

# Expression, characterization, and preliminary X-ray crystallographic analysis of recombinant murine Follistatin-like 1 expressed in *Drosophila* S2 cells

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## Summary

The matricellular protein Follistatin-like 1 (FSTL1) has been shown to negatively regulate bone morphogenetic protein (BMP)/Smad1/5/8 signaling by functioning as an antagonist and has been implicated in physiological and pathological events including organogenesis, immunity and cardiovascular disease. It is therefore an attractive target for potential therapeutic intervention studies. In this study, we established a high-level expression system in *Drosophila* S2 cells which could produce about 12.5 mg of recombinant murine Follistatin-like 1 protein (rFSTL1) per liter of culture medium. The recombinant protein was then purified to greater than 95% purity using Ni-NTA agarose affinity chromatography followed by HiLoad 16/60 Superdex 200 gel filtration. The biological activity of rFSTL1 was evaluated by its ability to negatively regulate BMP/Smad1/5/8 signaling in cultured mink lung epithelial cells. Furthermore, we crystallized a truncated form of rFSTL1 containing the follistatin-like domain using the sitting drop vapor diffusion method. In conclusion, we have generated and purified biologically active recombinant FSTL1 protein, which will be important for further protein structure and drug discovery studies.

**Keywords:** Follistatin-like 1 (FSTL1), *Drosophila* S2 cells, affinity chromatography, biological activity, crystallization

## 1. Introduction

Members of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily regulate diverse biological cellular functions, such as cellular growth, differentiation and development. The activities and cellular signaling of the TGF- $\beta$  superfamily members are regulated through multiple mechanisms. For example, multiple extracellular binding partners for the TGF- $\beta$  family, such as decorin (1), follistatin (2,3), chordin and noggin (4), have been characterized as regulators of TGF- $\beta$

signaling. Follistatin-like 1 (FSTL1) is also a TGF- $\beta$  superfamily binding protein; it has recently been identified as a bone morphogenetic protein 4 (BMP4) antagonist controlling embryonic development in mouse (5-8) and zebrafish models (9,10). *In vivo*, FSTL1 is expressed temporally and spatially and is generally associated with tissues undergoing remodeling, either during normal developmental processes or in response to injury. Evidence has implicated FSTL1 in a number of pathologic conditions, including inflammation (11), rheumatoid arthritis (12-16), tumorigenesis (17-19), and heart disease (20,21). Functions of FSTL1 in the extracellular milieu are diverse and remain elusive.

FSTL1 is a small, secreted glycoprotein belonging to a group of matricellular proteins that mediate cell-matrix interactions but whose primary function is not structural (22,23). Its protein sequence, which is highly conserved throughout vertebrate evolution (> 92% sequence identity) (24), consists of an N-terminal region homologous to follistatin (FS domain), and a

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domain containing two EF-hand calcium-binding sites (EC domain) followed by a C-terminal domain with homology to the von Willebrand factor type C-like (VWC) domain (23,25). The domain structure of murine FSTL1 is shown in Figure 1A. The structure analysis shows that FSTL1 is a member of the Fst-SPARC protein family, members of which possess an FS domain and a pair of EF-hands. Other members of this group of proteins include follistatin and BM-40/SPARC/osteonectin. However, unlike follistatin, the FS domain of FSTL1 does not bind to activin (26), nor does its EC domain functionally bind to collagen as is the case for SPARC (23). The lack of conservation of important functional features common to several other members of the Fst-SPARC family indicates that FSTL1, despite its sequence homology to others, has evolved to acquire distinct properties. The structural characterization of FSTL1 has not been elucidated.

