



Expression and purification of soluble single-chain Fv against human fibroblast growth factor receptor 3 fused with Sumo tag in *Escherichia coli*



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ABSTRACT

Background: Overexpression or mutated activation of Fibroblast growth factor receptor 3 (FGFR3) is involved in the pathogenesis of many tumors. More and more studies focus on the potential usage of therapeutic antibodies against FGFR3.

Results: In this study, a novel single-chain Fv (ScFv) against FGFR3 was prepared and characterized. To achieve the soluble expression, ScFv was fused with Sumo (Small ubiquitin-related modifier) by polymerase chain reaction (PCR), and cloned into pET-20b. The recombinant bacteria were induced by 0.5 mM Isopropyl-β-D-thiogalactopyranoside (IPTG) for 16 h at 20°C, and the supernatant liquid of Sumo-ScFv was harvested and purified by Ni-NTA chromatography. After being cleaved by the Sumo protease, the recombinant ScFv was released from the fusion protein, and further purified by Ni-NTA chromatography. The purity of ScFv was shown to be higher than 95% and their yield reached 4 mg per liter of bacterial culture. *In vitro* data showed that ScFv can significantly attenuate FGF9-induced phosphorylation of FGFR3.

Conclusion: We provide a novel method to produce soluble expression and bioactive functions of ScFv in *Escherichia coli*.

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1. Introduction

Fibroblast growth factors (FGFs), a family of more than 20 known members, play important roles in regulating cell proliferation, differentiation and survival during embryo development and adult stage [1]. FGF receptor (FGFR) mediated FGF-induced signaling pathway. As one of FGFRs, FGFR3 was involved in many diseases, especially cancer. Deregulation of FGFR3 was highlighted and defined in many types of cancer, such as multiple myeloma (MM) [2], hepatocellular carcinoma [3] and bladder cancer [4]. Enhancing FGFR3 signaling promotes tumor growth, metastasis and resistance to drug. Thus, FGFR3 has been considered as a novel therapy target for several tumors, such as multiple myeloma [5] and bladder cancer [5,6].

Antibody is considered as one of the most effective strategies against tumor antigens. The single chain Fv (ScFv), containing a variable heavy chain (V_H) and a variable light chain (V_L) from monoclonal antibody

had been considered as a novel therapeutic antibody, and used to kill tumors in recent years, such as anti-Her2 ScFv [7], anti-VEGF ScFv [8] and anti-CA125 ScFv against epithelial ovarian cancer [9].

In recent ten years, many monoclonal antibodies or ScFvs against FGFR3 have been reported on their anti-tumor activity *in vitro* and *in vivo*. One kind of monoclonal antibody against FGFR3 inhibits phosphorylation of FGFR3 and downstream signaling ERK activation in MM cells [10]. ScFv against FGFR3 was shown to block the binding of FGF9 and FGFR3, and inhibits proliferation of bladder cancer cell line RT112 *in vitro* in a dose- and FGF-dependent manner [11]. Immunotoxin (ScFv-fused toxin) against FGFR3 was reported to inhibit proliferation of bladder cancer cell line RT112 *in vitro* and exhibited significantly anti-tumor activity in RT112 tumor xenografts by inducing cell apoptosis [12].

The *Escherichia coli* expression system is the principle choice to get recombinant ScFv, however, recombinant ScFv often forms inclusion body using traditional *E. coli* expression method [13,14]. To overcome these shortcomings, we chose a novel expression system, small ubiquitin-related modifier (Sumo) molecular partner. Sumo covalently attached to other proteins to play roles of post-translational modifications [15], including significantly increasing

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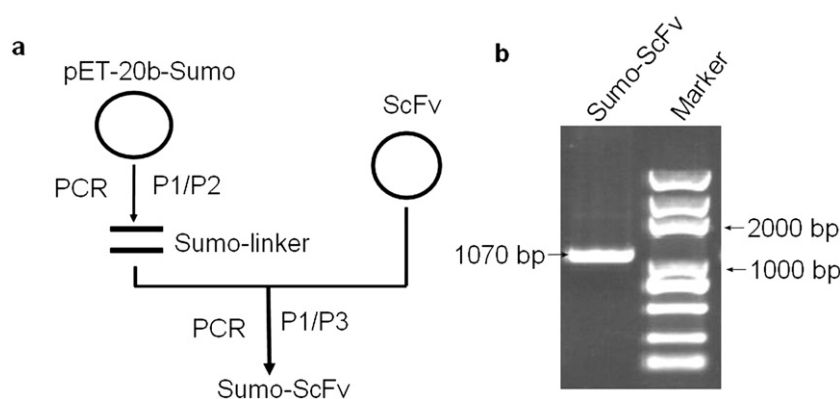


