Article published online: 2020-12-09

Plant-Produced Basic Fibroblast Growth Factor (bFGF) Promotes Cell Proliferation and Collagen Production

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Key word

cell proliferation, collagen, Fibroblast Growth Factor, *Nicotiana benthamiana*, Plant-produced recombinant protein, *Solanaceae*, Transient expression

received 09.07.2020
revised 08.09.2020
accepted 10.10.2020

Bibliography

Planta Med Int Open 2020; 7: e150–e157
DOI 10.1055/a-1289-1265
ISSN 2509-9264

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Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany

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ABSTRACT

Human fibroblast growth factor regulates a broad spectrum of biological functions, including cell proliferation and tissue differentiation, and has a wider application in tissue engineering. Here, we described the production of human basic fibroblast growth factor in plants by using a geminiviral vector system. In this study, we transiently expressed basic fibroblast growth factor containing a C-terminus 8X-Histidine with and without a barley alpha amylase signal peptide in *Nicotiana benthamiana*. The expression level of basic fibroblast growth factor without the signal peptide was found to be higher than the basic fibroblast growth factor with the signal peptide. Further, the recombinant basic fibroblast growth factor was purified from the plant crude extract by two-step purification viz., ammonium sulfate precipitation and Ni-affinity chromatography. Our results demonstrated that the purified plant-produced basic fibroblast growth factor was biologically active and promotes the proliferation of human periodontal ligament stem cells and human follicle dermal papilla cells in vitro. Moreover, the plant-produced basic fibroblast growth factor also induced collagen production in human dermal fibroblast cells. Our results suggest the potential use of plant-produced basic fibroblast growth factor as an antiaging and hair growth-promoting agent in the cosmetic industry.

Introduction

The mechanical, protective, and restorative properties of skin decline with aging [1]. Extrinsic factors, such as ultraviolet rays, may cause tissue damage as implicated from antioxidant depletion with increased production of reactive oxygen species [2]. Recently, growth factors have been used in tissue engineering due to their major role in skin rejuvenation. Growth factors regulate cell growth, proliferation, and differentiation via signaling pathways [3, 4]. Growth factors such as epidermal growth factor [5, 6], vascular endothelial growth factor [7], and acidic fibroblast growth factor [8] are used in cosmetic products intended for skin rejuvenation. Among the several human growth factors, basic fibroblast growth factor (bFGF) is one of the most well-known ingredients in the cosmetic industry. bFGF is one of the members of the FGF family that has significant roles in cell proliferation, migration, differentiation, and survival, which are often implied applied to wound healing and tissue repair functions [9, 10]. Five isoforms of human bFGF exist with a molecular mass (18, 22, 22.5, 24, and 34 kDa). The lower molecular weight isoform 18 kDa is considered the main prototype of the FGF family [10]. In clinical interpretations, bFGF treatment can enhance the quality of burned skin, reduce hardness, and promote wound healing.

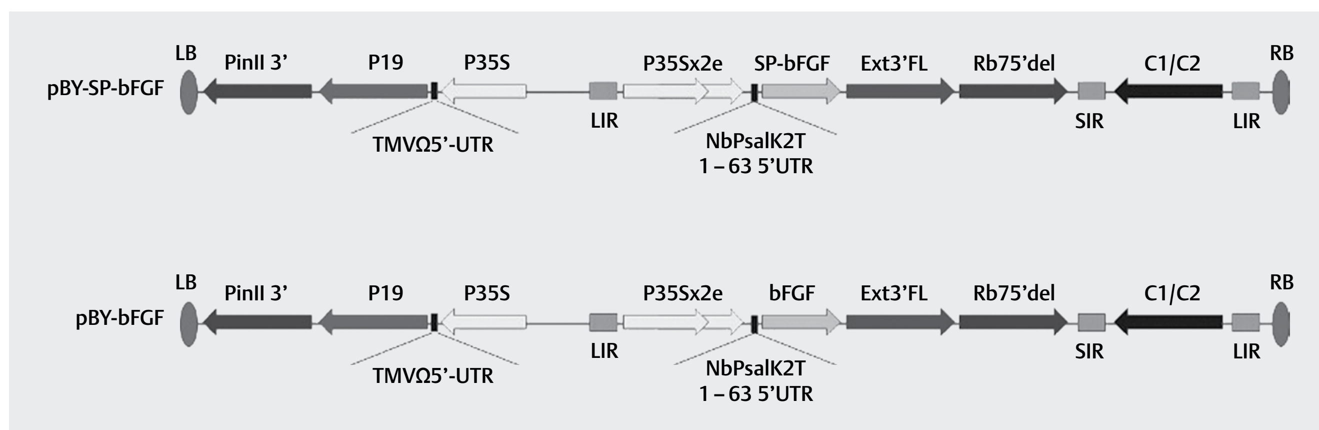
The recent advancement of cloning techniques has revolutionized molecular biology towards expressing commercially viable recombinant proteins in heterologous expression systems [11]. Recombinant bFGF is produced in a wide range of cell-based platforms, including bacteria [12, 13], yeast [14], insects [15], and plants [16, 17]. More recently, the plant expression system has gained increasing interest for the production of various recombinant and therapeutic proteins. Studies showed that plant-produced proteins show functional activity and efficacy both in *vitro* and *in vivo* [18–23]. Plant-based transient expression is one of the effective methods for recombinant protein production in a short time. It offers unique advantages over other conventional systems, especially cost-effective processing costs, production speed, scale-up capability, post-translational modification, and free from human pathogen contamination [24].