Involvement of FSTL1 in cardiovascular tissue regulation has been suggested (20,27-29). Its expression in adult heart is induced in response to injurious conditions that promote myocardial hypertrophy and heart failure (21,30,31). The systemic administration of an adenoviral vector expressing FSTL1 or overexpression of FSTL1 in mice protects the heart from ischemia/reperfusion injury or pressure overload-induced hypertrophy (20,21). Previous studies have shown that FSTL1 functions as an autocrine/paracrine regulatory factor and that the level of circulating FSTL1 is increased in patients with acute coronary syndrome, heart failure, and rheumatoid arthritis (16,31,32). Therefore, FSTL1 appears to be a clinically relevant secreted protein that has broad cardiovascular-protective activities. Targeting FSTL1 protein may provide a novel therapeutic approach for the treatment of patients suffering from the diseases mentioned above.

The objective of the current study is to generate and purify recombinant murine FSTL1 protein (rFSTL1) in an amount that is sufficient for biological studies, structural characterization and future antibody production. We report here the expression, purification, and characterization of full-length rFSTL1 expressed in *Drosophila* Schneider 2 (S2) cells. Moreover, we have obtained crystals of a truncated form of rFSTL1 that only contains the FS domain, which is critical for future structure study.

## 2. Materials and Methods

### 2.1. Materials

The PMT/BiP-HisA vector, pCoBlast vector, cellfectin, blasticidin, *Drosophila* S2 cells, and SFX-Insect medium were obtained from Invitrogen (Carlsbad, CA, USA). DNA polymerase was obtained from Roche Diagnostics (Basel, Switzerland). T4 DNA ligase, the pMD18-T vector, and NcoI and XhoI restriction

enzymes were purchased from Takara Biotechnology (Dalian, China). The Amicon Ultra centrifugal filters (10 kDa) used for buffer exchange and filtration of cell culture medium were from Millipore Corporation (Bedford, MA, USA). Ni-NTA-agarose beads were obtained from Qiagen GmbH (Hilden, Germany). BMP4 protein was purchased from PeproTech (Rocky Hill, NJ, USA). The BCA protein assay kit and ECL reagents were purchased from Pierce Biotechnology (Rockford, IL, USA). Anti-FSTL1, donkey anti-goat and goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-Smad1/5/8 and total-Smad5 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Screening kits were purchased from Hampton Research (Oklahoma city, OK, USA). All primers were synthesized by Invitrogen (Beijing, China).

### 2.2. Construction of murine *Fstl1* (*mFstl1*) expression plasmids

The *mFstl1* fragment, which was from 55 bp to 918 bp excluding the signal peptide sequence, was obtained by PCR amplifying using the pcDNA 3.1/myc-His (-) *A-mFstl1* plasmid (preserved by our laboratory and included a full-length *mFstl1* fragment) (6) as a template. An NcoI restriction site was added to the forward primer (5'-GAGGAGGAACCTAGAAAGCAA-3'), and an XhoI restriction site was added to the reverse primer (5'-GATCTCTTTGGTGTTCACCT-3'). The PCR reaction was carried out using the following reaction cycles: initial denaturation at 95°C for 5 min followed by 30 consecutive cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 7 min.

The amplified *mFstl1* gene was gel-purified using the High Pure PCR Product Purification Kit (Takara). After digestion of *mFstl1* with NcoI and XhoI, the purified product was inserted into the pMD18-T cloning vector. Positive clones were confirmed by restriction enzyme digestion and sequencing. pMD18-T-*mFstl1* plasmid was extracted from an overnight liquid culture derived from one positive clone. The plasmid was digested with NcoI and XhoI and cloned into the pMT/BiP-HisA vector (Figure 1A). This vector contains a metallothionein promoter, which allows for strong, expression of heterologous proteins in *Drosophila* S2 cells upon inducing with CuSO<sub>4</sub>. Finally, pMT/BiP-HisA-*mFstl1* plasmid was transformed into *Escherichia coli* (*E. coli*) DH5α strain for amplification of the recombinant plasmid, and positive colonies were selected. The pMT/BiP-HisA-*mFstl1* plasmid was purified and subjected to DNA sequence analysis.

