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SUMO fusion system facilitates soluble expression and high production of bioactive human fibroblast growth factor 23 (FGF23)

Xiaoju Liu · Yubin Chen · Xiaoping Wu · Haiyan Li · Chao Jiang · Haishan Tian · Lu Tang · Dezhong Wang · Ting Yu · Xiaokun Li

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Abstract As a key humoral regulator of phosphate homeostasis and its involvement in the pathogenesis of human disease, human fibroblast growth factor 23 (hFGF23) has become a particularly attractive therapeutic target. To prepare soluble and bioactive recombinant human FGF23 to meet the increasing demand in its pharmacological application, small ubiquitin-related modifier (SUMO)-FGF23 fusion gene and FGF23 non-fusion gene were amplified by standard PCR methods and cloned into vector pET-22b and pET-3c, then transformed into *Escherichia coli* Rosetta (DE3) and BL21 (DE3). The best combination of plasmid and host strain was screened, and only Rosetta (DE3)/pET-SUMO-FGF23 was screened for rhFGF23 protein expressed. The average bacterial yield and the soluble expression level of recombinant hFGF23 of three batches attained 687±18 g and 30±1.5%, respectively, after treatment with 0.4 mM isopropyl-thio-β-galactopyranoside for 19 h at 16 °C in a 30-L fermentor, after which it was purified by DEAE Sepharose FF and nickel nitrilotriacetic acid affinity chromatography. Once cleaved by the SUMO protease, the recombinant human FGF23 was released from the fusion protein. The purity of rFGF23 was shown by high performance liquid chromatography to be greater than 90% and the yield was 60±1.5 mg/L. In vitro data showed that the purified rFGF23 can induce the phosphorylation of mitogen-activated protein kinases in the glioma U251 cell. The results of in vivo animal experiments also showed that rFGF23 could decrease the concentration in the plasma of normal rats fed with a fixed formula diet.

Keywords SUMO fusion · Soluble expression · High production · Bioactive fibroblast growth factor 23 (FGF23)

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X. Liu · C. Jiang · T. Yu (✉) · X. Li (✉)
School of Public Health, Jilin University,
Changchun 130021, China
e-mail: yuting485@163.com
e-mail: proflxk@163.com

T. Yu
The Second Hospital of Jilin University,
Changchun 130041, China

X. Liu · Y. Chen · H. Li · C. Jiang · H. Tian · D. Wang · X. Li
Engineering Research Center of Bioreactor and Pharmaceutical Development, Ministry of Education,
Changchun, Jilin 130118, China

X. Liu · X. Wu · C. Jiang · H. Tian · L. Tang · D. Wang · X. Li
School of Pharmaceutical Science, Key Laboratory of Biotechnology and Pharmaceutical Engineering of Zhejiang Province, Wenzhou Medical College,
Wenzhou, Zhejiang 325035, China

Introduction

Human fibroblast growth factor 23 (hFGF23), a member of the FGF superfamily, was first identified in 2000 (Yamashita et al. 2000). The full-length hFGF23 consists of 251 amino acids with a signal peptide of 24 amino acids at the N-terminus, splicing a mature FGF23 polypeptide of 227 amino acids (Yamashita et al. 2000). FGF23 reduces serum phosphate by inhibiting both proximal tubular phosphate reabsorption and intestinal phosphate absorption (Shimada et al. 2004). This is concerning the FGF23 physiological function of regulating phosphate metabolism; on the other hand, mutations in FGF23 are implicated in a wide range of disorders. Both autosomal dominant hypophosphatemic

rickets and X-linked hypophosphatemic rickets were caused by excess FGF23, leading to hypophosphatemia (2000; Jonsson et al. 2003; Yamazaki et al. 2002). FGF23 levels are increased tenfold above controls in patients with tumor-induced osteomalacia, a tumor associated syndrome of renal phosphate wasting (Jonsson et al. 2003; Yamazaki et al. 2002). Circulating FGF23 levels are also increased and correlate with disease burden in patients with fibrous dysplasia, a disorder in which normal bone is replaced by fibro-osseous tissue (Riminucci et al. 2003). Reduced FGF23 signaling also causes pathology. Familial tumoral calcinosis is a disorder marked by hyperphosphatemia in which individuals develop calcified masses, often within the joints (Lyles et al. 1985). In addition, in patients with chronic kidney disease (CKD), FGF23 concentrations are elevated constitutively and increase progressively as kidney function worsens (Gutierrez et al. 2005), suggesting a direct link between increased FGF23 and the progression of CKD (Fliser et al. 2007).

It is a given that FGF23 has an important role in the physiological regulation of phosphate metabolism and its pharmacological significance in the pathogenesis of human disease. In the next step, it is necessary to prepare sufficient hFGF23 with activity for further mechanisms and pharmacological research. Yamashita et al (2000) has expressed FGF23 in CHO mammalian cell. It is well known that the mammalian cell expression system is more complicated and costly from the standpoint of culture requirements, making this strategy difficult to use in the development of large-scale fermentation processes. Furthermore, the preparation of sufficient amounts of virus for large-scale expression is time consuming. To overcome this shortcoming, Plotnikov et al. (2000) expressed FGF23 in *Escherichia coli* in the form of an inclusion body; however, because the inclusion bodies must be denatured and annealed, it was difficult to produce bioactive protein. Our previous experiments showed that FGF23 without using this fusion strategy was least effective in expression and solubility properties.