Fig. 1. Design and synthesis of Sumo-ScFv by PCR. (a) Sumo-ScFv was designed according to the schematic illustration. Firstly, Sumo-linker was created by PCR using P1 and P2. Secondly, Sumo-ScFv was then synthesized by PCR using Sumo-linker and ScFv as templates, P1 and P3 as primers. The final PCR product of Sumo-ScFv was shown in panel (b) and its molecular weight is about 1070 bp.

the yield of recombinant protein, correctly facilitating target protein folding, and promoting protein solubility [16,17], so Sumo has become an effective biotechnological tool as a fusion system in *E. coli*, by which many recombinant proteins, such as FGF21 [18], pramlintide [19], ScFv-9R [20], acquired better expression.

In the present study, we construct expression plasmid containing Sumo and ScFv against FGFR3. The recombinant expression plasmid was induced by IPTG in *E. coli* and purified by Ni-NTA. Further, ScFv was released from Sumo-ScFv cleaved by Sumo enzyme. Our results showed that Sumo is very helpful for promoting the soluble expression of ScFv in *E. coli*, and the resulting recombinant bioactive ScFv can be used for therapeutic applications and clinical diagnosis of patients in the future.

2. Materials and methods

2.1. Reagents, enzymes, antibodies, bacterial strains

Prime STAR®GXL DNA Polymerase was purchased from TaKaRa Company (Japan). Restriction enzymes *Nde*I and *Xho*I were provided by NEB (England). Ni-NTA agarose was purchased from GE healthcare (Sweden). Anti-His tag antibody was provided from Proteintech (Chicago). p-FGFR3 (Tyr724) antibody was purchased from Santa Cruz (USA). Anti-FGFR3 antibody was obtained from Abcam (USA). Anti-beta-actin antibody was purchased from Cell Signaling (USA). *E. coli* DH5α and *E. coli* BL21 (DE3) strains were obtained from Invitrogen (USA). The *E. coli* expression plasmid pET-20b-Sumo

containing Sumo fragment was kept in Biochemistry lab, College of Basic Medical Science, Jilin University. Dr. Qi Xiang from Jinan University generously supplied us with the Sumo protease.

2.2. Design of fusion genes and constructions of pET-Sumo-ScFv

The ScFv gene was constructed in a V_H -linker- V_L format according to the prior report [19] and US patent (8101721B2), and synthesized by ZoonBio Biotechnology Co (China). The primers used for the construction of fusion gene containing ScFv and Sumo fragment were designed as follows: P1 (GGAATTCATA TGCATCATCATCATCA TCACG) was designed according to the 5' terminal sequence of Sumo fragment; P2 (5'-CTCTGCTGCAGCTGCACCTGACCACC AATCTGTT CTCTGT3') contained 3' terminal sequence of Sumo and 5' terminal sequence of ScFv. P3 (CCGCTCGAGTTACTGGCCAGCACGGTCAGT) primer was designed according to the 3' terminal sequence of ScFv. As shown in Fig. 1a, Sumo-linker was created by PCR using P1 and P2 as forward and reverse primers, using pET-20b-Sumo as a template. PCR parameters consisted of 5 min of Prime STAR®GXL DNA Polymerase activation at 98°C, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 15 s, extension at 68°C for 30 s, and then a final single extension at 68°C for 5 min.

Further, the Sumo-ScFv was generated by PCR, using P1 and P3 as the forward and reverse primers, using Sumo-linker and ScFv as templates. The fusion gene was digested with *Nde*I and *Xho*I, and inserted into the pET-20b. Finally, the identified recombinant plasmid was confirmed by DNA sequencing (Sangon, Shanghai).