### 2.3. Expression of rFSTL1 protein

*Drosophila* S2 cells were stably transfected with

the pMT/BiP-HisA-*mFstl1* plasmid using cellfectin according to the manufacturer's instructions. The S2 cells stably transfected with pMT/BiP-HisA vector were used as a control. To permit the selection of positive cell lines, the S2 cells were co-transfected with the pCoBlast selection vector with a ratio of 1:9, which confers blasticidin resistance. Stably transfected positive lines were established after 3 weeks of selection with blasticidin at 25 mg/L. For large-scale production of rFSTL1, cell lines were seeded at a density of approximately  $3-5 \times 10^6$  cells/mL, and expression was induced with  $\text{CuSO}_4$  at a final concentration of 0.5 mmol/L. The conditioned medium was harvested after 3 days.

#### 2.4. Purification of rFSTL1 protein

The conditioned medium (1 L) from stably transfected *Drosophila* S2 cells was centrifuged for 10 min at 3,000 g to pellet cells. The supernatant was filtered with a 0.22  $\mu\text{m}$  membrane and concentrated with Amicon Ultra spin centrifugal filters (Amicon Stirred Cell Model 8003). Subsequently, buffer containing 50 mmol/L Tris and 500 mmol/L NaCl (pH 8.0) was added to the supernatant. The supernatant containing rFSTL1 protein was subjected to Ni-NTA agarose affinity chromatography. The column was washed with at least 10 column volumes of buffer containing 50 mmol/L Tris, 500 mmol/L NaCl and 10 mmol/L imidazole and then eluted with buffer containing 50 mmol/L Tris, 500 mmol/L NaCl and 500 mmol/L imidazole. Elutions were analyzed using 12% SDS-PAGE. The rFSTL1 protein purified using Ni-NTA agarose affinity chromatography was then applied to a HiLoad 16/60 Superdex 200 gel filtration column (GE Healthcare, Uppsala, Sweden) at a flow rate of 1 mL/min after the column had been equilibrated with buffer containing 50 mM Tris-HCl (pH 8.0) and 500 mM NaCl. Ten fractions of 1.0 mL each were collected in the peak region and were then subjected to SDS-PAGE and Western blot analysis.

#### 2.5. Cell culture

Mink lung epithelial (Mv1Lu) cells were obtained from State Key Laboratory of Biomembrane and Membrane Biotechnology at Tsinghua University (Beijing, China) with the original source from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were maintained in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS (Hyclone, Logan, UT) and antibiotics in 5%  $\text{CO}_2$  at 37°C in a humidified atmosphere. The cells were divided into the following two treatment groups: (1) after transfection with the pc-Fstl1 plasmid (1  $\mu\text{g}$ ) or pcDNA3.1 (1  $\mu\text{g}$ ) for 24 h, the cells were starved in serum-free medium for another 24 h, and then treated with 20 ng/mL of BMP4 for an additional 30 min; (2) 100% confluent cells were

starved in serum-free medium for 24 h, and then treated with 20 ng/mL BMP4 together with 100 ng/mL rFSTL1 protein for an additional 30 min.

#### 2.6. SDS-PAGE and Western blotting

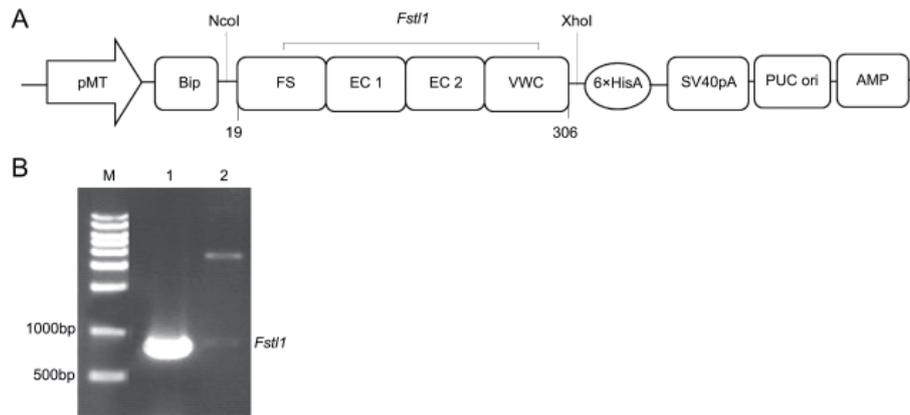
Protein samples were denatured under reducing conditions with  $\beta$ -mercaptoethanol at 100°C for 5 min and separated on a 12% SDS-PAGE gel followed by Coomassie blue staining.

