Increased production of human fibroblast growth factor 17 in *Escherichia coli* and proliferative activity in NIH3T3 cells

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Abstract. Fibroblast growth factor 17 (FGF17) is a novel member of the FGFs family, which is essential for cell development, tissue repair, tumor growth and invasion. The aim of the current study was to obtain a high expression level of recombinant human FGF17 (rhFGF17), including soluble proteins and inclusion bodies. An optimized rhFGF17 cDNA sequence was cloned into a pET3a vector, then the pET3a-hFGF17 vector was transformed into BL21(DE3)pLysS *Escherichia coli* cells. Expression was induced by optimizing the conditions using isopropyl β-D-1-thiogalactopyranoside (IPTG) and it was confirmed that a 24-h exposure to 0.8 mM IPTG at 16˚C provided the optimal condition for soluble hFGF17. Furthermore, for the inclusion bodies, the optimal condition was a 4-h exposure to 0.4 mM IPTG at 37˚C. Two forms of rhFGF17 protein were purified by heparin affinity and SP Sepharose Fast Flow chromatography. MTT assays demonstrated that the purified rhFGF17 exerted an important effect on the proliferative activity of NIH3T3 cells, although there was no significant difference when compared with standard rhFGF17. Thus, an optimal and economic expression system was created in the present study for rhFGF17 in *E. coli*. This expression strategy enables the preparation of sufficient and highly bioactive rhFGF17 for further investigation of underlying mechanisms.

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Introduction

The human fibroblast growth factors (FGFs) protein family consists of 22 members, which share a high affinity for heparin, as well as high-sequence homology within a central core domain of 120 amino acids (1). FGFs are essential in biological functions, such as angiogenesis, mitogenesis, cell differentiation and wound repair. FGF17 is a member of the heparin binding growth factor family (2), which is structurally the most homologous to FGF8 and FGF18. FGF8, FGF17 and FGF18 are highly conserved between human and mice, sharing 93% identity (2,3). Mouse FGF17 has three isoforms, while human FGF17 has just two: FGF17a and FGF17b, the latter of which has been selected as the canonical sequence (4). FGF17 is preferentially expressed in the embryonic brain and is highly associated with the nervous system (5).

Numerous studies have indicated that FGF17 may serve as a therapeutic agent to potentially treat certain types of disease. There is an increasing demand in the market to produce the FGF17 protein, and the large-scale production of bioactive human FGF17 is a challenging rate-limiting step. Given these factors, the development of a process that may enable significant preparation of sufficient, highly bioactive recombinant human (rh)FGF17 is considered to be a high priority for further investigations of the underlying mechanisms and clinical pathology. With the development of biotechnology, various expression systems are currently being used for expressing recombinant proteins for industrial production, as well as in research for structural and biochemical studies (6). *Escherichia coli*, with a short growth cycle, low cost, high stability and high transformation efficiency, is suitable for large-scale manufacture (7). In addition, *E. coli* are the most frequently used expression system for high-scale production of recombinant proteins (8-10).

As human (h)FGF17 is an important growth factor and, to the best of our knowledge, its non-tag expression in *E. coli* has not been reported, an rhFGF17 expression vector pET3a-rhFGF17, with a high expression level of rhFGF17 protein with soluble

protein and inclusion bodies, was constructed in the present study. Furthermore, the high purity of rhFGF17 protein was obtained via heparin affinity and SP Sepharose Fast Flow chromatography. In addition, the biological activity of rhFGF17, which may significantly increase the proliferative activity of NIH3T3 cells was examined. This novel expression strategy markedly enhanced the yield of rhFGF17 with high biological activity, which may meet the demand for fundamental research and therapeutic applications.

Materials and methods

Reagents and bacterial strain. The PCR purification, gel extraction and plasmid miniprep kits, and DNA Marker were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Goat anti-FGF17 polyclonal antibody (cat. no. sc-16826) and mouse anti-goat IgG-HRP (cat. no. sc-2354) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Heparin Sepharose column, SP Sepharose Fast Flow and AKTA purifier were purchased from GE Healthcare Life Sciences (Shanghai, China). The *E. coli* DH5α and BL21(DE3)pLysS component cells were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China).