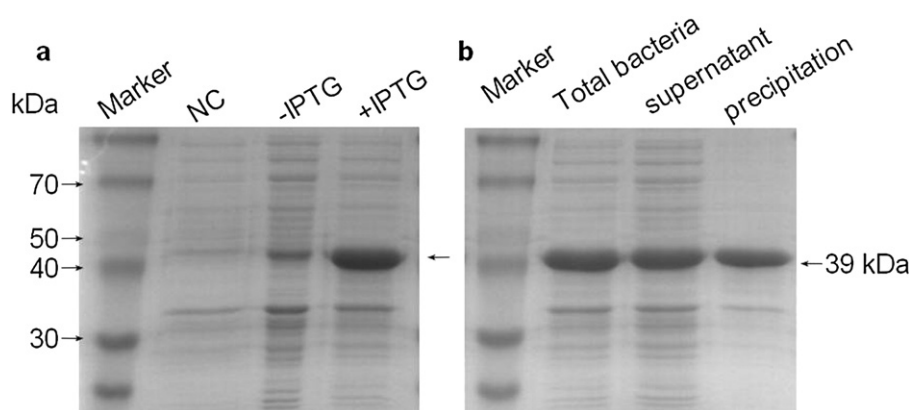


Fig. 2. The inducible expression of recombinant Sumo-ScFv and soluble analysis by SDS-PAGE. Bacterial cells containing Sumo-ScFv were induced by 0.5 mM IPTG for 16 h induction at 20°C, then the cell pellets were collected by centrifugation and protein was extracted by sonication and centrifugation. Both soluble and insoluble fractions were analyzed by 12% SDS-PAGE. Compared with negative control (NC) and without IPTG group, Sumo-ScFv highly expressed in recombinant bacteria by IPTG induction (panel (a)). The panel (b) showed total bacteria, supernatant and precipitation of bacterial containing Sumo-ScFv.

2.3. Expression and soluble detection of recombinant Sumo-ScFv

A single colony from *E. coli* BL21 (DE3) harboring pET-Sumo-ScFv was grown overnight in the LB medium (1% peptone, 1% yeast extract, and 0.5% sodium chloride, pH 7.0), and incubated in a shaker at 37°C. A 3 ml aliquot of resulting culture was inoculated into 300 ml fresh LB medium and incubated at 37°C and 220 rpm until OD₆₀₀ was 0.6 to 0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 0.5 mM final concentration for 16 h induction at 20°C. The cell pellets were collected by centrifugation and lysed by sonication. The supernatants were harvested by centrifugation at 12,000 × g for 30 min at 4°C, and the remaining pellets (insoluble fraction) containing inclusion bodies were resuspended into an equal volume of lysis buffer. Both soluble and insoluble fractions were analyzed by 12% SDS-PAGE [18].

2.4. Purification of Sumo-ScFv

The cell lysate of Sumo-ScFv was purified by Ni-NTA chromatography. The Ni-NTA resin was washed with wash buffer I (20 mM Tris-HCl, pH 8.0) until OD₂₈₀ of effluent reached base line. The proteins without 6His-tag were eluted from the column with wash buffer II (20 mM Tris-HCl containing 20 mM imidazole, pH 8.0). Finally, 6His-tagged Sumo-ScFv protein was harvested from the column with elution buffer (20 mM Tris-HCl containing 200 mM imidazole, pH 8.0). The purity of Sumo-ScFv was assessed by SDS-PAGE and its concentration was determined with BCA Protein Assay Kit.

2.5. Cleavage of Sumo from Sumo-ScFv and further purification

The Sumo-ScFv protein was diluted to a concentration of 1 mg ml⁻¹. 10U Sumo protease were added to the dilution in salt buffer (20 mM Tris-HCl, pH 7.0), and the mixture was incubated overnight at 4°C, then the cleaved sample was applied to a Ni-NTA resin column. The recombinant ScFv without His tag was washed directly, but Sumo and Sumo protease containing His-tag were bound to the Ni-NTA resin column. Finally, the recombinant ScFv was desalted overnight with dialysis bag at 4°C.

