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# Soluble expression of human insulin-like growth factor-1 with the assistance of SUMO fusion partner

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**ABSTRACT**

*Insulin-like growth factor-1 (IGF-1) has become a particularly attractive therapeutic target because of its role in various physiological processes, including the regulation of cellular proliferation. However, high-level expression and purification of recombinant IGF-1 (rIGF-1) in Escherichia coli (E. coli) is hindered by its incorporation into inclusion bodies in the bacteria. To overcome this problem, IGF-1 was fused to small ubiquitin-related modifier (SUMO) and subsequently transformed into E. coli Rosetta (DE3). After induction with 0.5 mM isopropyl-1-thio-β-galactopyranoside for 4 h at 37 ℃, SUMO-IGF-1 concentration reached 26.2% of the total protein. We then purified the fusion protein with affinity chromatography and used SUMO protease to release rIGF-1 from the column. The purity of rIGF-1 was shown by high performance liquid chromatography to be greater than 95%. Mitogenic activity assays showed that the purified rIGF-1 stimulated the proliferation of NIH-3T3 cells in a dose-dependent manner. These findings demonstrate that fusion of SUMO to IGF-1 enhances its solubility and purification. In conclusion, we acquired sufficient, soluble expression, bioactive of rIFG-1 in E.coli, which can then be used for clinical applications.*

**Key words:** SUMO fusion, soluble expression, Human insulin-like growth factor-1, Mitogenic activity

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## INTRODUCTION

Insulin-like growth factor-1 (IGF-1) is one of the most important growth factors in the growth hormone (GH)-IGF axis. IGF-1 is a single polypeptide protein consisting of 70 amino acids with a molecular weight of 7,649 Da. IGF-1 is an anabolic hormone produced in the liver that is known to stimulate proliferation and differentiation of many cell types and plays an important role in tissue renewal and repair[1]. Because of its regulation of somatic growth, it has been shown to be involved in various physiological processes in mammals[2-4] .

IGF-1 is currently used in treatment of dwarfism[5-7], diabetes[8] and osteoporosis[9]. Although the therapeutic effects of IGF-1 are promising, its treatment is very expensive. It is necessary to prepare sufficient bioactive recombinant IGF-1 (rIGF-1) for both clinical applications as well as biomedical research. Hunt et al was able to express IGF-1 in CHO mammalian cells[10], but it is clear that this expression system is more complicated and costly. The prokaryote *Escherichia coli* (*E. coli*) are the primary choice for recombinant protein expression because of its efficiency and cost-effectiveness. However, the expression of IGF-1 in *E. coli* has proven to be difficult because of its incorporation into inclusion bodies[11, 12] or disulfide-linked aggregates[13]. Yeast expression systems have the advantage of glycosylation, but the glycosylation is not always correct[13, 14]. Therefore, optimization of IGF-1 soluble expression from *E. coli* is likely the most effective option.

Recently, small ubiquitin-related modifier (SUMO) has become an effective biotechnological tool as a fusion system to enhance the soluble expression of proteins and decrease proteolysis degradation[15, 16]. After purification, SUMO is enzymatically cleaved from the desired protein by SUMO C-terminal hydrolases-isopeptidases[15] Various proteins, such as SARS virus protein[17], MMP13[18], EGF[16], metallothionein[19], KGF2[20], and FGF21[21], have been successfully expressed and purified using this fusion strategy.

Therefore, in this study we aimed to acquire a kind of soluble and more economical recombinant IGF-1 by fusing SUMO to human IGF-1 protein in *E. coli.*

## EXPERIMENTAL SECTION

### Reagents

Restriction enzymes, DNA polymerase, were purchased from Dalian Takara Co. Ltd (Dalian, china). All primers were synthesized by Beijing BGI Co. Ltd (Beijing, China). The pUC-57 vector containing human IGF-1 cDNA sequence was purchased from Proteintech Group Inc. (WuHan, China). DEAE-Sepharose Fast F, Ni-NTA and Sephadex G-25 were obtained from GE Health (Piscataway, U.S.A). Methylthiazoletetrazolium (MTT) and Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma-Aldrich (St. Louis, U.S.A). Fetal bovine serum was purchased from Hyclone (Los Angeles, U.S.A). Polyclonal mouse anti-IGF-1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### Construction of the Protein Expression Vector