Construction of rhFGF17 expression vector. The coding sequence of rhFGF17 (GenBank reference, NM_001304478.1) was obtained from the pUC57-FGF17 vector, previously constructed by our lab (unpublished data), using a Veriti™ Thermal Cycler (cat. no. 4375786; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with Phusion® High-Fidelity PCR Master Mix (cat. no. M0531S; New England BioLabs, Inc., Ipswich, MA, USA). Amplification conditions were as follows: Initial denaturation at 98˚C for 30 sec, followed by 30 cycles at 98˚C for 10 sec, at 65˚C for 20 sec and at 72˚C for 20 sec, with a final extension step at 72˚C for 10 min. The DNA fragment rhFGF17 was subsequently cloned into pET3a vector, using the *Nde*I and *Bam*HI restriction enzymes, to create the recombinant expression vector, pET3a-hFGF17, according to the manufacturer's protocol. *Nde*I (cat. no. R0111S) and *Bam*HI (cat. no. R0136S) were purchased from New England BioLabs, Inc.

Production of rhFGF17. The recombinant vector pET3a-hFGF17 was transformed into BL21(DE3)PLysS component cells. Briefly, 50 ng pET3a-hFGF17 vector were added to 100 μl thawed BL21(DE3)PLysS component cells. Cells were incubated for 30 min on ice, heat shocked at 42˚C for 90 sec, and then plated on a pre-warmed LB agar plate for further culture. The transformed colonies were cultured in 5 ml LB medium (tryptone 10 g/l, yeast extract 5 g/l, NaCl 5 g/l, in ddH_2O) containing 100 μg/ml ampicillin and 35 μg/ml chloramphenicol at 37˚C. When the optical density $(OD)_{600}$ reached 0.6-0.8, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. The cultures were incubated at 37˚C for 4 h under agitation (speed, 200 rpm). The colony with the greatest expression level was selected as the seed strain in subsequent experiments.

Optimizing the expression conditions for rhFGF17. The IPTG concentration for rhFGF17 expression yield was evaluated at 37˚C and 16˚C. Detection of soluble rhFGF17 was performed as follows: The seed strain was cultured overnight with agitation at 200 rpm in 20 ml LB medium containing 10 μg/ml ampicillin and 35 μg/ml chloramphenicol at 37˚C. Subsequently, the culture (6 ml) was transferred into two bottles, each containing 600 ml fresh LB medium with 100 μg/ml ampicillin and 35 μg/ml chloramphenicol for further growth. When OD_{600} reached 0.6-0.8, the IPTG was added to final concentrations of 0.4 and 1 mM, and cultured for 4 h at 37˚C (speed, 200 rpm) and 24 h at 16˚C (speed, 180 rpm). Cells were collected by centrifugation at 15,000 x g for 20 min at 4˚C. The cell pellets were resuspended in improved lysis buffer [20 mM Tris-HCl, 200 mM Nacl, 1% Triton X-100, 0.2% deoxysodium cholate, 1 mM EDTA, 5% glycerol, 0.2 M sucrose and 1 mM phenylmethylsulfonyl fluoride (PMSF; pH 7.5)]. Cells were lysed by sonication for 10 min in an ice bath. Following centrifugation at 15,000 x g for 20 min at 4˚C, the sediment and the supernatant were separated by 12% SDS-PAGE and the protein expression levels of rhFGF21 were determined, using western blot analysis.

Purification of soluble rhFGF17. The bacteria cells were harvested and lysed in lysis buffer (pH 7.5) containing 50 mM Tris-HCl, 2 mM EDTA, 300 mM NaCl, 1% Triton X-100, 0.2% deoxysodium cholate, 5-10% glycerol, 0.01 M sucrose and 1 mM PMSF. Supernatants were collected for subsequent purification. The following steps were all performed at 4˚C: First, the heparin-sepharose column was equilibrated with five bed volumes of binding buffer (20 mM Tris-HCl buffer, 25 mM NaCl and 1 mM EDTA; pH 7.5) at a rate of 1 ml/min. Subsequently, the supernatant was applied to the column. Following binding, the column was washed with binding buffer with gradients of 0.4, 0.6, 0.8 and 1.0 M NaCl. Further purification was performed using an SP Sepharose Fast Flow, where the methodology was the same as the heparin-sepharose purification. Finally, fractions were collected from the column according to the ultraviolet absorption peaks and conductivity curve. Then the elution fractions were determined using 12% SDS-PAGE.