In recent years, small ubiquitin-related modifier (SUMO) has become an effective biotechnological tool as a fusion system to enhance soluble expression of proteins and decrease proteolytic degradation, which could not be achieved by traditional expression systems (Butt et al. 2005; Sun et al. 2008); then, SUMO is post-translationally and enzymatically cleaved from the desired protein by SUMO C-terminal hydrolases–isopeptidases (Butt et al. 2005). Various proteins, such as SARS virus protein (Zuo et al. 2005), MMP13 (Marblestone et al. 2006), EGF (Sun et al. 2008), metallothionein (Huang et al. 2009), KGF2 (Wu et al. 2009), and FGF21 (Wang et al. 2010), have been expressed successfully and purified using this fusion strategy. Thus, we also cloned a SUMO fragment and constructed an expression plasmid-containing SUMO and human FGF23. The results show that this novel method of protein expression can promote significantly greater rFGF23 levels, facilitating the dissolution of rFGF23 in the soluble fraction for purification and producing native N-terminal recombinant protein with its bioactivity preserved.

Materials and methods

Primers, plasmids, and strains

All primers were synthesized chemically by Beijing Sunbiotech Co. Ltd (China). The pUC vector containing human FGF23 cDNA sequence (aa25-251, GenBank accession number BC069333 for expression of the protein without the first 24 amino acids) was purchased from Proteintech Group, Inc. All plasmids and the strains are obtained from the laboratory (Engineering Research Center of Bioreactor and Pharmaceutical Development, Ministry of Education, Jilin Agricultural University China): the *E. coli* strain DH5α was used as the host for all cloning experiments, while protein expression studies were performed using *E. coli* strain Rosetta (DE3) as host; the vector of pET3c, pET22b and the expression strain of BL21(DE3) were used for optimal expression and pET3c-SUMO was used as the template of the SUMO gene. The SUMO protease was provided by Dr. Yadong Huang from Jinan University.

Construction of four kinds of rFGF23 recombinant plasmid

The strategy for the construction of four kinds of rFGF23 recombinant plasmid is illustrated in Fig. 1. First, PCR was applied to amplify the DNA fragments encoding the core fragment of rFGF23 with the forward FL and reverse R23 primers, including *BamH I* sites, using the pUC vector

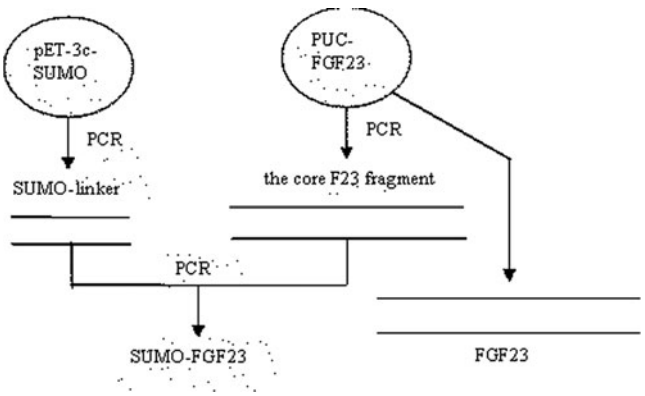

Fig. 1 Schematic illustration of SUMO-FGF23 and FGF23 synthesis. According to the human FGF23 mRNA coding sequence and the SUMO fragment C-terminal sequence, five primers were designed and synthesized. As shown in Fig. 1, SUMO-FGF23 and FGF23 were synthesized by PCR. The detailed steps are provided in the "Material and methods"

containing a human FGF23 coding sequence as the template. Then, the core FGF23 fragment was prepared for linking to SUMO; on the other hand, the FGF23 full-length gene without fusion of SUMO was created according to the procedure described above, except that the forward primer of F included the *Nde I* sites.

Second, the SUMO linker was generated using pET3c-SUMO (plasmid-containing SUMO fragment) as the template, P including *Nde I* sites and the SUMO linker (RL) as the forward and reverse primers, respectively. Then, using the SUMO linker and the core FGF23 fragment as the common template, P including *Nde I* sites and R23 primers including *BamH I* sites as the forward and reverse primers, respectively, the SUMO-FGF23 fusion gene construct was created using the same procedure described above.

Third, the FGF23 full-length gene and the SUMO-FGF23 fusion gene were digested with *Ned* I and *BamH* I, respectively, and then ligated into previously digested pET-3c, pET22b to create four kinds of new expression vectors, i.e., pET3c-FGF23, pET3c-SUMO-FGF23, pET22b-FGF23, and pET22b-SUMO-FGF23. Correct insertion of sequences was confirmed by DNA sequencing of each plasmid.

Small-scale expression and soluble screening of FGF23 in *E. coli*

The four kinds of FGF23 expression vector constructed above were transformed into competent cells of strains Rosetta(DE3) and BL21(DE3). The transformants were grown in 4-mL Luria–Bertani (LB) medium containing 100 μg/mL ampicillin and 1% glucose at 37 °C. When OD600 reached 0.6, isopropyl-thio-β-galactopyranoside (IPTG) was added to a final concentration of 1 mM. The culture was incubated at 37 °C for 4 h with shaking at 220 rpm. The expression of each culture was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the expression level of rhFGF23 was determined by densitometer scanning. The colony with the highest expression level was used in the subsequent experiments.