Plant-produced proteins can be purified by multiple techniques, such as precipitation, ion-exchange chromatography, and affinity chromatography. In particular, affinity chromatography is extensively used. It can purify proteins based on the interaction of ligands with a specific protein, for example, polyhistidine, HA-tagged, Fc domain of immunoglobulin, etc. The polyhistidine tag, which can vary from 6 to 10 histidine residues, is the most common protein tagging sequence utilized for chromatographic purification [8, 22, 25, 26].

In this study, we compare the expression of bFGF with and without the barley alpha amylase signal peptide in a plant-based expression system using transient expression. We utilized the geminiviral replicon system derived from the bean yellow dwarf virus [27, 28] for rapid production of human bFGF in *N. benthamiana*. The 8X-Histidine tag was added to bFGF at the C-terminus to facilitate purification by Ni-affinity chromatography. The plant-produced bFGF was further purified and characterized. Our results showed that the expression level of plant-produced bFGF without the barley alpha amylase signal peptide is higher than that of the plant-produced bFGF with a signal peptide. In addition, plant-produced bFGF can induce the proliferation of human periodontal ligament cells and human follicle dermal papilla cells, and also promote the collagen production in human dermal fibroblast cells.

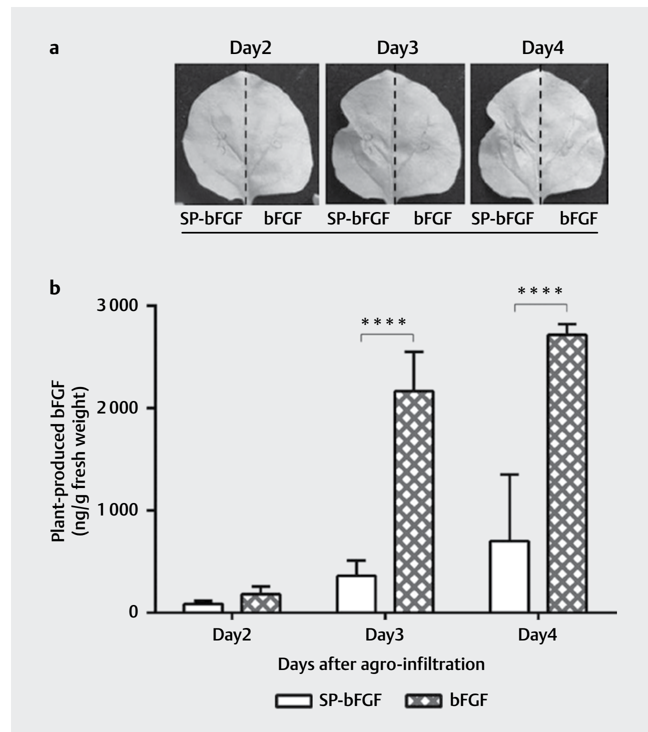
Results and Discussion

Human bFGF was transiently expressed in tobacco plants. To evaluate the effect of the signal peptide on expression efficiency, the signal peptide from barley alpha amylase was utilized for the present study. For the protein purification, 8X-Histidine tag was fused at the C-terminus. The bFGF was produced by transient expression in *N. benthamiana* leaves by using pBY-SP-bFGF and pBY-bFGF expression cassettes (▶ **Fig. 1**). Leaf necrosis was observed after 3 days post-infiltration for pBY-SP-bFGF construct whereas necrosis was observed after 4 days for the pBY-bFGF construct (▶ **Fig. 2a**).