Isolation and refolding of rhFGF17 inclusion bodies. Following fermentation, bacteria were harvested by centrifugation at 10,000 x g at 4˚C for 15 min, and wet bacteria (1 g) was resuspended in 20 ml lysis buffer. The inclusion bodies were collected following centrifugation at 10,000 x g at 4˚C for 15 min and resuspended in wash buffer (20 mM Tris-HCl, 200 mM NaCl, 1% Triton X-100 and 1 mM EDTA; pH10) by centrifugation at 10,000 x g at 4˚C for 15 min after ultrasonication in an ice bath. Subsequently, inclusion bodies (1 g) were resuspended in 20 ml denaturing buffer (8 M urea, 20 mM Tris, 150 mM Nacl, 3 mM EDTA, 5 mM DTT and 0.5 M arginine; pH 7.5). The protein was then refolded by a combination of dialysis and slow dilution. First, the denaturing buffer was dialyzed in dialysis buffer (20 mM Tris, 50 mM NaCl, 15% glycerol, 0.5 M arginine and 4 M urea; pH 7.5) until the urea concentration reached 4 M; subsequently, the buffer in the dialysis bag were collected by centrifugation at 10,000 x g at 4˚C for 15 min and then slowly diluted into appropriate volumes of renaturing buffer (20 mM Tris-HCl, 50 mM NaCl, 30% glycerol and 0.5 M arginine; pH 7.5).

Following centrifugation at 15,000 x g at 4˚C for 20 min, the supernatant was retained and prepared for inclusion in the heparin-sepharose column. Refolding of the protein was performed at 4˚C.

Purification of rhFGF17 inclusion bodies. According to the heparin affinity of rhFGF17, a heparin-sepharose column was selected for the purification. Purification procedures were the same as soluble rhFGF17. Refolding rhFGF17 protein was loaded onto the column that was equilibrated with wash buffer (the same as the soluble fraction) at a speed of 1 ml/min. The flow-through was collected. The protein was subsequently eluted using 0.4-1.0 M NaCl gradient in wash buffer at a speed of 1 ml/min. The elution fractions were collected and determined by Coomassie blue staining of 12% SDS-PAGE.

Western blot analysis. Protein concentration was determined using the Lowry protein assay. Purified rhFGF17 proteins (50 ng) were separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk for 20 min at room temperature, and then incubated with primary antibodies at 4˚C overnight, followed by incubation with the secondary antibody at room temperature for 30 min. Protein bands were visualized by enhanced chemiluminescence using the ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Goat anti-FGF17 polyclonal antibody served as the primary antibody (dilution, 1:1,000) and mouse anti-goat IgG-HRP was used as the secondary antibody (dilution, 1:8,000). The molecular sizes of the obtained protein were verified by comparison with the migration of pre-stained protein markers (cat. no. 26616; Thermo Fisher Scientific, Inc.).

Mitogenic activity of rhFGF17 assay. NIH3T3 cells ($2x10^3$ cells/well) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) in a 96-well plate at 37˚C for 24 h. The medium was then replaced with DMEM supplemented with 1% FBS and cells were starved overnight. The cells were treated with different concentrations of rhFGF17 or commercial rhFGF17 (R&D Systems China Co., Ltd., Shanghai, China) for 48 h and the number of viable cells was determined by adding 25 μl MTT (5 mg/ml) per well for 4 h. Finally, the medium was discarded and 150 μl dimethyl sulfoxide was added to each well to dissolve the crystals by agitation at room temperature for 10 min; the absorbance was immediately measured at a wavelength of 600 nm using the GENESYS™ 10S UV-Vis Spectrophotometer (Thermo Fisher Scientific, Inc.).

Results

Construction of the rhFGF17 expression vector. To produce the rhFGF17 protein, an expression vector containing the optimized hFGF17 gene was constructed. The hFGF17 fragment was obtained (Fig. 1A), then digested with *Nde*I and *Bam*HI and cloned into the pET3a vector to create the pET3a-rhFGF17 recombinant plasmids, which were then confirmed by restriction enzymatic analysis (Fig. 1B and C) and automated DNA sequencing.