Western blotting was performed as described previously (33). Equal amounts of conditioned medium from stably transfected *Drosophila* S2 cells was precipitated with 100% Trichloroacetic acid (TCA), washed with acetone twice, and then denatured under reducing conditions. The cells were rapidly washed with PBS and lysed using RIPA lysis buffer containing 1% NaF. Protein concentrations were determined using the BCA protein assay. Equal amounts of total protein were denatured under reducing conditions. Samples were separated on a 12% SDS-PAGE gel. The gels were electroblotted onto a PVDF membrane (Millipore). The membranes were blocked with 5% skim milk for 1 h at RT, before incubation with different primary antibodies at 4°C overnight. The antibodies were used to recognize the following proteins: FSTL1 (1:200), phospho-Smad1/5/8 (1:1000), and Smad5 (1:1000). Horseradish peroxidase-conjugated antibodies (goat anti-rabbit for phospho-Smad1/5/8 and Smad5, 1:5000; donkey anti-goat for FSTL1, 1:1500) were used as the secondary detection reagents and incubated with the immunoblots for 2 h at RT. Bands were visualized using ECL reagents.

#### 2.7. Crystallization and X-ray diffraction of rFSTL1

The rFSTL1 fusion protein expressed in *Drosophila* S2 cells contains a C-terminal 6xHis tag cleavable by TEV protease. After purification using Ni-NTA agarose affinity chromatography, the rFSTL1 fusion protein was digested with TEV protease to remove the 6xHis tag, and then separated with HiLoad 16/60 Superdex 200 gel filtration chromatography. The rFSTL1 protein without the C-terminal tag was used for crystallization trials.

Crystallization conditions were initially obtained by screening with a variety of screening kits using the sitting drop vapor diffusion technique at 290 K. Crystals were optimized from the initial conditions using the hanging drop vapor diffusion technique. Diffraction-quality crystals were obtained from a protein stock solution (2 mg/mL protein in a buffer containing 50 mM Tris (pH 8.0) and 0.2 M NaCl) that had been mixed with an equal volume of a reservoir solution (consisting of 0.1 M Tris, pH 8.5 and 1.2 M sodium citrate tribasic dehydrate). Additional cryoprotectants were not used prior to data collection. High-quality diffraction data were collected in an in-house X-ray facility at 100 K at a wavelength of 1.5418 Å using a Rigaku MM-007HF



**Figure 1. Construction of the *mFstl1* expression plasmid.** (A), Schematic representation of the pMT/BiP-HisA-*mFstl1* expression plasmid. The murine *Fstl1* cDNA sequence is preceded by the *Drosophila* metallothionein promoter (pMT). The secretion signal sequence of the *Drosophila* immunoglobulin, BiP, is included to promote secretion. Full-length murine FSTL1 is a 306 residue protein, while the fragment cloned into the expression vector is from 19 to 306 residues excluding the signal peptide. In addition, FSTL1 is composed of a follistatin-like (FS) domain, two EF-hand calcium-binding sites (EC domain), and a von Willebrand factor type C-like (VWC) domain. (B), Agarose gel electrophoretic analysis of the PCR product of the *Fstl1* gene and double enzyme digestion of the recombinant plasmid. Lane M, PCR DNA ladder; lane 1, the 876 bp PCR product of *Fstl1* without the signal peptide; lane 2, the 864 bp fragment produced by digestion of the pMT/BiP-HisA-*mFstl1* plasmid with NcoI and XhoI.

X-ray source equipped with an R-AXIS HTC image plate detector. The data set was processed using the HKL-2000 package.