▶ **Fig. 1** Schematic diagram of the geminiviral vector containing the hFGF gene. LB and RB: the left and right borders of the agrobacterium T-DNA region; P35S: cauliflower mosaic virus (CaMV) 35S promoter; TEV 5′: tobacco etch virus 5′ UTR; P19: P19 gene from the tomato bushy stunt virus (TBSV); Pin II 3’: 3’ of the proteinase inhibitor II gene; LIR: long intergenic region of the BeYDV genome; NbP 5’: 5’ of the nicotiana photosystem I reaction center subunit psaK; Ext3’FL: 3’ of the nicotiana extension gene; Rb7 5’: 5’ of the Rb7 matrix attachment region/scaffold attachment region; SIR: short intergenic region of the BeYDV genome; C1: bean yellow dwarf virus (BeYDV) ORFs C1 and C2, which encode for the replication initiation protein (Rep) and RepA; SP-bFGF: human fibroblast growth factor gene with 8X-Histidine residues at C-terminus and signal peptide at N-terminus; bFGF: human fibroblast growth factor gene with 8X-Histidine residues at C-terminus.

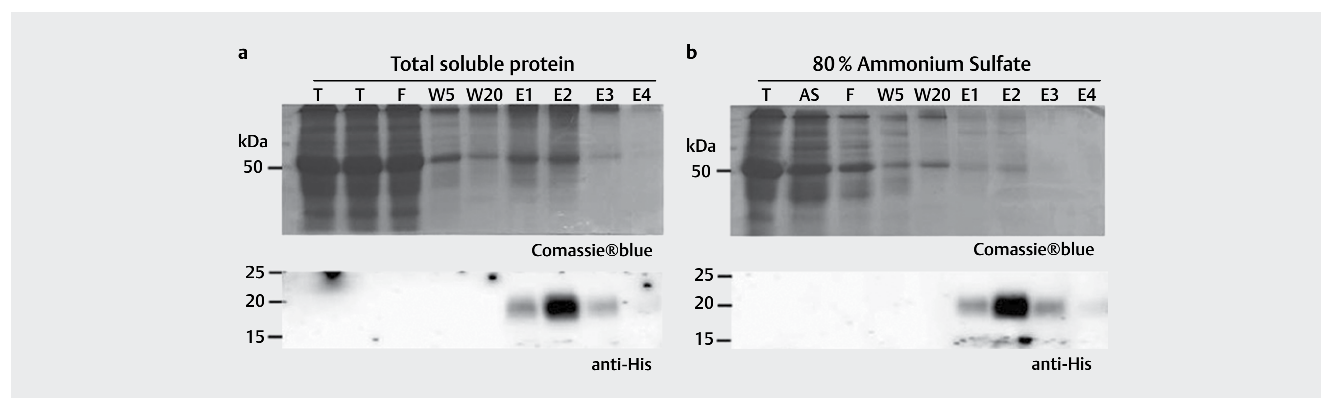
The results showed that both constructs have a similar protein expression level on day 2 after agroinfiltration (▶ **Fig. 2b**). For pBY-bFGF, the protein expression level was significantly increased on day 3 and day 4 post-infiltration, accumulating protein levels up to 2.16 and 2.72 µg/g fresh weight, respectively (▶ **Fig. 2b**). The expression level of bFGF produced from transient expression in the plant was lower compared to other expression systems [12–16]. This phenomenon might be due to the rapid necrosis exhibited by infiltrated leaves. Relatively, tissue necrosis is postulated to affect protein expression and stability [29, 30]. In comparison between the two constructs, the expression level of bFGF from the pBY-bFGF cassette was approximately 5-fold higher than the pBY-SP-bFGF construct. The difference in the expression level of bFGF with and without the signal peptide varied depending on the stability of the protein in different locations. Based on the results, the pBY-bFGF gene construct was used for further experiments to produce bFGF.