An analysis was performed to determine the effects of the three factors (IPTG concentration, temperature, and time after induction) on the expression yield and productivity of soluble FGF23 in Rosetta(DE3)/pET22b-SUMO-FGF23. The recombinant bacteria were harvested by centrifugation at 30,000 × *g* for 5 min at 4 °C. Cell pellets were suspended in Tris–HCl buffer at a concentration of 20 mM and dissolved by sonication. The suspensions were centrifuged at 30,000 × *g* for 30 min at 4 °C. The clear supernatant (soluble fraction) was collected, and the remaining pellets (insoluble fraction) containing inclusion bodies were resuspended in an equal volume of lysis buffer. Both soluble and insoluble fractions were analyzed by 15% SDS-PAGE.

Purification of SUMO-FGF23

An overnight culture of *E. coli* strain Rosetta(DE3) transformed with the expression vector pET22b containing the SUMO-FGF23 gene was diluted 1:50 into 2 L of growth medium (LB medium containing 100 μg/mL of ampicillin). The culture was incubated at 37 °C until its optical density at 600 nm was 0.6. Protein expression was induced by the addition of 0.4 mM IPTG at 16 °C. After 19 h of additional incubation, the cells were harvested, resuspended in 20 mM Tris–HCl buffer (pH=8.0) containing 2 M urea, and sonicated. The resulting cell lysate was cleared of bacterial debris by ultracentrifugation, and the fusion protein with his_6-tagged N-terminal SUMO was purified by DEAE Sepharose FF column chromatography. SUMO-FGF23 flows through the DEAE column with binding buffer (20 mM Tris–HCl+2 M urea, pH=8.0), followed by further purification with nickel nitrilotriacetic acid resin (Ni-NTA, Invitrogen). The Ni-NTA resin was washed with binding buffer (20 mM Tris–HCl+2 M urea, pH=8.0) until OD280 of the effluent reached baseline conditions. Contaminating proteins were eluted from the column with wash buffer (20 mM Tris–HCl+2 M urea containing 25 mM imidazole, pH=8.0). Finally, his_6-tagged SUMO-FGF23 protein was collected from the column with elution buffer (20 mM Tris–HCl+2 M urea containing 250 mM imidazole, pH=8.0). Samples taken at the elution peak were pooled. The purity of SUMO-FGF23 was assessed using SDS-PAGE, and the concentration was evaluated by the Bradford method.

Release of the target protein by SUMO protease cleavage

To remove imidazole and urea, the fusion protein obtained from the affinity column was subjected to a buffer change to TBS using dialysis in 20 mM Tris buffer (pH=8.0) through a semipermeable membrane (cutoff, 10,000 Da). The resulting protein solution was treated with SUMO protease to release the recombinant FGF23. In a standard cleavage reaction, 50 μg of fusion protein (37.6 kDa) in a 50-μl digestion buffer (50 mM Tris–HCl, pH=8.0, 0.15 M NaCl, 1 mM DTT) was incubated overnight with five units of SUMO protease (provided by Dr. Yadong Huang from Jinan University) at 4 °C. The result of the cleavage reaction was monitored by SDS-PAGE. The target protein could be visualized by conventional SDS-PAGE.

Purification and the authenticity assay for rFGF23

After SUMO protease cleavage, the reaction mixture contained his_6-tagged N-terminal SUMO, FGF23 target protein, SUMO-FGF23 fusion protein, and SUMO protease. Then, the reaction mixture was passed through a miniature Ni-IMAC column. The SUMO, SUMO-FGF23, and SUMO

protease stayed bound to the Ni-NTA resin, and only rFGF23 flowed through the column with the digestion buffer; these products were desalted with a Sepharose G50 column.

The purified rFGF23 was immunoblotted with a polyclonal mouse anti-FGF23 antibody (Santa Cruz Biotechnology, Inc.,) according to the manufacturer's protocol. Immunoreactive bands were visualized using a DAB kit (Boshide, Inc., Wuhan, China). Furthermore, the target protein were identified by matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI-TOF/TOF) mass spectrometry analysis (technical services provided by the Shanghai Applied Protein Technology Co. Ltd.).

For high performance liquid chromatography (HPLC) analysis, the purified rFGF23 was desalted and then loaded onto a C18 column. The elution was conducted using a linear gradient of 30–70% acetonitrile at a flow rate of 0.8 mL/min in the presence of 0.1% trifluoroacetic acid. The fractions containing rFGF23 were pooled. The concentration of rFGF23 was evaluated by the Bradford method.

In vitro activity assay of rFGF23

To assess the biological activity of rFGF23 proteins at the cellular level, we studied the ability of the protein to activate 44/42 MAP kinase in the glioma U251 cell line, which expresses abundantly FGF23 cognate receptors FGFR1, compared to commercial FGF23, which is also expressed in *E. coli*. The glioma U251 cells (1,000,000 cells/dish) were serum starved for 16 h and then stimulated with rFGF23 (100 ng/mL), commercial FGF23 (100 ng/mL), or PBS for 10 min. After stimulation, the cells were lysed, and cellular proteins were resolved on SDS-polyacrylamide gels and transferred electrophoretically to nitro cellulose membranes; then, the protein blots were probed with antibodies to phosphorylated 44/42 MAP kinase and non-phosphorylated 44/42 MAP kinase according to the manufacturer's protocol. The protein levels of p-ERK, ERK, were examined by Western blot with actin as a loading control. All antibodies were from Santa Cruz Biotechnology.