Expression of rhFGF17 in BL21(DE3)pLysS. The recombinant plasmid was transformed into BL21(DE3)pLysS. The SDS-PAGE demonstrated that rhFGF17 was induced by 1 mM IPTG and the apparent molecular band was ~23 kDa, corresponding to the predicted molecular weight (22.6 kDa; Fig. 2A). The greatest expression level of rhFGF17 was ~30% of total protein.

Optimizing the expression of soluble rhFGF17. To establish the optimal culture conditions, the following concentrations of IPTG were evaluated: 0.2, 0.4, 0.8 and 1.0 mM at 37˚C or 16˚C, under agitation at 180 rpm. When rhFGF17 was induced by 0.4 mM IPTG at 37˚C or 0.8 mM IPTG at 16˚C, the rhFGF17 yield reached the highest level with ratios of ~30 and ~20% of the total protein, respectively according to the SDS-PAGE results (Fig. 2B). Following fermentation in a 2-liter flask under the above-mentioned optimized conditions, the yield of bacteria was ~9 g/l at 37˚C and 6 g/l at 16˚C. Soluble detection was performed by lysis. SDS-PAGE analysis of the lysate supernatant and sediment indicated that the recombinant protein was marginally soluble at 16˚C, but inclusion bodies appeared to be formed at 37˚C (Fig. 2C).

Purification of soluble rhFGF17. The soluble product was purified with improved lysis buffer and more soluble proteins were obtained with almost no sediment (Fig. 3A). Heparin-affinity column chromatography combined with SP-Sepharose column chromatography was used for purification of the soluble fraction of proteins. rhFGF17 was eluted with 1.0 M NaCl in elution buffer from the two columns (Fig. 3B and C), and the yield was 1 mg/g (1 mg rhFGF17 from 1 g bacteria cells).

Purification and identification of rhFGF17 inclusion bodies. rhFGF17 inclusion bodies were predominantly produced from the culture condition of 37˚C for 4 h. rhFGF17 was denatured by urea and refolded in the dialysis buffer by dialysis and then renaturing buffer by dilution at pH 7.5 (Fig. 4A). As indicated in Fig. 4A denaturing buffer dissolved the majority of the rhFGF17. The concentration of total protein in the denaturing buffer was ~41 mg/ml and in the renaturing buffer prior to applying it to the heparin-sepharose column, total protein was decreased to ~2 mg/ml. As demonstrated in Fig. 4B the fractions containing rhFGF17 were finally eluted by heparin affinity chromatography using 20 mM Tris-HCl containing 1.0 M NaCl. The purified rhFGF17 protein yield reached 8 mg/g (8 mg rhFGF17 from 1 g bacteria cells).

The purified soluble rhFGF17 and rhFGF17 inclusion bodies were homogenous and their purity was >95%. Western blot analysis demonstrated that the purified rhFGF17 had good immunoreactivity with the anti-human FGF17 antibody (Fig. 4C).

Mitogenic activity of rhFGF17. To assess the biological activity of purified rhFGF17, the proliferative effect of rhFGF17 was determined using a standard MTT assay on NIH3T3 cells and compared with commercial rhFGF17 (the positive control). As shown in Fig. 5, the two purified soluble forms and inclusion