▶ **Fig. 2** Comparison of bFGF expression using pBY-SP-bFGF and pBY-bFGF vectors. Typical phenotype of *N. benthamiana* leaves expressing recombinant bFGF using pBY-SP-bFGF (left) and pBY-bFGF (right) on days 2, 3, and 4 after infiltration **a**. *N. benthamiana* leaves were harvested and bFGF protein expression was determined by ELISA **b**. Data represent means ± SD, $^{****}p < 0.0001$.

To establish a simple and efficient purification protocol for plant-produced bFGF, two-step purification by ammonium sulfate precipitation and Ni-affinity chromatography were carried out. One-step purification with Ni-affinity chromatography showed that the purified protein contained some of the plant proteins, especially rubisco, which is the most abundant protein in plants (▶ **Fig. 3a**). However, preceding purification by ammonium sulfate precipitation, specifically after the addition of 35 % ammonium sulfate salt, showed the removal of more than 50 % of rubisco (▶ **Fig. 3b**). Previous studies have also shown that 35 % ammonium sulfate precipitation can effectively remove most of the rubisco protein [31]. The bFGF was precipitated at 80 % ammonium sulfate and subsequently purified by Ni-affinity column chromatography (▶ **Fig. 3b**). With this two-step purification, the purity of the plant-produced bFGF was enhanced.

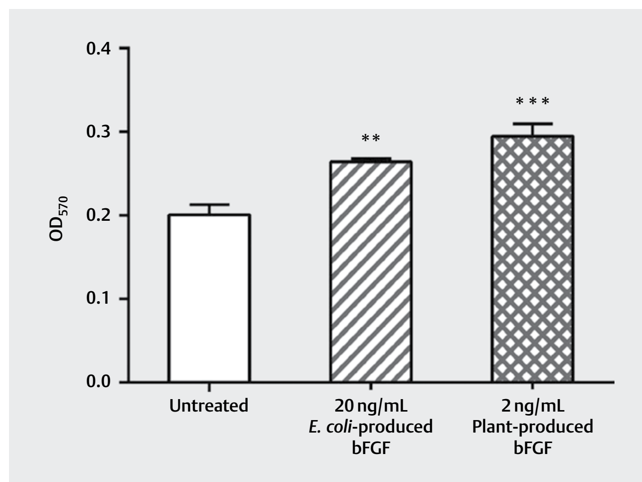
Human periodontal ligament stem cells (hPDLSCs) play a key role in tissue engineering. Previous studies demonstrated that bFGF is necessary for hPDLSCs proliferation [32]. To investigate the effect of plant-produced bFGF protein in the proliferation of hPDLSCs, the cells obtained from three different donors were treated with 2 ng/mL of plant-produced bFGF and 20 ng/mL of *E. coli*-produced bFGF for 3 days prior to the cell viability measurement. Our results showed that plant-produced bFGF significantly induced the proliferation of hPDLSCs compared to untreated cells (▶ **Fig. 4**). Both plant-produced and *E. coli*-produced bFGF can activate the prolif-



▶ **Fig. 3** Purification of bFGF from the plant crude extract by ammonium sulfate precipitation. Total soluble protein **a** or plant protein precipitated at 35-80 % ammonium sulfate saturation **b**. Plant-produced bFGF was purified using Ni-NTA affinity chromatography. The purified protein was separated by SDS-PAGE and stained with Coomassie blue stain; Western blot with anti-histidine antibody conjugated with HRP. T: total soluble protein from the plant crude extract; AS: plant proteins precipitated at 35-80 % ammonium sulfate saturation; F: flow though; Wash 5 (W5): fraction collected during washing with buffer containing 5 mM imidazole; Wash 20 (W20): fraction collected during washing with buffer containing 20 mM imidazole; E1: elution fraction 1; E2: elution fraction 2; E3: elution fraction 3; E4: elution fraction 4.

eration of hPDLSCs, but the dose of plant-produced bFGF was 10 times lower (▶ **Fig. 4**). Our results confirmed that the plant-produced bFGF can be used for the maintenance of stemness in hPDLSCs.