The strategy for the construction of two kinds of rIGF-1 recombinant plasmid is illustrated in Figure 1. First, we amplified the histidine-SUMO fusion gene (SUMO fused to a histidine tag) from the pET-28a-SUMO plasmid using S1 and S2 as the forward primer and reverse primer, respectively. Second, the IGF-1 gene (GenBank accession NO: CAA01954.1) was obtained from the pUC-57 plasmid using I1 and I3 as the forward and reverse primer pair. Finally, using the recovery fragments as the templates, the full-length fusion gene was amplified using S1 as the forward primer and I3 as the reverse primer. This amplicon was digested with Nde I and BamH I and then ligated into previously digested pET3c to create the SUMO-IGF-1 fusion protein expression vector: pET3c-SUMO-IGF-1.

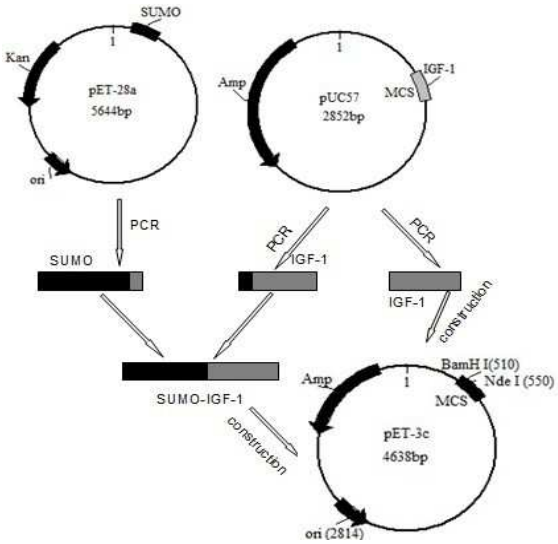


**Fig. 1 Schematic illustration of the generation of SUMO-IGF-1 and IGF-1. From cDNAs comprising human IGF-1 and SUMO, five primers were designed and synthesized. As shown in Fig. 1, SUMO-IGF-1 and IGF-1 were generated by PCR. The detailed steps are provided in the "Material and methods". Briefly, IGF-1 and SUMO were amplified using PCR, from which SUMO-IGF-1 was further amplified in a second round of PCR. The final IGF-1 and SUMO-IGF-1 constructs were digested and ligated into the pET-3C expression**

The second IGF-1 recombinant plasmid was generated by amplifying the IGF-1 gene without fusion to SUMO using I2 as the forward primer and I3 as the reverse primer. The amplicon was digested with Nde I and BamH I and then ligated into previously digested vector pET3c to create the IGF-1 fusion protein expression vector: pET3c-IGF-1. Automated DNA sequencing was performed to confirm the accuracy of the inserted DNA segment.

### Expression and soluble screening of IGF-1 in E. coli

The two IGF-1 expression vectors constructed above were transformed into competent Rosetta (DE3) cells. The transformants were grown in 5 mL Luria broth (LB) medium containing 100 μg/mL ampicillin at 37°C. When the $OD_{600}$ reached 0.8, isopropyl-thio-β-galactopyranoside (IPTG) was added to a final concentration of 0.5 mM. The

culture was incubated at 37℃ 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 rIGF-1 was determined by densitometer scanning. The colony with the highest expression level was used in the subsequent scale-up culture.

After induction, the bacteria were harvested by centrifugation at 20000 rpm for 5 min at 4℃. Cell pellets were suspended in Tris–HCl buffer at a concentration of 20 mM and lysed by sonication. The suspensions were centrifuged at 20000 rpm for 30 min at 4℃. 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 (20mM Tris-HCl buffer and 8M urea, pH 7.8). Both soluble and insoluble fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