In vivo activity assay of rFGF23

All described procedures that involved animals and their care were approved by the Institutional Animal Care and Use Committee of Jilin University and were performed in accordance with institutional guidelines for animal experiments. Wistar adult male rats (weighting 220–250 g) were divided randomly into three groups: the treatment group (rFGF23, $n=10$), control group (commercial FGF23, $n=10$), and the negative control group (NC, saline $n=10$). The phosphaturic activity of rFGF23 was examined in normal rats by a published protocol (Goetz et al. 2007). Briefly, the animals were fed with a complete, fixed-formula diet containing 0.94% phosphate for 2 weeks, and, then, they fasted overnight for 8–12 h. Three hours before the injection, 0.5 mL of blood samples were collected from the tail. After the i.v. administering 0.1 μg/kg of body weight of either rFGF23, commercial FGF23, or saline, we took blood samples at 1, 2, and 3 h. Phosphate concentrations in the serum were determined using a phosphomolybdic acid detection kit (Nianjing Jiangcheng Co.).

Statistical analysis

Values are expressed as mean ± standard error of mean. Comparisons of mean values between two time points were performed using the Student's *t* test. Significant differences were considered at $P<0.05$. Cell assay were performed in triplicate. All experiments were repeated at least three times.

Results

Synthesis of FGF23 and SUMO-FGF23 gene and construction of four kinds of expression vector

To synthesize the full-length FGF23 and SUMO-FGF23 fusion gene composed of SUMO and the core FGF23 fragment, five special primers were designed (Table 1). The synthesis strategy is described in Fig. 1, and the detailed procedure is included in the "Materials and methods" section. The PCR products of SUMO, FGF23, and SUMO-FGF23 are shown in Fig. 2. The final PCR products (the full-length FGF23 and SUMO-FGF23) were digested with two restriction enzymes (*Ned* I and *BamH* I) and cloned into the expression vectors pET-3c and pET22b, respectively. The sequence of the target gene was confirmed by automated DNA sequencing. The results of PCR products showed that the sequences of human

Table 1 PCR primers for amplifying FGF23 and SUMO-FGF23 fusion gene

Primer name (size)	Sequence (5′–3′)
P(32 bp)	GGAATTCCATATGCATCATCATCATCATCACG
FL(41 bp)	ACAGAGAACAGATTGGTGGTTATCCCAATGCCTCCCCACTG
RL(41 bp)	CAGTGGGGAGGCATTGGGATAACCACCAATCTGTTCTCTGT
R(27 bp)	CGGGATCCTTACTAGATGAACTTGGCG
F(32 bp)	GGGAATTCCATATGTATCCCAATGCCTCCCCA

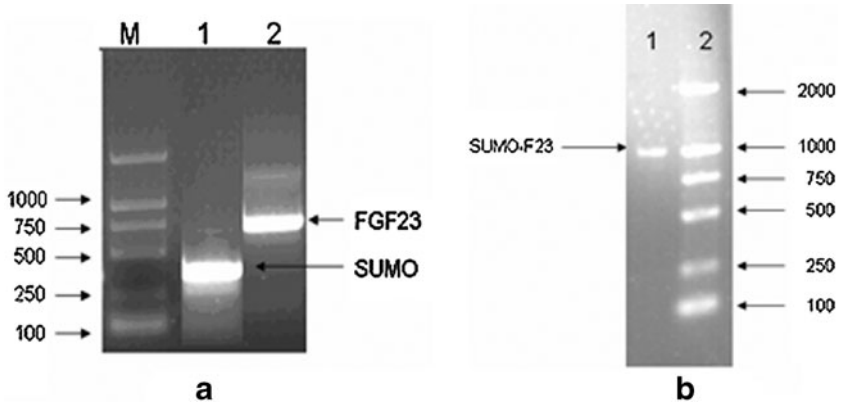

Fig. 2 Synthesis of FGF23 and SUMO-FGF23 by PCR. The strategy for synthesizing FGF23 and SUMO-FGF23 was described in the "Material and methods." The molecular weight of the PCR fragment containing SUMO and FGF23 was shown in **a**. *Lane 1* SUMO fragment (340 bp); *lane 2* FGF23 fragment (705 bp). The PCR product of the SUMO-FGF23 fusion gene was shown in **b**. It was 1,018 bp in length

FGF23 (705 bp) and SUMO-FGF23 (1,018 bp, SUMO 340 bp) conformed with the expected sequence.

Small-scale expression screening and optimization of induction conditions for productivity of soluble rFGF23

To achieve greater productivity of rFGF23, we performed several small-scale expression studies. In the experiments, we used host bacteria Rosetta(DE3) and BL21(DE3) and four kinds of recombinant plasmid for expression screening. The results showed that, except for the combination of pET22b-SUMO-FGF23 and pET3c-SUMO-FGF23, the two kinds of plasmid, and the Rosetta(DE3) host strain (Fig. 3a), all other combinations had no target protein expressed.