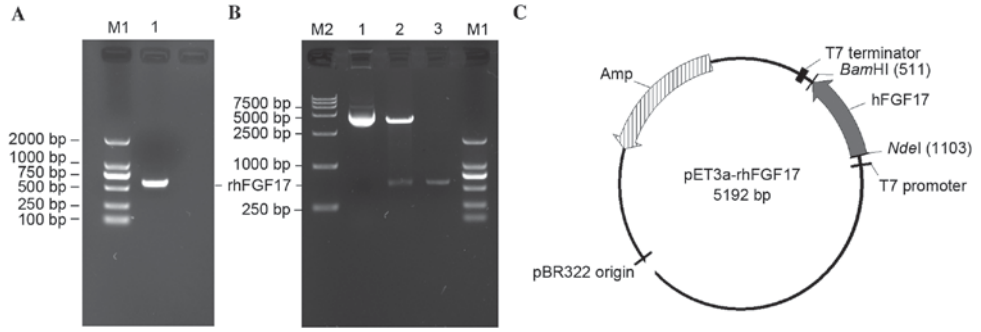

Figure 1. Construction of the pET3a-rhFGF17 expression vector. (A) rhFGF17 fragment obtained from pUC57-FGF17 by polymerase chain reaction amplification. Lane 1, FGF17 (597 bp); lane M1, DNA marker 1. (B) Identification of recombinant plasmid by enzyme digestion (*Nde*I and *Bam*HI); Lane 1, pET3a-rhFGF17; lane 2, restriction products of recombinant plasmid pET3a-rhFGF17; lane 3, rhFGF17 fragment control; lane M1, DNA marker 1; lane M2 DNA marker 2. (C) Structure of the pET3a-rhFGF17 vector. rhFGF17, recombinant human fibroblast growth factor 17.

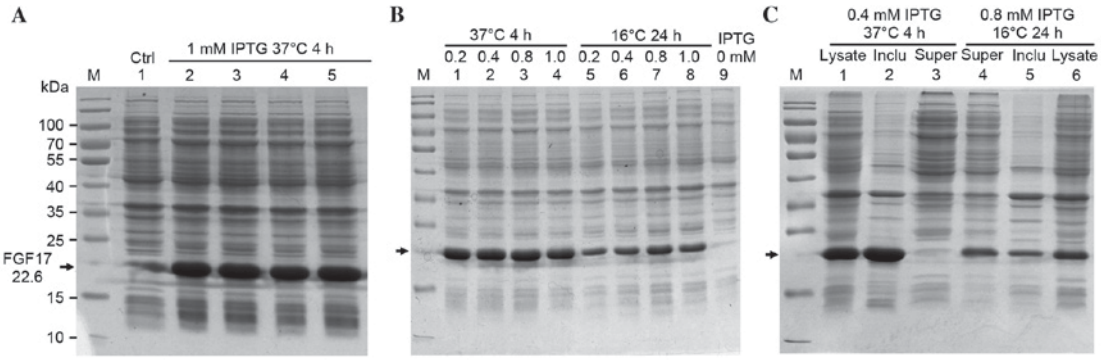

Figure 2. Optimizing the expression conditions of rhFGF17. (A) SDS-PAGE analysis of rhFGF17 expression in BL21(DE3)PLysS induced by 1 mM IPTG for 4 h at 37˚C. Lane 1, served as a control and was not induced with IPTG; lanes 2-5, induced with IPTG; (B) Optimizing the expression conditions of rhFGF17. Lanes 1-4 and 5-8: 0.2, 0.4, 0.8, 1.0 mM IPTG induced at 37˚C for 4 h and 16˚C for 24 h, respectively. (C) Distribution of rhFGF17. Lanes 1-3, 37˚C culture (lane 1, induced BL21(DE3)PLysS/pET3a-rhFGF17; lane 2, inclusion bodies of bacteria following ultrasonication; and lane 3, supernatant). Lanes 4-6, 16˚C culture (lane 4, supernatant of bacteria following ultrasonication; lane 5, inclusion bodies; and lane 6, induced BL21(DE3)PLysS/pET3a-rhFGF17. rhFGF17, recombinant human fibroblast growth factor 17; IPTG, isopropyl β-D-1-thiogalactopyranoside; Ctrl, control.

