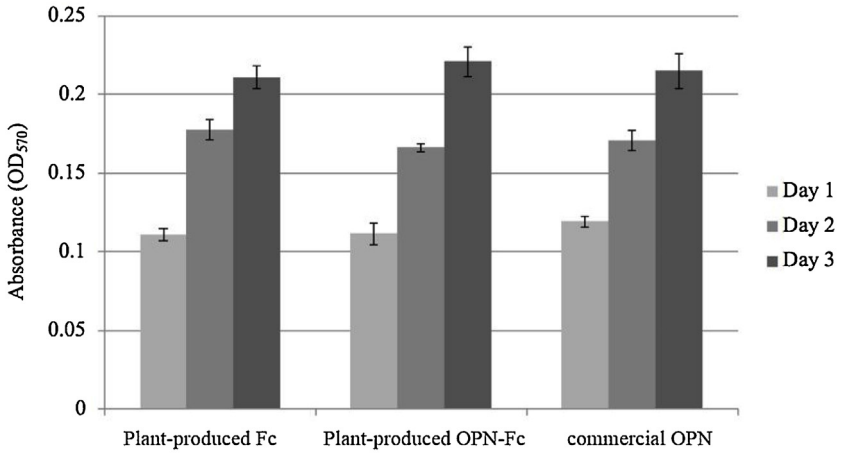

Fig. 3. The OPN-Fc fusion protein showed no effect on human periodontal ligament cells (PDL) proliferation. Three PDL cell lines established from three different donors were cultures on plates coated with 300 ng of OPN-Fc or Fc proteins. The experiments were done in triplicated. Cell survival was evaluated by MTT assay on day1, 2 and 3. Data represented the absorbance at 540 nm. Data are means of 3 independent replicate samples +/−SD. Control in this experiment is the untreated cells. (*$p \leq 0.05$).

2.9. Alizarin red S staining

The hPDL cells were cultured in general media (GM: 10% DMEM supplemented with 10% FBS, 1% Glutamax I supplement, 1% antibiotic-antimycotic) or osteogenic media (OM: 10% DMEM supplemented with 50 mg/ml ascorbic acid, 10 mM B-glycerophosphate, 100 nM Dexamthasone) for 21 days. Subsequently, the cells were fixed with cold methanol for 20 min. After washed with deionized water, the cells were incubated with 2% (W/V) Alizarin red S (Sigma-Aldrich, St. Louis, MO) staining solution for 5 min at room temperature. Each of the stain specimens was washed by deionized water and air-dried.

3. Results

3.1. Expression and Purification of Recombinant OPN-Fc fusion protein

Transient expression of OPN-Fc fusion protein was achieved with Geminiviral vector [21] designed to contain the OPN at the N-terminus. *Agrobacterium tumefaciens* contained the pBYOPN-Fc vector were co-infiltrated *A. tumefaciens* contained p19 vector (Fig. 1A) into *N. benthamiana* leaves. The OPN-Fc construct was generated, human OPN protein with the human IgG1 Fc domain at its C terminus. These two domains were separated by a $(Gly4Ser)_3$ linker to ensure correct protein folding and function (Fig. 1B).

Infiltrated leaves were harvested 4 days post-infiltration. The expression of OPN-Fc fusion protein was detected by Western blot using anti-OPN antibody. We compared the protein expression levels of OPN and OPN-Fc fusion protein. The result of Western blot using anti-OPN antibody confirmed that the expression level of OPN protein increased when the protein was fused to Fc (Fig. 2A). To confirm the expression of both OPN and Fc parts in the fusion protein, Western blot using both anti-OPN and anti-human IgG antiserum was performed. The protein A affinity purified OPN-Fc fusion protein was assessed by Coomassie-stained SDS-PAGE gel (data not shown) and by immunoblotting with anti-OPN antiserum (Fig. 2B) and anti-human IgG1 antiserum (Fig. 2C).

3.2. The Effect of plant-produced OPN-Fc fusion protein on hPDL cell proliferation

The OPN-Fc fusion protein was studied for the effect on hPDL cell proliferation, comparing to OPN protein. In this study, the hPDL cells were established from 3 different donors. The cells were