We chose the Rosetta(DE3)/pET22b-SUMO-FGF23 for optimization of induction conditions for soluble rFGF23. Recombinants were inoculated in fresh LB medium and incubated in a shaking incubator at 37 °C until the OD600 was 1.0 to 1.2. Then, IPTG was added to a final concentration of 0.4 mM for 19 h at 16 °C for the induction of expression. Recombinant bacterial cells were collected by centrifugation and lysed by sonication. The supernatants and pellets were collected and analyzed using 15% SDS-PAGE. The results showed that the molecular weight of the expression product was 37 kDa, which corresponds to the predicted size of SUMO-FGF23. The target protein is more than 35% of the total cellular protein, and the soluble fraction was as much as 80% of the total expressed recombinant proteins (Fig. 3b).

Concerning optimal cell growth conditions for protein production, we screened for suitable IPTG concentration, temperature, and time after induction for the expression yield and productivity of soluble FGF23 in Rosetta(DE3)/pET22b/SUMO-FGF23. We found that lower temperature (16 °C), lower IPTG concentration (0.4 mM), and longer induction (19 h) can express FGF23 protein significantly in soluble form, while most of the SUMO-FGF23 protein was

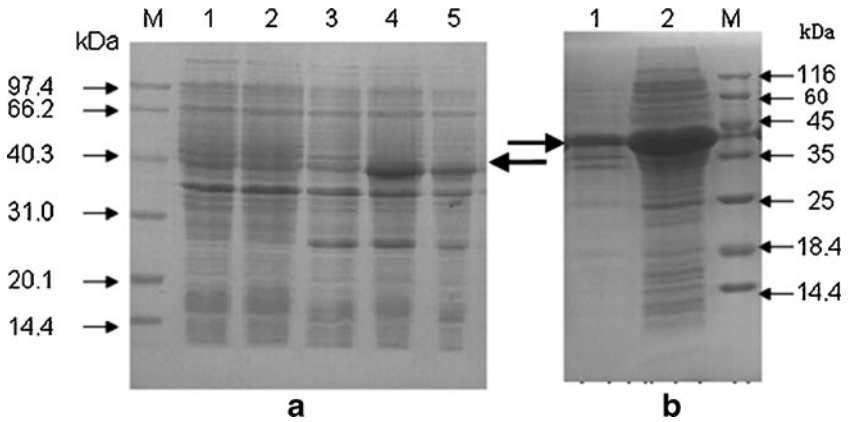

Fig. 3 SDS-PAGE analysis of SUMO-FGF23 expression screening and optimization of induction conditions for productivity of soluble SUMO-FGF23. Four kinds of recombinant plasmid were transformed into *E. coli* strain Rosetta(DE3) and BL21(DE3) for expression screening. The results showed that, the Rosetta(DE3) host strain, which were transformed by either pET22b-SUMO-FGF23 or pET3c-SUMO-FGF23, were effective for target protein expressed, and the fusion protein molecular mass was approximately 37.6 kDa, corresponding to the predicted molecular weight. **a** *M* protein molecular weight marker (in kilodalton, Takara); *lanes 1–2* uninduced and induced Rosetta(DE3)/pET22b, respectively, as control; *lanes 3 and 4* uninduced and induced Rosetta (DE3)/pET22b-SUMO-FGF23, respectively; *lane 5* induced Rosetta (DE3)/pET3c-SUMO-FGF23. **b** *Lanes 1–2* the precipitation and supernatant of Rosetta(DE3)/pET22b-SUMO-FGF23 were induced by 0.4 mM IPTG for 19 h at 16 °C; *M* protein molecular weight marker (in kilodalton, Fermentas). Analysis of the supernatant using 15% SDS-PAGE showed that the soluble SUMO-FGF23 was expressed 80% at 16 °C for 19 h in total supernatant protein

expressed in the inclusion body at 37 °C and 25 °C in our previous experiment (data not shown).

Purification and cleavage of SUMO-FGF23

According to the isoelectric point of fusion protein, DEAE Sepharose FF was chosen for the purification of SUMO-FGF23. Small amounts of host proteins were removed from SUMO-FGF23 after it was purified with a DEAE Sepharose FF column, except for most of the host DNA (ESM Fig. S1). A Ni-NTA affinity column was chosen for further purification. Proteins without 6× his tags were removed from the Ni-NTA resin using washing buffer containing 25 mM imidazole; SUMO-FGF23 was eluted using elution buffer containing 250 mM imidazole. SDS-PAGE analysis of samples taken from this step showed that the purity of SUMO-FGF23 reached 94% (Fig. 4a).

A SUMO protease recognition sequence immediately upstream of the target peptide allowed the latter to be released from the fusion with SUMO without leaving any unwanted residues upon treatment with SUMO protease. To achieve maximal release of FGF23 protein, the cleavage reaction was performed overnight at 4 °C. The efficiency of the cleavage was monitored by SDS-PAGE, and the slow disappearance of the band of the fusion protein at 37 kDa (Fig. 4b) was observed.

Isolation of recombinant rFGF23 from the cleavage mixture and further characterization of rFGF23 by Western blot and HPLC

When the target protein was fused directly to the C-terminus of SUMO, cleavage by SUMO protease 1 resulted in the release of the target protein with the desired N-terminal amino acid sequence. In our studies, after the purified fusion protein was diluted and cleaved by SUMO protease 1, the cleavage mixture was purified by Ni-NTA resin. SUMO, SUMO protease 1, and SUMO-FGF23 fusion protein containing his tags that were affiliated with Ni-NTA resin, but only rFGF23, flow through the column with the digestion buffer. Our results showed that rFGF23 was highly purified by SDS-PAGE (Fig. 5a) and could react with the human FGF23 polyclonal antibody by Western blot (Fig. 5b). HPLC analysis of the target protein showed a major peak of rFGF23, with a retention time of 14.770 min; the purity exceeded 90% (Fig. 5c). Also FGF23 protein was successfully identified by MALDI-TOF/TOF. Details of the protein was listed in ESM Fig. S2.