**Purification of SUMO-IGF-1**

All purification procedures were carried out at 4°C. The frozen cell pellet was thawed and resuspended in ice-cold 20 mM Tris–HCl buffer (pH 7.8) containing 1 mM EDTA– 2Na and 0.05% Tween 80 at a ratio of 1 g cell pellet to 8 ml Tris–HCl buffer. The cell suspension was sonicated in an ice bath and the insoluble pellet was removed by centrifugation at 20,000rpm for 15 min. The supernatant was filtered through a 0.45-μm membrane and loaded onto a DEAE Sepharose FF column .The column was washed with three bed volumes of 20 mM Tris–HCl buffer (pH 7.8) until the $OD_{280}$ of the eluent reached baseline conditions. Proteins were eluted by 20 mM Tris–HCl buffer (pH 7.8) with step gradients of 0.2 and 0.4 M NaCl. Pooled fractions were applied to a nickel nitrilotriacetic acid resin (Ni-NTA). The column was washed with three bed volumes of 20 mM Tris–HCl buffer (pH 7.8) containing 0.3M NaCl. Contaminating proteins were eluted from the column with wash buffer (20 mM Tris–HCl containing 0.3 M NaCl and 25 mM imidazole, pH=7.8). Finally, SUMO-IGF-1 protein was eluted from the column with elution buffer (20 mM Tris–HCl containing 0.3 M NaCl and 200 mM imidazole, pH=7.8). Samples taken at the elution peak were pooled. The purity of SUMO-IGF-1 was assessed using SDS-PAGE, and the concentration was tested by the Bradford method.

**Release of the target protein by SUMO protease cleavage**

Fractions were pooled and further desalted with a Sephadex G-25 column. The resulting protein solution was treated with SUMO protease to release the recombinant IGF-1. In a standard cleavage reaction, 50 μg of fusion protein (25 kDa) in a 50-μl digestion buffer (50 mM Tris–HCl containing 0.15 M NaCl and 1 mM DTT, pH=7.8,) was incubated overnight with five units of SUMO protease at 4℃. The result of the cleavage reaction was monitored by SDS-PAGE. After SUMO protease cleavage, the reaction mixture contained N-terminal SUMO, IGF-1 target protein, SUMO-IGF-1 fusion protein, and SUMO protease. The reaction mixture was passed through a Ni-NTA column to remove the SUMO, SUMO-IGF-1, and SUMO protease. Free rIGF-1 was desalted with a Sephadex G-25 column and analyzed with western blot analysis using a polyclonal mouse anti-IGF-1 antibody for immunoblotting according to the manufacturer's protocol.

For high performance liquid chromatography (HPLC) analysis, the purified rIGF-1 was loaded onto a $C_{18}$ column. The elution was conducted using a linear gradient of 30–70% acetonitrile at a flow rate of 1 mL/min in the presence of 0.1% trifluoroacetic acid.

**Bioassay for Mitogenic Activity of IGF-1**

NIH-3T3 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml ampicillin and streptomycin. $8\times10^3$cells per well were transferred to a 96-well plate and incubated at 37°C for 24 h. The medium was replaced with DMEM supplemented with 0.4% FBS and the cells were cultured for 24 h. The cells were treated with1280ng/ml IGF-1 and commercial IGF-1 diluted with serum-free DMEM to a serial of concentrations ranged from 0.3 to 1200ng/ml, and incubated for 48 h. The number of viable cells was determined by adding 20 μl methylthiazoleterazolium (MTT) (5 mg/ml) to each well and incubated for 4 h. After removal of the medium, 150 μl dimethyl sulfoxide (DMSO) was added to each well. The plate was kept at room temperature for 30 min, and then the $OD_{570}$ was measured immediately.

Statistical analysis values are expressed as mean ± standard error of the mean. Comparisons of mean values between two time points were performed using the Student's *t*-test. *P*-values less than 0.05 were considered significantly different. All experiments were repeated at least three times.

## RESULTS AND DISCUSSION

### Generation of IGF-1 and SUMO- IGF-1 expression vectors

**Table 1 PCR primers for amplifying the IGF-1 and SUMO- IGF-1 fusion genes**

| Prime name (size) | Sequence(5'-3') |
|---|---|
| S1 (32) | GGAATTC*CATATG*CATCATCATCATCATCACG |
| S2 (32) | TTTCCGGGCCCATACCACCAATCTGTTCTCTG |
| I 1 (27) | GATTGGTGGTATGGGCCCGGAAACCCT |
| I 2 (30) | GGAATTC*CATATG*GGCCCGGAAACCCTGTG |
| I 3 (24) | CG*GGATCC*TTATCATGCCGATTTC |

To generate the full-length IGF-1 and the SUMO-IGF-1 fusion gene, we designed five specific primers (Table 1). Our strategy is described in Figure 1. The PCR products of IGF-1, SUMO, and SUMO-IGF-1 are shown in Figure 2. The results showed that the human IGF-1 (219 bp) and SUMO-IGF-1 (559 bp, SUMO = 340 bp) were their expected size. The final PCR products (the full-length IGF-1 and SUMO-IGF-1) were digested with two restriction enzymes (Nde I and BamH I) and ligated into the expression vector pET3c. The sequence of the target gene was confirmed by automated DNA sequencing.