2.6. Western blot analysis

The immunogenic activity of purified Sumo-ScFv was assayed by Western blot. Total cellular protein was boiled in an equal volume of sample loading buffer, a Tris-HCl buffer (pH 6.8) containing 20% glycerol, 2.5% SDS, 10% β-mercaptoethanol and 0.005% bromophenol blue. Protein samples were electrophoresed on 12% of SDS-PAGE, and then electrophoretically transferred onto PVDF membrane. The nonspecific binding of transferred membrane was blocked with 5% non-fat milk powder overnight. The membrane was incubated with a polyclonal anti-His tag antibody (1:1000), then washed and incubated with a 1:1000 dilution of secondary HRP-conjugated antibody. Immunoreactive bands were visualized using an ECL kit.

2.7. Mass spectrometry analysis

The specific band of ScFv from the SDS-PAGE gel was pooled and analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS). This technical service was supported by Beijing Protein Institute Co. Ltd.

2.8. Inhibition of FGFR3 signaling by ScFv in RT112 cells

RT112 cells were grown overnight in DMEM medium containing 10% FBS, 100 U ml⁻¹ ampicillin and 100 U ml⁻¹ streptomycin at 37°C of cell incubator. Before stimulation, cells were firstly starved with DMEM medium without FBS in the absence or presence of ScFv for 14 h, and then stimulated with 10 ng ml⁻¹ FGF9 for 30 min. Then total

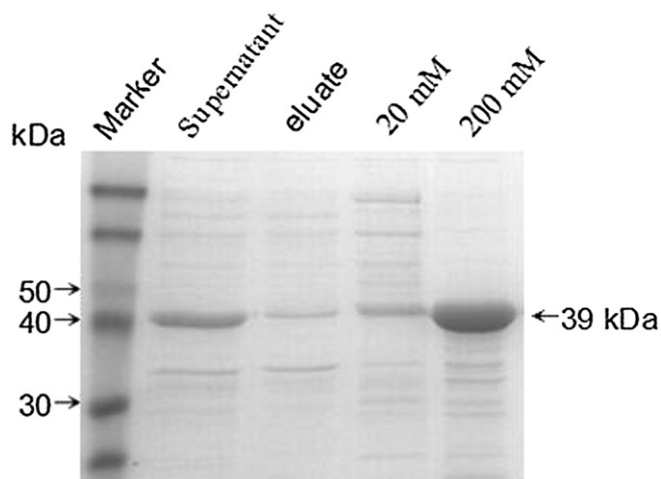


Fig. 3. The purification of recombinant Sumo-ScFv through Ni-NTA. The recombinant protein of Sumo-ScFv supernatant shown was loaded on a Ni-NTA and washed with different concentrations of imidazole. SDA-PAGE analysis showed the purification of recombinant Sumo-ScFv from eluant of 20 mM and 200 mM imidazole, as well as Sumo-ScFv supernatant and balance elute.

protein from different treated RT112 cells was harvested and analyzed by SDS-PAGE, and followed by Western blot with specific antibody against p-FGFR3 and FGFR3. Beta-actin was detected as a loading control.

3. Results and discussion

3.1. Construction of pET-Sumo-ScFv expression plasmid

The gene sequence of ScFv was synthesized by Zoonbio Biotech Co. (China) and its size is 738 bp. The fusion gene containing Sumo and ScFv was generated according to the design of Fig. 1a by PCR. As shown in Fig. 1b, the PCR product of Sumo-ScFv is about 1070 bp. Further, Sumo-ScFv was double digested and cloned into pET-20b plasmid. The Sumo-ScFv sequence which came from the recombinant plasmid was confirmed by DNA sequencing, and results showed that it