In vitro and in vivo activity assay of rFGF23

To test the activity assay of the rFGF23, we stimulated the glioma U251 cells with both rFGF23 and commercial FGF23 and then examined whether rFGF23 protein can induce the phosphorylation of 44/42 mitogen-activated protein (MAP) kinase (p44/42 MAPK) in the glioma U251 cells. As illustrated in Fig. 6a, the phosphorylation of MAPK were activated by either rFGF23 or commercial FGF23; also, the rFGF23-induced phosphorylation of ERK (141% activation, compared with negative-treated group) was more than that of commercial FGF23 (116% activation, compared with negative-treated group). The results suggested that the rFGF23 produced by SUMO fusion method had better biological activity than the commercial FGF23. Here, we speculate that the commercial FGF23 may be

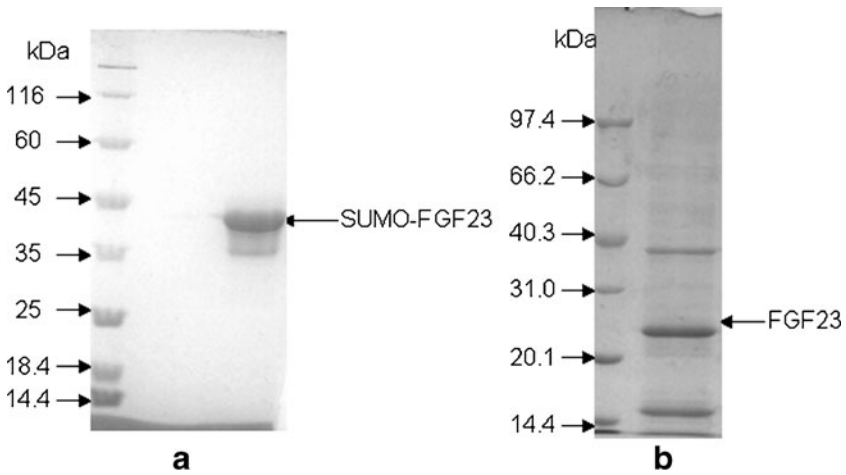

Fig. 4 SDS-PAGE analysis of purification and cleavage of SUMO-FGF23. After recombinant bacteria of Rosetta(DE3)/pET22b-SUMO-FGF23 were treated by sonication and centrifugation, the supernatants were loaded on a DEAE Sepharose FF and Ni-NTA orderly. The purification results for SUMO-FGF23 are shown in the **a**. **a** *M* protein molecular weight standard (in kilodalton, Fermentas); purified SUMO-FGF23 eluted from Ni-NTA column; the purified SUMO-FGF23 was digested by SUMO protease at 4 °C overnight. As seen in **b**, rFGF23 was cut from the SUMO-FGF23 by SUMO protease. **b** *M* protein molecular weight standard (in kilodalton, Takara); recombinant His_6-tagged SUMO-FGF23 fusion protein, rFGF23, and His_6-tagged SUMO as cleavage product, respectively