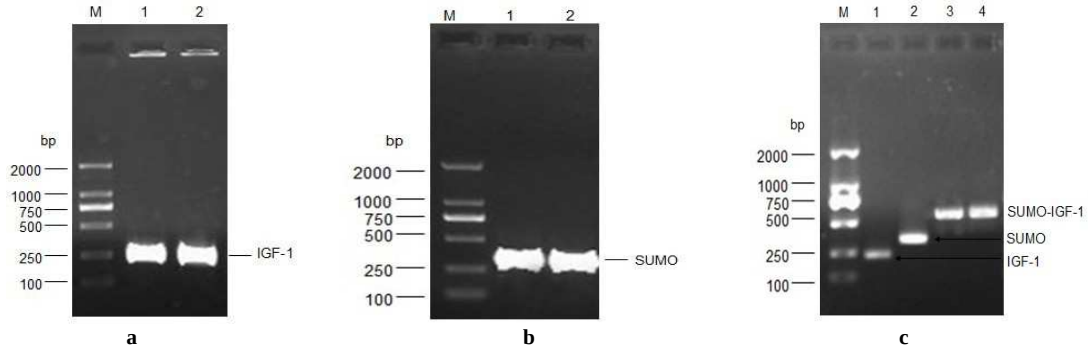


**Fig. 2 Verification of IGF-1 and SUMO-IGF-1 by PCR analysis. After amplification by PCR, the sizes of IGF-1 and SUMO-IGF-1 were verified. (a) The molecular weight of the IGF-1 PCR product and control IGF-1. Lane 1, PCR product; lane 2, control IGF-1 (219 bp). (b) The PCR product of SUMO. Lane 1, PCR product; lane 2, control SUMO (340bp). (c) Re-amplification of IGF-1 (lane 1) and SUMO (lane 2), and synthesis of SUMO-IGF-1 fusion gene (lanes 3 and 4, 559 bp)**

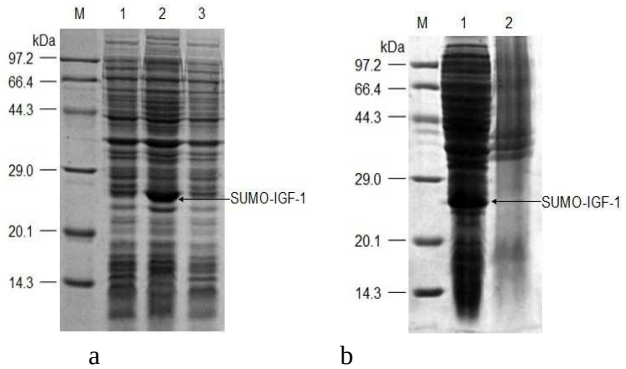


**Fig. 3 Expression screening of SUMO-IGF-1 during the optimization of induction conditions. (a) IGF-1 and SUMO-IGF-1 were transformed into E. coli strain Rosetta (DE3). Four hours after IPTG induction, bacterial lysates were analyzed by 12% SDS-PAGE and CBB staining. Lane 1 was un-induced Rosetta (DE3)/pET3c-SUMO-IGF-1; lane 2 was induced Rosetta (DE3)/pET3c-SUMO-IGF-1; lane 3 was induced Rosetta (DE3)/pET3c-IGF-1. The molecular weight of SUMO-IGF-1 is correct at approximately 25 kDa. (b) SUMO-IGF-1 was found exclusively in the soluble fraction after 4 h induction with 0.5 mM IPTG at 37°C. Lanes 1 and 2 were the soluble and insoluble faction, respectively**

### Expression screening and optimization of induction conditions for soluble IGF-1

In these experiments, we screened for the expression of IGF-1 and SUMO-IGF-1 in Rosetta (DE3)-transformed cells. The results demonstrated that SUMO-IGF-1 expressed markedly better than IGF-1 alone (Fig. 3a). We then optimized the induction conditions for soluble rIGF-1. Recombinants were inoculated in fresh LB medium and

incubated in a shaking incubator at 37℃ until the $OD_{600}$ reached 0.8–1.0. IPTG was the added to a final concentration of 0.5 mM for 4 h at 37℃ to induce expression. The cells were collected by centrifugation and lysed by sonication. The supernatants and pellets were collected and analyzed using 12% SDS-PAGE. The results showed that the molecular weight of the expression product was 25 kDa, which corresponds to the predicted size of SUMO-IGF-1. The target protein was more than 25% of the total cellular protein, and the soluble fraction was as much as 95% of the total expressed recombinant protein (Fig. 3b).