A previous study showed that FGFs, including bFGF, can promote hair growth in mice [33]. Dermal papilla cells are one of the regulators of the hair cycle [34]. To investigate the effect of recombinant bFGF in the proliferation of human follicle dermal papilla cells (hFDPCs), the cells were treated with 50, 100, and 200 ng/mL of plant-produced bFGF or *E. coli*-produced bFGF for 24 h. Our results showed that both plant-produced and *E. coli*-produced bFGF significantly induced the proliferation of hFDPCs compared to untreated cells (▶ **Fig. 5**), suggesting that plant-produced bFGF might be a good candidate for activating the hair cycle and hair growth.



▶ **Fig. 4** The effect of plant-produced bFGF on human periodontal ligament stem cell (hPDLSC) proliferation evaluated by the MTT assay. Cells were treated with 2 ng/mL of plant-produced bFGF, and 20 ng/mL of *E. coli* produced bFGF for 3 days prior to cell viability measurement. Data represent means ± SD; $^{**}p < 0.01$ and $^{***}p < 0.001$.

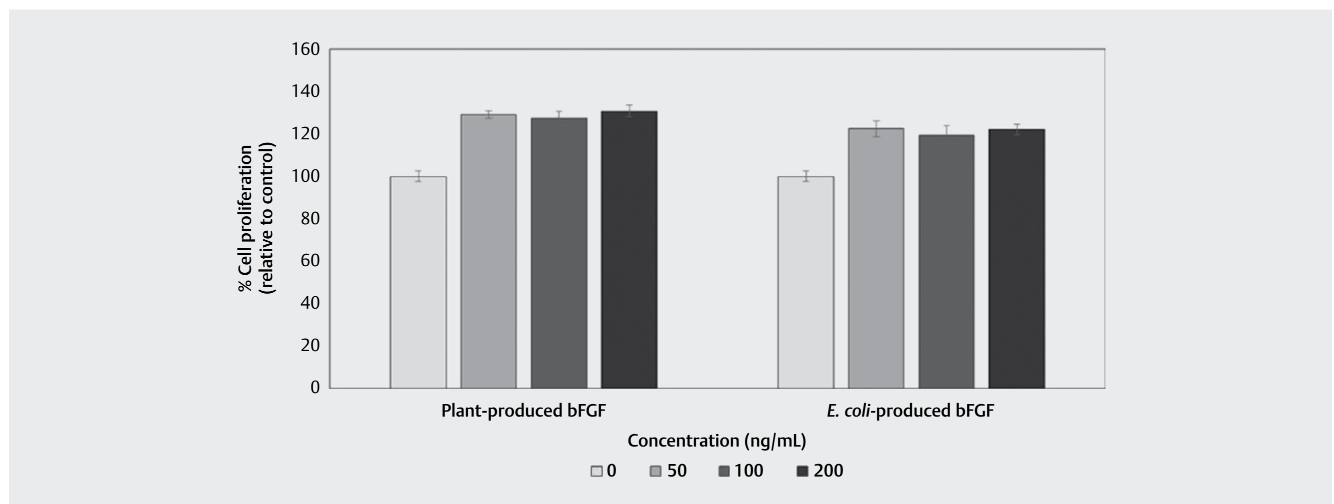
Reduction of collagen stimulates the manifestation of wrinkles in skin [35]. Therefore, the induction of collagen production is essential for antiaging products. bFGF has a role on collagen-related gene expression [35–37]. To investigate collagen induction in primary dermal fibroblast cells (hDFBCs), the cells were treated with 25 ng/mL of plant-produced bFGF and vitamin C (VitC, control) for 6 days. The results showed that both plant-produced FGF and vitamin C induce cell proliferation compared to untreated cells (▶ **Fig. 6a**). The collagen in the cells was stained with direct red 80 in picric acid. Our results showed that the hDFBCs treated with plant-produced bFGF produced more collagen than the untreated cells. The percentage of total stained collagen confirmed that the plant-produced bFGF significantly induced collagen production in hDFBCs (▶ **Fig. 6b**, **c**). This result suggests that plant-produced bFGF might be an effective ingredient in antiaging skin care, supposing its induction capacity for collagen production in hDFBCs.