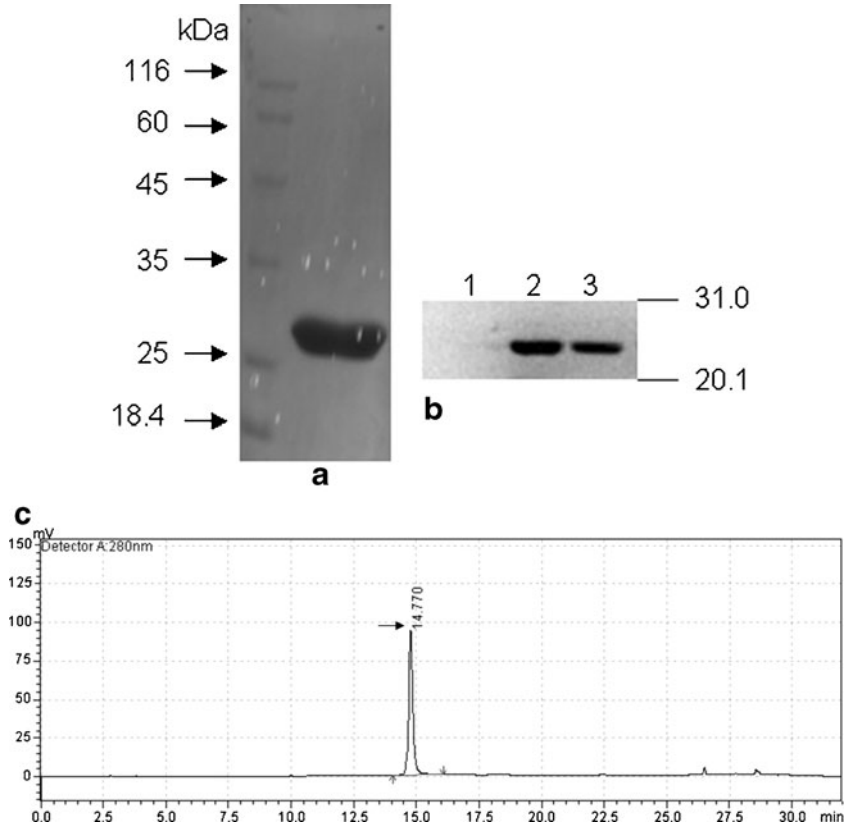

Fig. 5 SDS-PAGE analysis of purified rFGF23 and its characterization by Western blot and the purity by HPLC. **a** *Lane 1* protein molecular weight marker (in kilodalton, Fermentas); *lane 2* purified rFGF23. **b** Western blot analysis of the rFGF23. Following SDS-PAGE, Western blot analysis was performed as described in "Materials and methods" section; *lane 1* bacterial lysate of transformants uninduced by ITPG, as negative control; *lane 2* bacterial lysate of transformants induced by ITPG; *lane 3* purified rFGF23. **c** HPLC analysis of the purified rFGF23. The purity of rhFGF23 was further evaluated by HPLC analysis using a C18 column. As seen from the chromatogram, the *y*-axis indicates the absorbance, while the *x*-axis represents elution time (in minutes). The main peak eluted at 14.770 min. The purity of purified rhFGF23 was greater than 90%

expressed in the inclusion body in *E. coli* or in some other form that was not fused with SUMO.

To evaluate further the biological activity of rFGF23, we constructed the rat models as described in the "Materials and methods" section. According the reference of Goetz (Shimada et al., 2004), mice were administered with either rFGF23 or commercial FGF23 at 0.1 μg/kg (i.v.) in the experiments. Blood samples were collected 3 h before and 1, 2, and 3 h after injection. The plasma levels of phosphate were determined by a phosphomolybdic acid detection kit. The statistical results are described in the column graph shown in Fig. 6b. We can see that comparison of the phosphate levels before and after treatment disclosed that both rFGF23 and commercial FGF23 had hypophosphatemic effects over 1–3 h. Compared to commercial FGF23, however, rFGF23 afforded a better hypophosphatemic effect (Fig. 6b). The better hypophosphatemic effect of rFGF23 than commercial FGF23 was considered due to the increased bioactivity of rFGF23 produced by SUMO fusion method.

Discussion

The discovery of FGF23 not only revealed that there is a regulatory system of serum phosphate but also expanded the spectrum of endocrine diseases. It is necessary to develop methods for abundant production of FGF23 with specific bioactivity for specific treatment of FGF23-related diseases in the future; therefore, we have reported a novel strategy to express and purify recombinant hFGF23 greatly.

In our experiment, we have proved that FGF23 is least effective in expression properties as an unfused protein, regardless of either of the two host strain (data were not shown). Whereas, FGF23, when fused with SUMO, showed higher amount of expression, but only in the host strain of Rosetta(DE3) not BL21(DE3). Because on the one hand, SUMO have a chaperone-like activity, the translational fusion of SUMO to other proteins often results in significantly improving expression of those proteins in *E. coli* (Butt et al. 2005; Malakhov et al. 2004; Marblestone et al. 2006; Panavas et al. 2009; Zuo et al., 2005); on the other hand, Rosetta(DE3) strain can provide additional tRNAs for *E. coli* rare codons (AUA, AGG, AGA, CUA, CCC, GGA), thereby increasing FGF23 gene expression. Taken together, for protein efficient expression, both were indispensable.

Our data also showed that soluble SUMO-FGF23 concentration was over 25% of all protein at optimal expression conditions (induced by 0.4 mM IPTG for 19 h at 16 °C), compared to that when induced by 1 mM IPTG at 37 °C or 25 °C, most of SUMO-FGF23 protein was expressed in inclusion body (data were not shown). In fact lower