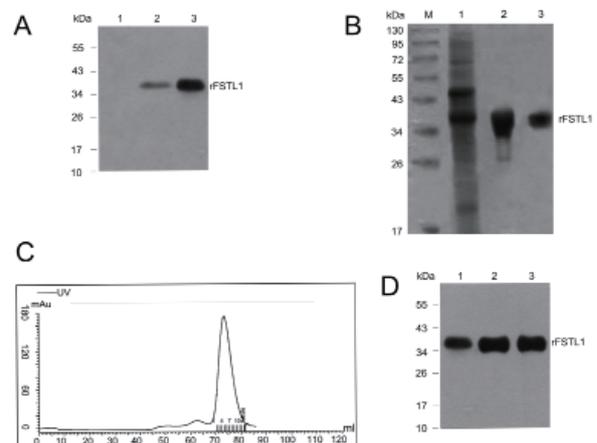
### 3. Results

#### 3.1. Construction of the rFSTL1 expression plasmid

We designed the expression plasmid, pMT/BiP-HisA-*mFstl1*, to encode the functional domain sequence of murine *Fstl1* without the signal peptide sequence. The overall design of this expression plasmid is illustrated in Figure 1A. A PCR product (876 bp) of the coding region of the *Fstl1* gene (Figure 1B, lane 1) was N-terminally fused in-frame to the vector-derived BiP secretion signal peptide rather than using the native signal sequence of the *Fstl1* gene. Constructing the plasmid in this way allows for secretable expression of the *Fstl1* gene driven by the signal sequence for the natural *Drosophila* BiP protein. The recombinant plasmid was able to produce the rFSTL1 protein with a C-terminal 6xHis tag fusion, which is convenient for the detection of rFSTL1 protein using anti-His antibodies. The polyhistidine tag of rFSTL1 can bind to Ni-NTA agarose and is used for effective purification. The identity of the recombinant plasmid pMT/BiP-HisA-*mFstl1* was confirmed by restriction enzyme analysis (Figure 1B, lane 2) and DNA sequencing. The sequence was identical to that published in GenBank (GenBank accession no. NM\_008047.5).

#### 3.2. Expression and purification of the rFSTL1 protein

Expression of the rFSTL1 protein was carried out using *Drosophila* S2 cells as the host system. *Drosophila* S2 cells stably overexpressing rFSTL1 were grown



**Figure 2. Expression and purification of rFSTL1 protein.** (A), Western blot analysis of rFSTL1 protein expression in *Drosophila* S2 cells. Lane 1, conditioned medium from control S2 cells; lane 2, conditioned medium from S2 cells expressing rFSTL1; lane 3, conditioned medium from CuSO<sub>4</sub>-treated S2 cells expressing a high level of rFSTL1. (B), SDS-PAGE analysis of the two-step purification of rFSTL1 protein. Lane M, protein molecular weight marker; lane 1, conditioned medium from CuSO<sub>4</sub>-treated S2 cells expressing a high level of rFSTL1; lane 2, rFSTL1 protein after Ni-NTA agarose affinity chromatography; lane 3, rFSTL1 protein after Ni-NTA agarose affinity chromatography followed by gel filtration using a HiLoad 16/60 Superdex 200 gel filtration column. (C), Size-exclusion column profile of the rFSTL1 protein. (D), Western blot analysis of purified rFSTL1 protein. Lane 1, conditioned medium from CuSO<sub>4</sub>-treated S2 cells expressing a high level of rFSTL1 detected by anti-mouse FSTL1 antibody; lane 2, rFSTL1 protein after Ni-NTA agarose affinity chromatography detected by anti-mouse FSTL1 antibody; lane 3, purified rFSTL1 protein after Ni-NTA agarose affinity chromatography followed by gel filtration using a HiLoad 16/60 Superdex 200 gel filtration column detected by anti-mouse FSTL1 antibody.

in serum-free medium, and rFSTL1 was induced by treatment with CuSO<sub>4</sub> for 3 days. Samples of the conditioned medium were harvested and subjected to Western blot analysis. As shown in Figure 2A,

a band representing rFSTL1 protein was detected with a molecular weight of about 37 kDa, the same molecular weight as previously reported (20). The robust production of rFSTL1 (approximately 12.5 mg/L) was evident in the conditioned medium of cells treated with CuSO<sub>4</sub>. No signs of degradation of rFSTL1 were visible on the Western blots of the conditioned medium.