Overall, the present study demonstrates that recombinant bFGF produced transiently in *N. benthamiana* leaves promotes the proliferation in hPDLSCs and hFDPCs, even in a minimal concentration, compared with *E. coli*-produced bFGF. Interestingly, it also stimulates collagen production in hDFBCs. Our results strongly support the promising potential of plant-produced bFGF as an antiaging and anti-hair loss agent in the cosmetic industry.

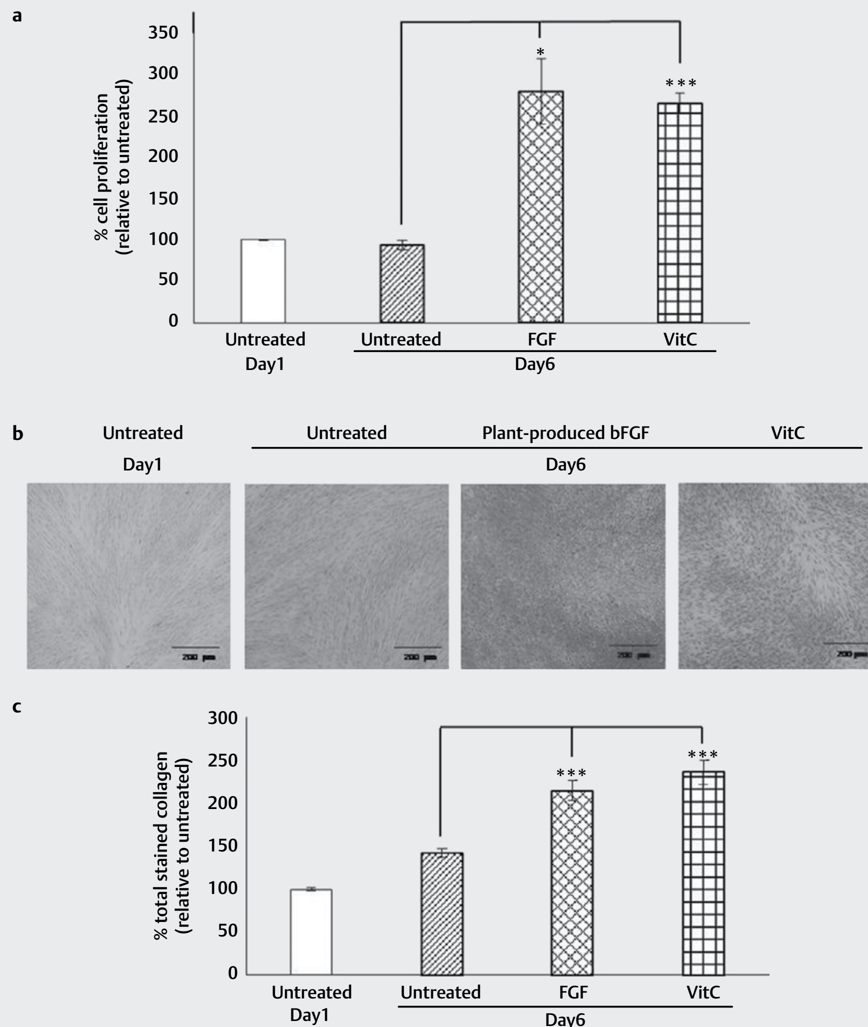
Materials and Methods

Vector construction

The nucleotide sequence of bFGF (Genbank Accession No: AAQ73204.1) linked with 8X-Histidine at C-terminus was plant codon optimized and synthesized by GeneArt gene synthesis (Thermo Scientific). The gene was cloned in two constructs either with or without barley alpha amylase signal peptide at N-terminus, SP-bFGF, and bFGF, respectively. The genes were cloned in a geminiviral



▶ **Fig. 5** The effect of plant-produced bFGF on human follicle dermal papilla cell (hFDPCs) proliferation was evaluated by the Cell Titer-Glo ATP assay. The cells were treated with 50, 100, and 200 ng/mL of plant-produced bFGF and *E. coli*-produced bFGF for 24 h. Cell proliferation was tested by using Cell Titer-Glo reagent. Data represent means ± SD; $^{**}p < 0.05$.