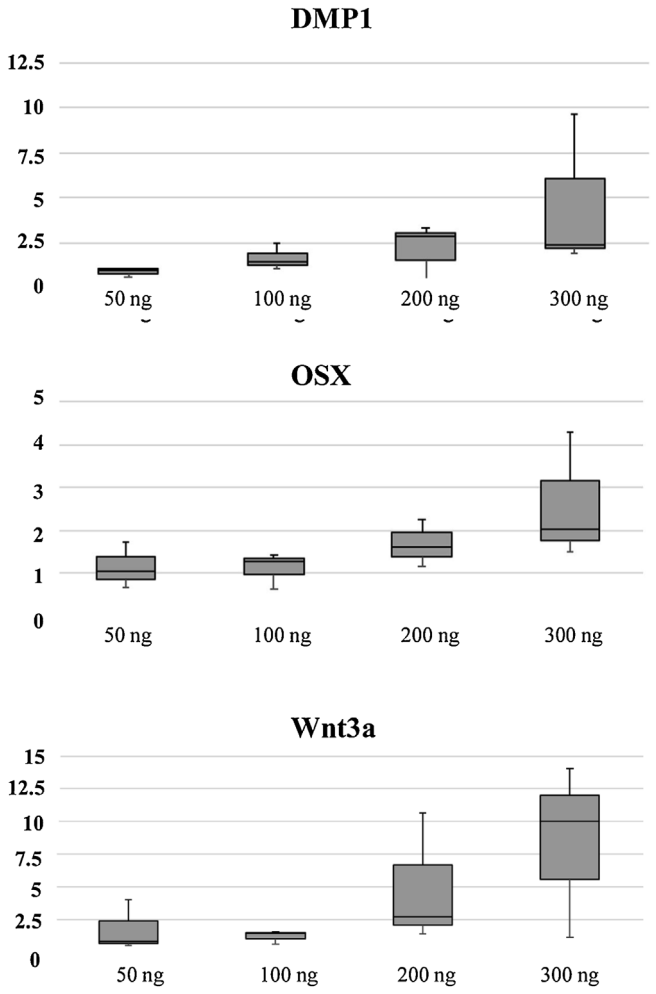

Fig. 4. Effect OPN-Fc fusion protein on the induction of osteogenic related genes in human periodontal ligament cells (PDL). The hPDL cells from three different donors were cultured on plates coated with 50 ng, 100 ng, 200 ng, and 300 ng of plant-produced OPN-Fc fusion protein hOPN for 24 h. Total RNA was extracted and real time PCR was performed using primer sets for human *OSX*, *DMP1*, and *Wnt3a* genes. Relative mRNA expression values were calculated normalized to the cells cultured on 300 ng of plant-produced Fc-coated plate. The values obtained for control cells cultured on non-coated plate were set at 1 for subsequent fold change calculation.

cultured on the plate coated with OPN-Fc coated. The cell number was determined by MTT assay after cultured for 1, 2 and 3 days. The results showed that the OPN-Fc fusion protein does not affect the proliferation of the hPDL cell, similar to the OPN protein and the plant-produced Fc negative control (Fig. 3).

3.3. The effect of plant-produced OPN-Fc fusion protein on the activation of osteogenic related genes

The hPDL cells were cultured on the plates coated with 50, 100, 200, and 300 ng of OPN-Fc fusion protein for 72 h. The mRNA was extracted and tested for the expression of *OSX*, *DMP1*, and *Wnt3a* genes by qRT-PCR. Relative mRNA expression values were calculated normalized to the cells cultured on plant-produced Fc-coated plate. Our results showed that 300 ng of OPN-Fc protein can induce the expression of *OSX*, *DMP1*, and *Wnt3a* genes (Fig. 4). The OPN-Fc induces these three genes in a dose-dependent manner.

3.4. The effect of plant-produced OPN-Fc fusion protein on the activation of calcification

The hPDL cells were grown in the general medium and osteogenic medium for 21 days. The hPDL cells were stained with Alizarin red at the end of the experiment. Fig. 5 showed that the hPDL cells cultured in general media (GM) was not red, but the cells were light red when they were cultured in osteogenic medium (OM). When the cells were cultured in OM and treated with the plant-produced Fc protein as the negative control (Fc), the cells were light red, similar to OM. The cells cultured in OM and treated with plant-produced OPN-Fc protein showed bright red, indicating the calcium deposit in these cells. The OPN-Fc protein modulate the calcification of hPDL cells in a dose-dependent manner (Fig. 5).

4. Discussion

The Fc fusion platform is the attractive technology for drug development. The advantages of the Fc fusion is to increase half-life of the proteins [10,22] and allow easier expression with protein A affinity purification [23]. Fc based fusion proteins are composed of Fc portion of antibody attached to the protein of interest through a linker peptide. Moreover, the Fc domain can improve the solubility and stability of the recombinant protein by independent protein folding [24,25].

In this study, a geminiviral-based transient expression system [26] was used to express recombinant OPN-Fc fusion protein in Agrobacterium infiltrated *N. benthamiana* leaves. Fusion of the Fc fragment of the human IgG1 antibody [20] and the ER retention signal KDEL to the C-terminus of Fc facilitated the expression and purification of the OPN protein. When compared the Fc fusion protein with the OPN protein on a Western blot (Fig. 2A), the expression level of OPN-Fc fusion protein was higher than OPN alone. In addition, the presence of both OPN and Fc portion was confirmed with Western blot using anti-OPN (Fig. 2B) and anti-human IgG (Fig. 2C) antiserum.