After the 3-day CuSO<sub>4</sub> treatment, the culture medium was centrifuged, and the supernatant was collected for rFSTL1 purification. The supernatant was first filtered (0.22 μm) to remove particulate matter and was then concentrated by ultrafiltration using a 10 kDa molecular weight cut-off membrane. The ultrafiltration step effectively concentrated the supernatant, and also removed small impurities, such as pigment and aggregates. A two-step procedure was used to perform a large-scale purification of rFSTL1 from the concentrated supernatant of the culture. Ni-NTA agarose affinity chromatography was first used to capture rFSTL1 protein from the supernatant *via* binding to the 6xHis tag of the fusion protein (Figure 2B, lane 2). A HiLoad 16/60 Superdex 200 gel filtration column was then used to remove imidazole and other chemicals in the elution buffer (Figure 2B, lane 3). The position of the eluting peak from the gel filtration column indicated that rFstl1 existed as a dimer in solution (Figure 2C). Purification of 1 L of culture medium by Ni-NTA agarose affinity chromatography and HiLoad 16/60 Superdex 200 gel filtration yielded 3.75 mg of purified rFSTL1 protein, which represents a recovery yield of about 30% (Table 1). Analysis of a Coomassie-stained SDS-PAGE gel with a Tanon Gis digital image gel analytical system demonstrated that protein purity was > 95% (Figure 2B, lane 3). The protein recovery and purity of rFSTL1 at different purification steps are shown in Table 1. SDS-PAGE analysis showed one broad band of approximately 37 kDa (Figure 2B). The identity of the purified recombinant fusion protein was further confirmed by Western blot analysis with an anti-mouse FSTL1 antibody (Figure 2D).

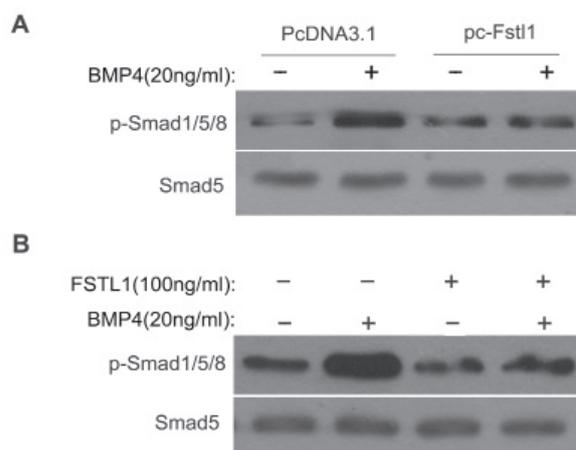
### 3.3. Biological activity of rFSTL1 protein

FSTL1 can function as a BMP4 antagonist and can negatively regulate BMP/Smad1/5/8 signaling during mouse embryonic lung development (6). To evaluate the anti-BMP4/Smad1/5/8 activity of our purified rFSTL1 protein, we used *in vitro* cultured Mv1Lu cells. The results were then compared with those obtained using FSTL1 overexpression. As shown in Figure 3, BMP4 activated downstream Smad1/5/8 signaling, as indicated by the increased level of phosphorylated-Smad1/5/8. As expected, BMP4-induced phosphorylation of Smad1/5/8 was inhibited in Mv1Lu cells that had been transiently transfected with the eukaryotic expression plasmid pcDNA3.1/myc-His(-)A-*mFstl1* (pc-Fstl1) (Figure 3A). BMP4-induced Smad1/5/8 phosphorylation was

**Table 1. Analysis of the rFSTL1 purification process from 1 L conditioned medium from *Drosophila* S2 cells**

Purification step	rFSTL1 (~ mg)	Purity (~ %)	Recovery (~ %)
Supernatant	12.50	ND	100
Ultrafiltration	10.40	ND	83
Ni-NTA agarose affinity chromatography	8.30	85	66
HiLoad 16/60 Superdex 200 gel filtration column	3.75	95	30

ND: Not determined.