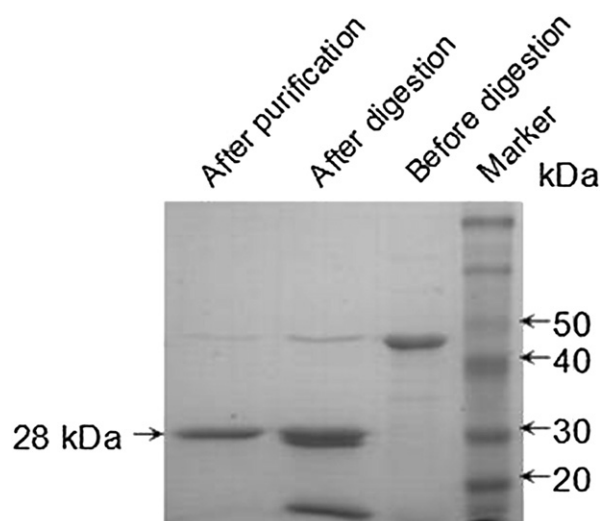


Fig. 4. The digestion of recombinant Sumo-ScFv by sumo protease and further purification through Ni-NTA. The purified Sumo-ScFv was digested by Sumo protease for 10 h at 4°C, and then applied to the Ni-NTA column. The Sumo and Sumo protease containing 6His-tag were affiliated by Ni-NTA resin, only ScFv was eluted from the column when washed off the resin with elution buffer. The results of digestion and purification were shown by SDS-PAGE.

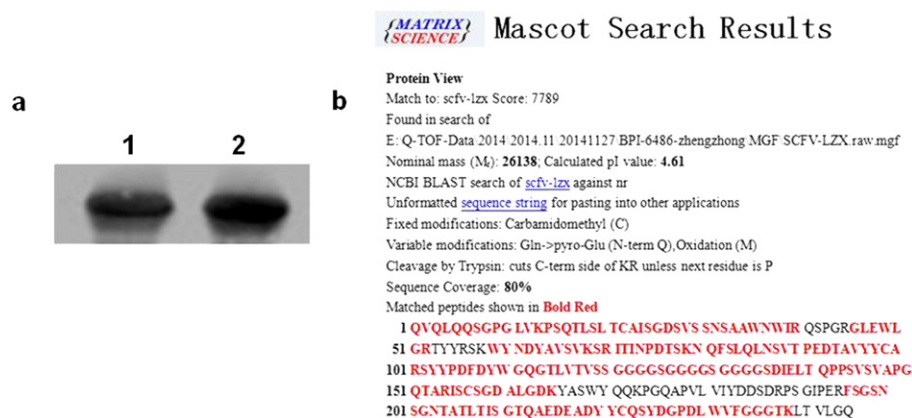


Fig. 5. The identification of recombinant Sumo-ScFv and ScFv. The recombinant Sumo-ScFv was detected with anti-His tag antibody by Western blot (panel (a)). Both lane 1 and lane 2 are recombinant Sumo-ScFv. As shown in panel (b), the ScFv which was removed Sumo by sumo protease was assayed by Mass spectrometric method. The peptides in bold red highly match with recombinant ScFv, in which its sequence coverage reaches 80%.

was in conformity with the expected sequence. The sequence of fusion gene can be found in GenBank (accession number KP405837).

3.2. Expression and purification of Sumo-ScFv

E. coli cells harboring Sumo-ScFv were induced by 0.5 mM IPTG for 16 h at 20°C, then the cell pellets were harvested by centrifugation and protein was extracted and separated by sonication and centrifugation. The supernatants and precipitate were collected and performed to 12% SDS-PAGE analysis. As shown in Fig. 2a, the expression of a 39 kDa protein which is similar to the predicted size was induced by IPTG, compared with negative control (blank plasmid) or recombinant bacteria without IPTG induction.

To detect the soluble expression of recombinant Sumo-ScFv, the supernatant and precipitate of cell lysate were both analyzed by SDS-PAGE and the results showed Sumo-ScFv highly expressed in the fraction of both supernatant and precipitate, but soluble fractions (supernatant) exceed 50% of total target protein. Previous studies showed that recombinant ScFv often forms inclusion body using traditional *E. coli* expression method [13,14]. Our results suggested that Sumo may be very helpful to promote soluble expression of recombinant ScFv.

To acquire high purity recombinant protein, Ni-NTA was applied to the purification because 6His-tag was located in the N-terminal of Sumo. Different concentrations of imidazole were tried to elute the recombinant protein. The results showed that Sumo-ScFv was efficiently eluted from Ni-NTA column using elution buffer containing

200 mM imidazole. SDS-PAGE analysis showed that the purity of Sumo-ScFv exceeds 90% (Fig. 3).