▶ **Fig. 6** The effect of plant-produced bFGF on collagen production in human dermal fibroblast cells (hDFBCs). Cells were treated with 25 µg/mL of plant-produced FGF and VitC for 6 days. Cell proliferation **a**, picrosirius red staining images of human dermal fibroblasts showing collagen **b**, and total collagen production **c**. Data represent means ± SEM; $^{*}p < 0.05$ and $^{***}p < 0.005$.

vector (pBY2eK2Md) with XbaI and SacI restriction sites (▶ **Fig. 1**). *Agrobacterium tumefaciens* strain GV3101 was transformed with plasmid pBY-SP-bFGF and pBY-bFGF for transient expression in *N. benthamiana*.

Recombinant protein production in Nicotiana benthamiana

To compare the expression level of both constructs, *A. tumefaciens* cells containing either pBY-SP-bFGF or pBY-bFGF were infiltrated with OD600 0.2 in *N. benthamiana* leaves. The infiltrated leaves were harvested on days 2, 3, and 4 post-infiltration. The leaves were gound, extracted with extraction buffer (5 mM imidazole, 20 mM Tris-HCl pH 7.4, 50 mM NaCl), and centrifuged at 26 000 *g* at 4 °C for 15 min. The supernatant was quantified for the amount of bFGF using the human FGF basic ELISA Kit (R&D System). Experiments were conducted in triplicate and all the data are presented as the mean ± SD.

Purification of plant-produced basic fibroblast growth factor

For large scale protein expression, the *Agrobacterium* strain harboring bFGF (OD600 0.2) was vacuum infiltrated into 6- to 8-week old *N. benthamiana* plants. Infiltrated plants were kept under a 16-h light/8-h dark cycle at 28 °C for 3 days. Next, the leaves were harvested (400 g) and homogenized using a blender with the extraction buffer. The crude extract was centrifuged at 26 000 *g* at 4 °C for 45 min. The supernatant was purified by ammonium sulfate precipitation and Ni-affinity chromatography. For ammonium sulfate precipitation, ammonium sulfate salt was initially added into the supernatant until 35 % saturation and centrifuged at 26 000 *g* at 4 °C for 10 min. Then, ammonium sulfate salt was further added into the 35 % saturated supernatant until 80 % saturation and centrifuged at 26 000 *g* at 4 °C for 10 min. The 35 and 80 % resulting pellets were resuspended with extraction buffer for Ni-NTA column

purification (35 % AS and 80 % AS). All solutions were filtered with a 0.4-µm filter and loaded into a Ni-affinity column. After washing with 10 CV washing buffer 5 (Wash 5; 5 mM imidazole, 20 mM Tris-HCl pH 7.4, 50 mM NaCl) and 10 CV washing buffer 20 (Wash 20; 20 mM imidazole, 20 mM Tris-HCl pH 7.4, 50 mM NaCl), the purified protein was eluted with elution buffer (250 mM imidazole, 20 mM Tris-HCl pH 7.4, 50 mM NaCl) and analyzed by SDS-PAGE and Western blot probed with HRP-conjugated rabbit polyclonal anti-6x His tag antibody (Abcam). The concentration of plant-produced bFGF was determined by ELISA.

SDS-PAGE and Western blot

The protein samples were mixed with loading buffer [125 mM Tris-HCl pH 6.8, 12 % SDS, 10 % (v/v) glycerol, 22 % (v/v) *β*-mercaptoethanol, 0.001 % (w/v) bromophenol blue] and denatured for 5 min. The denatured protein was separated on 15 % SDS-PAGE and stained with Coomassie brilliant blue R-250 (AppliChem). For Western blot analysis, separated proteins were transferred to a nitrocellulose membrane (Bio-Rad) and probed with HRP-conjugated rabbit polyclonal anti-6x His tag (Abcam). The membranes were developed by chemiluminescence using ECL plus detection reagent (GE Healthcare).