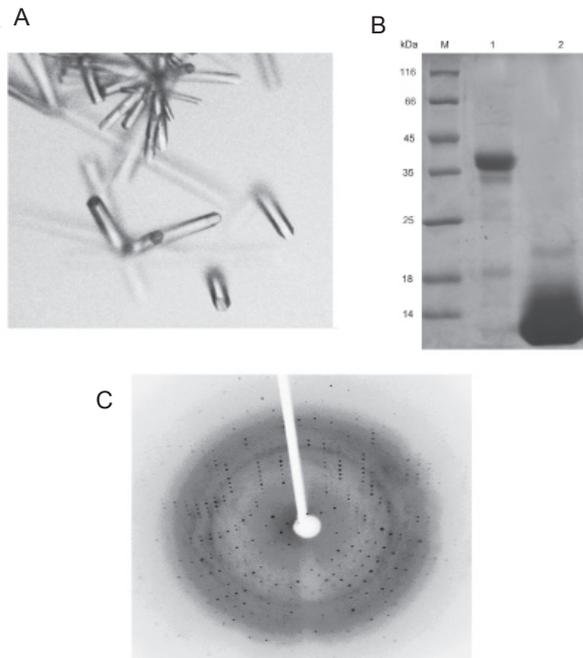
**Figure 3. Biological activity analysis of the purified rFSTL1 protein in Mv1Lu cells. (A)**, BMP4-induced phosphorylation of Smad1/5/8 was inhibited in Mv1Lu cells overexpressing pc-Fstl1 (1 μg). **(B)**, BMP4-induced phosphorylation of Smad1/5/8 was inhibited by application of exogenous purified rFSTL1 protein (100 ng/mL) in Mv1Lu cells.

also inhibited in cultured Mv1Lu cells treated with exogenous rFSTL1 protein (100 ng/mL) (Figure 3B), suggesting that the 37 kDa form of rFSTL1 can function in a similar manner to endogenously overexpressed FSTL1. Thus, the ability of rFSTL1 to negatively regulate BMP4/Smad1/5/8 signaling is similar to that of FSTL1.

### 3.4. Crystallization and X-ray diffraction of rFSTL1

Crystal screening of the full-length form of rFSTL1 was performed using the sitting drop vapor diffusion method. Diffraction-quality crystals appeared after approximately 4 days under the conditions described in the methods section. In a SDS-PAGE check of resolubilized crystals of rFSTL1, the degradation of protein was obvious (Figures 4A and 4B). The band corresponded to a degradation fragment of about 10 kDa derived from the 37 kDa full length protein. By N-terminal amino acid sequencing, this fragment was confirmed to cover the follistatin-like domain of rFSTL1.

The best crystals of the truncated form of rFSTL1 diffracted to approximately 2.5 Å using an in-house Rigaku X-ray source (Figure 4C). The crystals were



**Figure 4. Crystals and diffraction pattern of the truncated form of rFSTL1.** (A), Crystal of the truncated form of rFSTL1. (B), SDS-PAGE analysis of the dissolved crystals. Lane M, protein molecular weight marker; lane 1, full-length rFSTL1; lane 2, the truncated form of rFSTL1. (C), Diffraction pattern of a crystal of the truncated form of rFSTL1.

hexagonal, belonging to the space group  $P6_3$ , and had one molecule per asymmetric unit; the unit cell dimensions were  $a = b = 45.58 \text{ \AA}$ ,  $c = 68.97 \text{ \AA}$ ,  $\alpha = \beta = 90^\circ$ , and  $\gamma = 120^\circ$ . Assuming that each molecule was 10 kDa in the asymmetric unit, the solvent content was approximately 42%.