3.3. Further purification and determination of ScFv after cleavage of Sumo

To cleave Sumo fragment from the fusion protein, the purified Sumo-ScFv was digested by Sumo protease for 10 h at 4°C, and then applied to the Ni-NTA column. The Sumo and Sumo protease containing 6His-tag were affiliated by Ni-NTA resin, only ScFv was eluted from the column when washed off the resin with elution buffer. As shown in Fig. 4, SDS-PAGE analysis showed that more than 90% recombinant Sumo-ScFv was efficiently cleaved by Sumo protease. Because ScFv has no His tag, Sumo fragment from Sumo-ScFv could be separated from ScFv by Ni-NTA column. SDS-PAGE analysis showed a 26 kDa band of ScFv, and its purity exceeds 95%. Since the 6His-tag and start codon were put in the N-terminal of Sumo-ScFv, the recombinant ScFv has a natural amino acid sequence without any tags after cleavage of Sumo. This showed that Sumo expression system is a feasible method to acquire natural recombinant protein without any tags.

3.4. Identification of recombinant Sumo-ScFv and ScFv

To assay the authenticity of fusion protein, the immunoactivity of purified Sumo-ScFv was performed with anti-his tag antibody by Western blot due to 6His-tag designed in the N-terminal of Sumo. The results revealed that Sumo-ScFv (Fig. 5a, lane 1 and lane 2) could specifically react with anti-his tag antibody.

The immunoactivity of ScFv could not be performed because there are no specific antibodies or antigens against recombinant ScFv. To confirm the authenticity of ScFv, ScFv was successfully identified by Mass spectrometric method and detailed data was shown in Fig. 5b. The results indicated that the data of score and sequence coverage both match the amino acid sequence of ScFv.

3.5. ScFv specifically inhibits FGFR3 signaling in RT112 cells

To investigate whether ScFv has bioactivity against FGFR3 positive cells, the bladder cancer RT112 cells which highly expressed FGFR3 were stimulated by FGF9 in the absence or presence of ScFv. The phosphorylation of FGFR3 which represents receptor kinase activity was detected by Western blot with specific antibody. The results showed that FGF9 can stimulate FGFR3 phosphorylation significantly and ScFv efficiently attenuates FGF9-induced FGFR3 phosphorylation (Fig. 6). The results also displayed that ScFv can specifically inhibit FGFR3 signaling pathway by competing FGFR3 with FGFs. In Martínez-Torrecuadrada's [11] study, several human ScFvs specifically bound FGFR3 were found

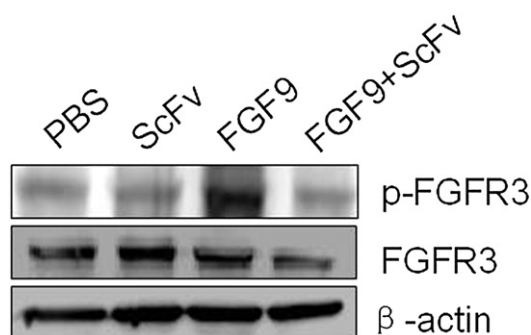


Fig. 6. Inhibition of FGFR3 signaling pathway by ScFv-9R. RT112 cells were starved with DMEM medium without FBS in the absence or presence of ScFv for 14 h, and then stimulated with 10 ng ml⁻¹ FGF9 for 30 min. Then total protein from different treated RT112 cells was harvested and analyzed by SDS-PAGE, and followed by Western blot with specific antibody against p-FGFR3 and FGFR3. Beta-actin was detected as a loading control.

and had competition activity with FGF9 by blocking FGFR3 activation and inhibited RT112 cell proliferation. Next, we also deeply study ScFv functions in blocking proliferation, migration and invasion of FGFR3 positive cancer cells. In recent years, tumor-specific immunoliposome has become a novel delivery tool to carry siRNA drug or chemical drug [21]. It's possible that our ScFv will be used to make immunoliposome for tumor target therapy.

4. Conclusions

In this study, we report that the expression, purification and characterization of a novel ScFv antibody against FGFR3. This procedure which fused with Sumo tag promotes the soluble expression of recombinant ScFv and acquires natural and bioactive protein.

Conflict of interests

We declare that there is no conflict of interest regarding the publication of this work.

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