**Purification and cleavage of SUMO-IGF-1**

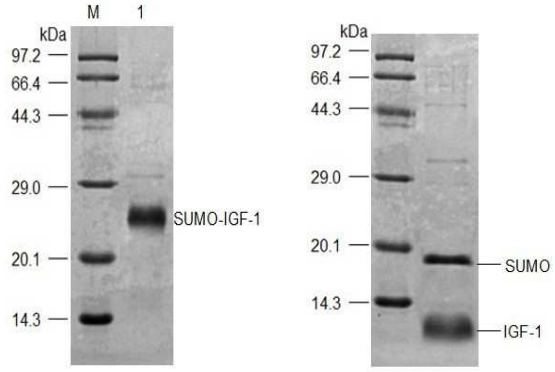


**Fig. 4 Analysis of purified and cleaved SUMO-IGF-1 by SDS-PAGE. After 4 h IPTG induction at 37 ℃, Rosetta (DE3)/ pET3c-SUMO-IGF-1 were sonicated and centrifuged. The supernatants were loaded onto DEAE Sepharose FF and Ni-NTA columns sequentially. (a) The purification of SUMO-IGF-1 was verified by 12% SDS-PAGE and CBB staining. M was protein molecular weight standard (kDa); lane 1 was purified SUMO-IGF-1 eluted from the Ni-NTA column. (b) The purified SUMO-IGF-1 was digested by SUMO protease 1 at 4 ℃overnight, efficiently releasing IGF-1 from SUMO-IGF-1. M was protein molecular weight standard (kDa); lane 1 was Histamine-tagged SUMO and IGF-1**

According to the isoelectric point of the fusion protein, DEAE Sepharose FF was chosen for the purification of SUMO-IGF-1. Approximately half of the host proteins were removed from SUMO-IGF-1 after it was purified with a DEAE Sepharose FF column. Because SUMO was histidine-tagged, a Ni-NTA affinity column was used for further purification. Contaminating proteins were removed from the Ni-NTA resin using a wash buffer containing 25 mM imidazole. SUMO-IGF-1 was eluted from the resin using an elution buffer containing 200 mM imidazole. SDS-PAGE analysis of samples taken from this step showed that the purity of SUMO-IGF-1 reached 95% (Fig. 4a). A SUMO protease recognition sequence immediately upstream of the target peptide allowed IGF-1 to be released from the fusion protein by cleavage with SUMO protease. To achieve maximal cleavage, the reaction was performed overnight at 4°C. The efficiency of the cleavage was monitored by SDS-PAGE (Fig. 4b). Isolation of recombinant rIGF-1 from the cleavage mixture and further characterization of rIGF-1 by western blot and HPLC .After cleavage with SUMO protease 1, the cleavage mixture was incubated with Ni-NTA resin. SUMO, SUMO-IGF-1, and SUMO protease 1 were bounded to the Ni-NTA resin. And only rIGF-1 flowed through the column with the digestion buffer. Our results showed that rIGF-1 was highly purified (Fig. 5a) and could react with the human IGF-1 polyclonal antibody by western blot (Fig. 5b). HPLC analysis of the target protein showed a major peak of rIGF-1, with a retention time of 11.534 min; the purity exceeded 95% (Fig. 5c).