The follistatin-like domain of rFSTL1 contains 10 cysteine residues constituting 5 pair of disulfide bonds. By taking advantage of the anomalous signal of sulfur, the structure was solved by the single wavelength anomalous diffraction (SAD) method. Preliminary analysis of the structure revealed that the follistatin-like domain of rFSTL1 folded similarly to that of follistatin. A dimer formed through a 2-fold symmetric axis could be identified, but its correlation with its function needs to be further clarified. The detailed analysis of the crystal structure will be discussed elsewhere (Li *et al.*, unpublished data).

#### 4. Discussion

The targeting of TGF- $\beta$ /BMP signaling may be a favorable clinical strategy for the treatment of various diseases. However, inhibition of TGF- $\beta$  signaling may have adverse effects because TGF- $\beta$  superfamily members are highly pleiotropic cytokines. Recently, a significant effort has been made to identify and target tissue- or disease-specific mechanisms of activation of TGF- $\beta$  superfamily members for use in clinical

therapies. FSTL1 is a TGF- $\beta$ -induced protein and functions diversely in the extracellular milieu, which makes it an excellent candidate as a drug target for potential therapeutic intervention. In the current report, we have generated a *Drosophila* S2 cell line stably expressing functional domains of murine FSTL1, and we have purified the recombinant protein using Ni-NTA agarose affinity chromatography followed by gel filtration using a HiLoad 16/60 Superdex 200 gel filtration column. The resulting protein was greater than 95% pure with a yield of 3.75 mg/L. Characterization of the purified product showed that it possessed BMP4 antagonist activity. Furthermore, we performed crystal screening of rFSTL1; a truncated form of rFSTL1 containing the follistatin-like domain was crystallized using the sitting drop vapor diffusion method.

Previous reports have described the expression and purification of recombinant FSTL1 (12,23,24,27). *E. coli* strains were used as the expression host in some of these reports; however, in some of these studies, the expressed recombinant FSTL1 protein had less biological activity due to incomplete post-translational modification of FSTL1 in the prokaryotic system (12,24,27). Recombinant forms of FSTL1 were also expressed in human cells and used to compare their structural and functional properties with those described for other members of the FST-SPARC protein family (23), but the biological activity assay for recombinant FSTL1 was not performed. Recently, Ouchi and colleagues, as well as our group, produced active recombinant FSTL1 protein in insect cell lines (*Lepidopteran* Sf9 or *Drosophila* S2 cells) respectively (34). In this study, we reported in detail the production of large amounts of highly purified and functional rFSTL1 using *Drosophila* S2 cells. Expression of recombinant proteins in insect cell hosts is advantageous because it permits production of posttranslationally modified eukaryotic proteins in large amounts and in a relatively short period of time. Moreover, in the present *Drosophila* S2 expression system, the pMT/BiP-HisA expression vector contained the metallothionein (MT) promoter, which allowed for high levels of FSTL1 expression when induced by copper sulfate ( $\text{CuSO}_4$ ) (35). In addition to the MT promoter, the vector also contained a BiP secretion signal, which promoted secretion of FSTL1 containing proper posttranslational modifications, such as glycosylation (36).

We have previously evaluated the role of rFSTL1 protein in the negative regulation of BMP4/Smad1/5/8 signaling in human alveolar epithelial (A549) cells (6). Here, we demonstrated the role of rFSTL1 in another lung epithelial cell line, Mv1Lu cells, suggesting that the biological activity of rFSTL1 is not dependent on cell type. We prepared rFSTL1 protein in amounts sufficient for use in biochemical characterization studies and for future antibody production.

rFSTL1 proteins expressed in *Drosophila* S2 cells

contain highly flexible, glycosylated fragments that dramatically interfere with crystallization. In this report, even with thorough screening, only a degraded fragment of FSTL1 could be crystallized until now. Structure determination of FSTL1 is currently in progress using the molecular replacement method. It will be necessary to thoroughly optimize the crystallization conditions to obtain crystals of the full-length protein. The success in identifying crystallization conditions for rFSTL1 will be essential to our efforts to characterize FSTL1 both structurally and functionally and to elucidate the mechanisms by which FSTL1 exerts its biological functions.

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