The OPN protein was expressed in plants and showed the ability to induce the osteogenic related genes in human periodontal stem cells [8]. However, the purification of recombinant protein in plants using Ni affinity chromatography was not efficient. There were plant contaminated protein in the purified OPN protein. In this study, protein A affinity chromatograph was used to purify the OPN-Fc fusion protein.

Previous studies showed that fusion of the protein antigen with the Fc increases the immunogenicity of the antigens [27–29]. This is the first study showing that this approach can use to improve the expression of therapeutic protein. The purified OPN-Fc fusion protein was tested on the human periodontal ligament cells and showed no significantly different on the proliferation compared to the commercial OPN protein and non-treated protein (Fig. 3). However, the OPN-Fc protein could induce the osteogenic-related genes such as DMP1, OSX, and Wnt3a (Fig. 4) and stimulated the calcium deposition in the hPDL cells (Fig. 5). The calcium deposition was induced by the plant-produced OPN-Fc protein in a dose-dependent manner. This suggested that the OPN-Fc could induce osteoblast differentiation in hPDL cells. In this study,

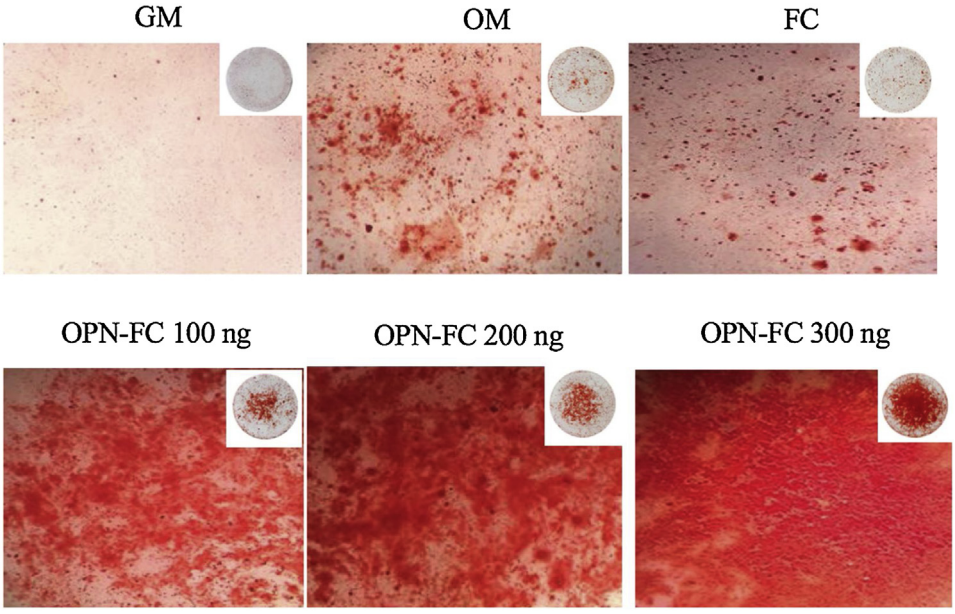

Fig. 5. Effect of OPN-Fc fusion protein on the induction of the calcium deposition in hPDL cells. Calcification was detected in the hPDL cells that seeding 50,000 cells/well and cultured in the osteogenic medium for 21 days. The hPDL colonies were stained with Alizarin red S solution at the end of the experiment. GM: Control cells growing in general medium, OM: Control cells growing in osteogenic medium, Fc: Cells treated with the plant-produced Fc protein in osteogenic medium, OPN-Fc 100 ng: Cells treated with 100 ng of plant-produced OPN-Fc protein in osteogenic medium, OPN-Fc 200 ng: Cells treated with 200 ng of plant-produced OPN-Fc protein in osteogenic medium, OPN-Fc 300ng: Cells treated with 300 ng of plant-produced OPN-Fc protein in osteogenic medium.

periodontal ligament stem cells were used as a model. Periodontal ligament stem cells possess the mesenchymal stem cells characteristics with the potential to differentiate into osteoblast-like cells and has been proposed to be the major cell types involved in periodontal regeneration [30].

Our studied showed that the Fc fusion platform can be applied to use with the recombinant protein in tissue engineering. The Fc fusion platform can increase the protein expression level and also improve the purification process. Moreover, it does not affect the function of the interested protein. This approach can be applied for other recombinant proteins that have difficulties in the expression level and purification process.

In summary, we report a strategy for the production of effective and inexpensive OPN protein from plants. In this study, the OPN-Fc fusion protein was expressed in *N. benthamiana* leaves using Geminiviral vectors. The OPN-Fc fusion protein facilitated the purification process and also increased the expression level. Our findings showed that the plant-produced OPN-Fc protein induced the osteogenic related genes and the calcium deposition in hPDL cells. These findings suggest that the plant-produced OPN-Fc has potential application in tissue engineering in the future. However, further studies are required to clarify the underlying mechanism.

Conflict of interest

The authors declare no conflicts and informed consent.

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