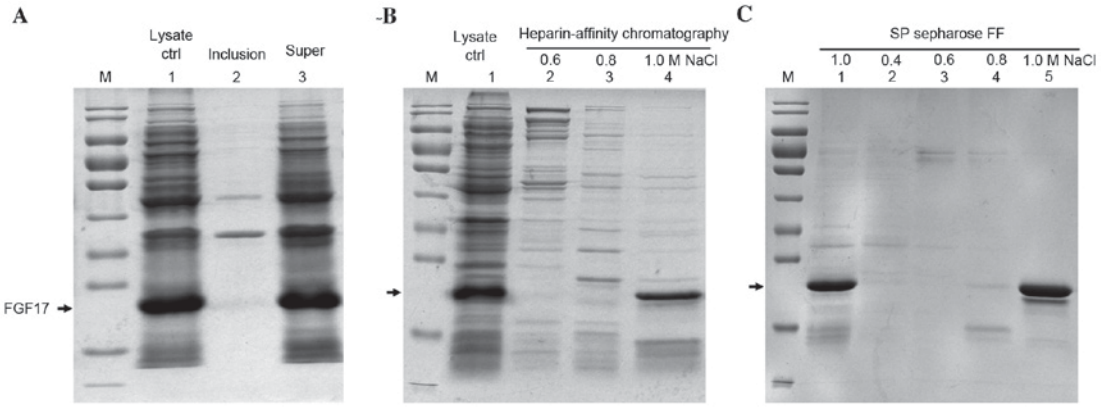

Figure 3. SDS-PAGE analysis of the purification of soluble rhFGF17 using improved lysis buffer. (A) Lane 1, lysate control; lane 2, inclusion bodies of bacteria following ultrasonication; lane 3, supernatant. (B) Purification of soluble rhFGF17 with heparin-affinity chromatography (lanes 2-4). Lane 1, supernatant of bacteria after ultrasonication; lane 2-4 eluted with 0.6, 0.8, 1.0 M NaCl from heparin-affinity chromatography, (C) Lanes 1-5, SP Sepharose Fast Flow of rhFGF17 eluted with different NaCl concentrations. Lane1 and 5, 1.0 M NaCl; lanes 2-4, 0.4, 0.6 and 0.8 M NaCl. The arrow indicates the rhFGF17 band site. rhFGF17, recombinant human fibroblast growth factor 17; Ctrl, control; FF, fast flow.

bodies of rhFGF17 demonstrate similar mitogenic activity in NIH3T3 cells, which is consistent with the findings of a previous study (11). Additionally, compared with the commercial rhFGF17, the rhFGF17 protein formed during the present study exhibited improved biological activity. Furthermore, rhFGF17 was found to have a dose-dependent effect on the viability of NIH3T3 cells, whereas the negative control did not. Finally, the results demonstrated that the soluble and inclusion body forms of rhFGF17 had a marked biological effect on NIH3T3 cells.

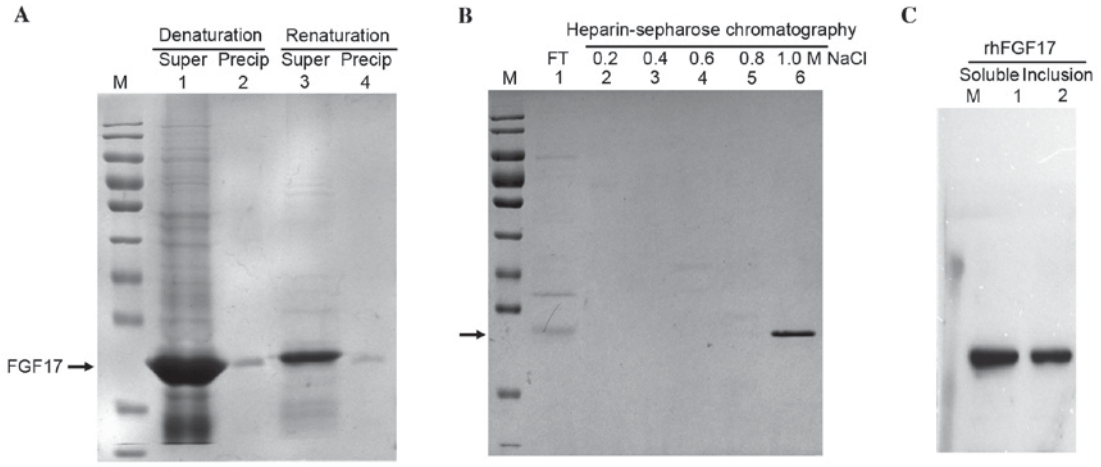

Figure 4. SDS-PAGE analysis of the purification of rhFGF17 inclusion bodies. (A) Lanes 1 and 3, Supernatant rhFGF17 and precipitation rhFGF17 in denaturation and renaturation buffer; lanes 2 and 4, Precipitation rhFGF17 in denaturation and renaturation buffer. (B) Heparin-sepharose chromatography. Lane 1, FT; lanes 2-6, eluted rhFGF17 with 20 mM Tris-HCl containing 0.2, 0.4, 0.6, 0.8 and 1.0 M NaCl, respectively. (C) Western blot analysis of rhFGF17. Lane 1, purified soluble rhFGF17; lane 2, purified rhFGF17 inclusion bodies. rhFGF17, recombinant human fibroblast growth factor 17; FT, flow through.