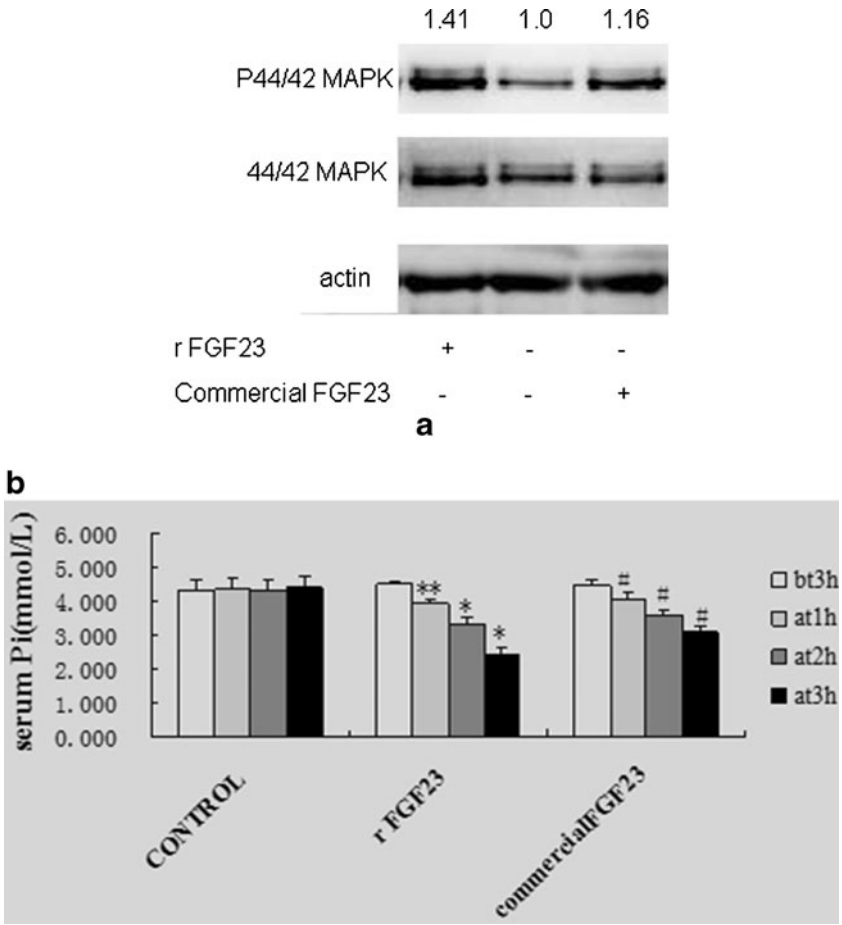

Fig. 6 In vitro and in vivo activity assay of rFGF23. **a** In vitro activity assay of rFGF23. The glioma U251 cell lines were treated with either rFGF23 or commercial FGF23 for 10 min. The protein levels of phosphorylation of 44/42 MAP kinase was examined by Western blot with actin as a loading control (*values on the top* of Western blots represent the mean optical density ratio in three independent experiments, vs. negative control group). **b** In vivo activity assay of rFGF23. After either rFGF23 or commercial FGF23 (0.1 μg/kg, i.v.) was administrated to Wistar adult male rats, blood samples were collected at 1, 2, and 3 h. Plasma phosphate concentrations were measured as described in "Materials and methods." Data point represent the mean±SD (n=10). (*p< 0.05, **p<0.01, vs. 3 h before the rFGF23 injection; #p<0.05 vs. 3 h before the commercial FGF23 injection; *bt* before treatment; *at* after treatment)

temperature and lower IPTG concentration indeed make protein fold significantly decelerated and increase soluble protein level. In addition, a long induction period is also necessary.

During the purification, in the presence of 2 M urea denaturants, both SUMO-FGF23 solution and its affinity with Ni-NTA were significantly altered, whereas, in the absence of 2 M urea, SUMO-FGF23 is not well soluble in solution, but also all flew through nickel column (our previous experiment have proved this point, data were not shown). We speculate that, most likely, FGF23 with 227 amino acids covered the six histidine sites before the SUMO tag with 108 amino acids. Adding 2 M urea into the buffer made the six histidine sites before the SUMO tag exposed, thereby increasing the affinity for Ni-NTA resin.

Because FGF23 is a systemic humoral factor, its renotropic function indicates that the kidney may bear a unique receptor specific for FGF23. Despite our previous attempts with the use of human kidney cell lines, phosphorylation responses were not well observed after rFGF23 treatment. We tried the glioma U251 cells for overexpressing FGFR1 to evaluate rFGF23 biological activity, and a better response is obtained (seen Fig 6a) although in the case of the absence of a Klotho receptor. Recent etiological studies indicate that high FGF23 levels are associated with increased mortality and cardiovascular dysfunction, both in patients with CKD and also in subjects with preserved renal function (Juppner et al. 2010; Shimada et al. 2004). Whether FGF23 has a direct action on cardiovascular tissues because Klotho is also not expressed there, the study of cell assay strategy is a good answer to the hypothesis put forward.

Many experiments have documented that FGF23 reduces serum phosphate mainly by the two mechanisms of both inhibiting proximal tubular phosphate reabsorption and intestinal phosphate absorption. Specific explanation is that FGF23 suppresses the expression of type 2a and 2c sodium–phosphate cotransporters which mediate the physiological phosphate reabsorption in proximal tubules (Shimada et al. 2004); the other is that FGF23 decreases the expression of 25-hydroxyvitamin D-1α-hydroxylase and at the same time enhances 25-hydroxyvtamin D-24-hydroyxlase expression (Shimada et al. 2004). By modifying the expression of these vitamin D metabolizing enzymes, FGF23 acts to reduce serum 1,25-dihydroxyvitamin D [1,25(OH)2D], which stimulates intestinal calcium and phosphate absorption. In our studies, we can also see that the changes in plasma phosphate levels all showed significant differences after either injecting rFGF23 or commercial FGF23, compared to

negative control, but administration of rFGF23 resulted in a time-dependent better reduction in circulating levels of phosphate than commercial FGF23. We speculated that different effects might be due to different expression form in *E. coli*.

In summary, rFGF23 was successfully expressed in *E. coli* Rosetta(DE3) from SUMO-FGF23 fusion gene. The resulting fusion protein could be cleaved by SUMO protease to give free rFGF23 and then isolated by the Ni-NTA affinity column. The identity of the purified protein was confirmed by Western blot, and its purity was determined by HPLC analysis. The recombinant FGF23 can induce phosphorylation of 44/42 MAP kinase (p44/42 MAPK) in the glioma U251 cell line. The results of animal experiments in vivo also showed that rFGF23 produced by using this method can decrease the concentration of plasma phosphate in normal rats fed with a fixed formula diet. This study demonstrated that SUMO, when fused with FGF23, was able to promote the soluble expression of the latter in *E. coli*, making it convenient to purify rFGF23 with its bioactivity preserved.

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