Culturing of human periodontal ligament stem cells and cell proliferation assay

hPDLSCs were isolated according to a previous study [38]. The protocol was approved by the Ethical Committee (Study code: HREC-DCU 2018-054; May 31, 2018), Faculty of Dentistry, Chulalongkorn University and informed consent was obtained from each donor. The hPDLSCs were removed from the middle one-third of the root. The explants were cultured in DMEM containing 10 % FBS, 2 nM L-glutamine, 100 units/mL of penicillin, 100 mg/mL of streptomycin, and 5 mg/mL of amphotericin B. Media and supplements were supplied by Gibco (Thermo Fisher Scientific). Cells were incubated at 37 °C under 5 % CO_2. Once the cell confluence was obtained, the cells were trypsinized with 0.25 % trypsin-EDTA and subcultured in a ratio of 1:3 on 60 mm tissue culture dishes (Passage 1). The cells from three different donors were used for the experiments. For the hPDLSC proliferation experiment, the hPDLSCs were seeded at the density of 5 × 104 cell per well in a 24-well plate and incubated for 24 h. After incubation, cells were treated with either 2 ng/mL of plant-produced bFGF or 20 ng/mL of *E. coli*-produced bFGF (Cat. No.: 100-18B; Peprotech) as the positive control. Cells were harvested 3 days after treatment. Cell viability was determined by the MTT assay and detected by spectrophotometry at 570 nm using a reference wavelength of 630 nm on an ELX800UV universal microplate reader (Bio-Tek Instruments Inc.). The experiments were done in triplicate and all data are presented as the mean ± SD.

Culturing of human follicle dermal papilla cells and cell proliferation assay

hFDPCs were procured from PromoCell (C-12071). Cells were cultured in follicle derma papilla cell growth medium (PromoCell) supplemented with 10 % FBS, 1 % penicillin (100 units/mL) and 100 µg/mL streptomycin, and 1 % nonessential amino acid. Supplements were supplied from Gibco (Thermo Fisher Scientific). Cells were incubated at 37 °C under 5 % CO_2. For the hFDPCs study, the CellTiter-Glo luminescent cell viability assay (Promega) was performed to evaluate the cellular ATP levels. Primary dermal fibroblasts were seeded at 2 × 104 cells/well into a 96-well plate and incubated for 24 h. After incubation, cells were treated either with 50, 100, and 200 ng/mL of plant-produced bFGF or *E. coli*-produced bFGF (positive control) for 24 h. Then, 1 × cell culture lysis reagent (Promega) was added. After 10 min, Cell Titer-Glo reagent was added and incubated at room temperature for 10 min. Next, luminescence was measured using a microplate luminometer (SpectraMax L, Molecular Devices). The percentage of cell viability was calculated using the following equation:

$$\text{Cell proliferation (\%)} = \frac{\text{Luminescence of treated cells} \times 100}{\text{Luminescence of untreated cells}}$$

The experiments were performed in triplicate and all data are presented as the mean ± SD.

Determination of collagen content

hDFBCs were procured from ATCC (PCS-201-010 TM). Cells were cultured in DMEM supplemented with 10 % FBS, 1 % penicillin (100 units/mL) and 100 µg/mL of streptomycin, and 1 % nonessential amino acid. Media and supplements were supplied from Gibco (Thermo Fisher Scientific). Cells were incubated at 37 °C under 5 % CO_2 until reaching 90 % of confluency. For collagen content determination, collagen content in the primary hDFBCs was investigated by the Picrosirius-polarization method [39]. Briefly, the cells were seeded and treated with 25 µg/mL of plant-produced FGF and vitamin C (VitC, Sigma; Cat. No.: A0278; control) for 6 days (media was replaced every 2 days). After treatment, cells were washed with 1 × PBS 3 times and fixed with 4 % PFA for 10 min. The cells were stained with 0.1 % (W/V) direct red 80 in saturated picric acid for 10 min. The excess dye was removed by 0.01 N HCl in 70 % ethanol. Stained collagen in cell culture was dissolved with 0.5 N NaOH and absorbance was measured at 540 nm using a microplate reader (Synergy H1, BioTeK). The percentage of total stained collagen was calculated using the following equation:

$$\text{Cell proliferation (\%)} = \frac{\text{Luminescence of treated cells} \times 100}{\text{Luminescence of untreated cells}}$$

The experiments were performed in triplicate and all data are presented as the mean ± SD.

Funding

This work was supported by Thailand Research Fund grant RSA628 0006. The authors would like to thank the Graduate School, Ratchadapisek Somphot Fund (KR) and The Second Century Fund (C2F), Chulalongkorn University (BS) for providing financial support.

Acknowledgements

We thank Associate Professor Hugh Mason (Arizona State University, United States) for providing geminiviral vectors.

Conflict of Interest

The authors declare they have no conflict of interest.

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