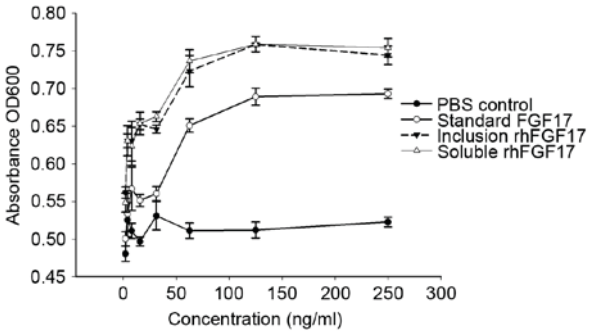

Figure 5. Mitogenic activity of rhFGF17 on NIH3T3 cells. Data are expressed as the mean ± standard deviation. rhFGF17, recombinant human fibroblast growth factor 17; OD, optical density; PBS, phosphate-buffered saline.

Discussion

As a novel member of the FGF family, numerous pharmacological studies have demonstrated that FGF17 is a key factor in neuropsychiatric diseases due to its important roles in the patterning of the cerebellum and cortex (12). As a potential carcinogen, FGF17 is predominantly associated with prostate cancer (13) and hematopoietic tumors (14). Therefore, it is necessary to investigate FGF17 and develop strategies for abundant production of FGF17 with high bioactivity. As reported previously, the recombinant form of FGF17 protein was produced using insect cells; however, compared with prokaryotic expression systems, eukaryotic systems are considered unsuitable for large-scale purification (15). To date, there are few reports regarding the expression of hFGF17, particularly in *E. coli* expression systems. The low level of soluble production and difficulty purifying inclusion bodies, particularly denaturing and refolding, has restricted further research and application. There are certain methods used to overcome these limitations, including fusion systems to enhance target protein expression, such as Halo-tag fusion (16). However, the method for obtaining target protein requires that the fused tag must be removed, which involves an expensive cleavage restriction enzyme and may impact the bioactivity of the target protein.

Low temperatures increase the expression levels of soluble proteins and reduce the aggregation of recombinant proteins, thus reducing the formation of inclusion bodies (17). In addition, low agitation speeds will reduce the speed of bacteria proliferation, but increase the amount of soluble proteins. Therefore, in the current study, the culture conditions at 37˚C and 200 rpm, and 16˚C and 180 rpm would yield high levels of inclusion bodies and soluble proteins, respectively. To further improve the production levels of the target protein, the expression conditions were optimized according to the IPTG concentration, thus the expression level of inclusion bodies and soluble protein reached >30 and >20% of total protein, respectively with 0.4 mM IPTG for 4 h at 37˚C and 1 mM IPTG for 24 h at 16˚C. Meanwhile, the lysis buffer was improved by the addition of more Tris, glycerol and sucrose; thus, a soluble protein was obtained with almost no sediment (Fig. 3A). Taken together, the conditions were improved and high production levels of target protein were achieved for purification.

Based on an isoelectric point of 10.43 and the heparin binding ability for rhFGF17, the non-fusion rhFGF17 protein was efficiently separated by heparin-sepharose chromatography and SP Sepharose Fast Flow (18,19). The purified rhFGF17 proteins were biologically active *in vitro* and exerted a dose-dependent effect on the proliferation of NIH3T3 cells; inclusion-bodies were demonstrated to have a biological activity similar to the soluble proteins. Thus, the soluble proteins and inclusion bodies obtained using the culture conditions at 37˚C and 200 rpm, and 16˚C and 180 rpm, respectively, are efficiently produced and are characterized by high levels of bioactivity. Furthermore, FGF17 has previously been reported to be involved in Kallmann syndrome (20) and causes tamoxifen resistance *in vitro* (21). Therefore, whether there is a direct association between FGF17 and breast cancer requires further investigation.

In conclusion, soluble and inclusion bodies of rhFGF17 were successfully expressed in *E. coli*. The current study indicates that the non-tagged expression of either soluble proteins

or inclusion bodies of rhFGF17 is simple, viable and highly effective, making it convenient for high-efficiency expression and purification of proteins, whilst preserving the high biological activity levels.

Acknowledgements

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