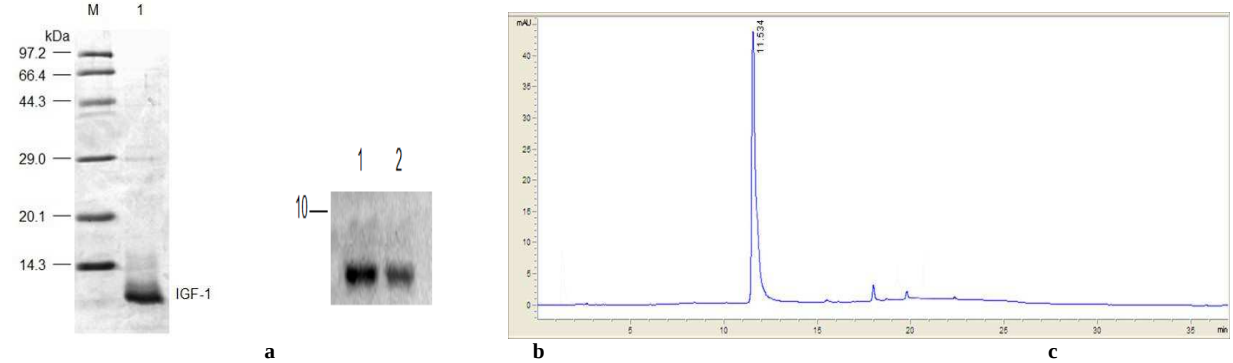


**Fig. 5 SDS-PAGE analysis of rIGF-1 and its characterization by HPLC. (a) rIGF-1 was analyzed by 12% SDS-PAGE and CBB staining. M was protein molecular weight marker (kDa); lane 1 was rIGF-1. (b) Western blot analysis of rIGF-1. Following SDS-PAGE, Western blot analysis was done used an anti-IGF-1 antibody. Lane 1 was commercial IGF-1, as a positive control; lane 2 was rIGF-1. (c) The purity of rIGF-1 was further evaluated by HPLC analysis using a $C_{18}$ 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 11.534 min. The purity of purified IGF-1 was greater than 95%**

**Mitogenic activity assay**

The biological activity of rIGF-1 was determined by its ability to stimulate the proliferation of NIH-3T3 cells. We found that rIGF-1 was able to stimulate the proliferation of NIH-3T3 cells in a dose-dependent manner (Fig. 6), comparable to that achieved with commercial IGF-1. These results also demonstrate that the biological activity of rIGF-1 was approximate to the commercial IGF-1.

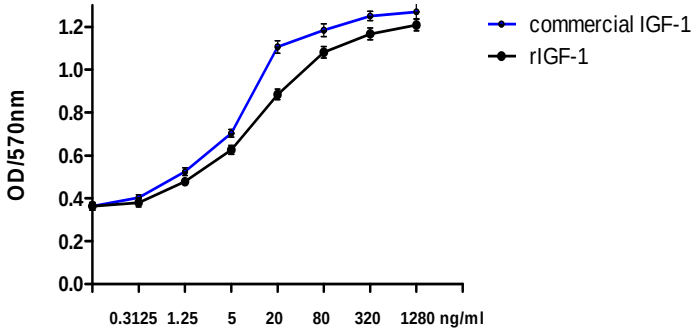


**Fig. 6 Proliferative activity of IGF-1 on NIH-3T3 cells. NIH-3T3 cells were incubated with commercial IGF-1 or rIGF-1 at the indicated concentrations for 48 h. Cell proliferation was determined by the MTT cell proliferation assay. Data presented are the mean (±SDs) of triplicate tests**

## DISCUSSION

Given its important function for growth and metabolism, as well as its association with a spectrum of disorders, IGF-1 has received much attention. Studies showed that low doses of IGF-1 significant improved intestinal absorption[22], hypogonadism[23], and liver functions[24] in liver cirrhosis rats . However, its needs a daily doses of 1.5 to 2 mg IGF-1 in liver cirrhosis patients replacement therapy[25].Thus, a liver cirrhosis patient needed a good deal of IGF-1. Since it's very difficult to obtain from nature, its treatment was costly. In recent years, a lot of researchers tried to acquire IGF-1 by other methods. Hunt et al. expression IGF-1 in CHO cells[10], Henry et al. acquired IFG-1 in transgenic chloroplasts[25].They were either expensive or needing a long production cycle. Kim et al. expressed IGF-1 in Escherichia coli[26].However, its protein presented as inclusion bodies. And its difficulty in purification has hindered its clinical applicability. Therefore, we have reported a novel strategy to enhance the expression and purify of recombinant IGF-1. Compared with native IGF-1, SUMO-IGF-1 showed markedly higher protein expression. This is likely due to the chaperone-like activity of SUMO, which maintains IGF-1 in a soluble state. The fusion of SUMO to other proteins has also shown improved expression of those proteins in *E. coli*[27]. Otherwise, in this study after IGF-1 was released from the fusion protein, it didn't have methionine at the N-terminal of IGF-1. Thus it's highly similar to native IGF-1.

pET vectors were extensively used in prokaryotic expression system. They adopted T7 RNA polymerase to selective activate T7 phage promoter. T7 RNA polymerase is about five-fold faster than E.coli RNA polymerase when they transcribed mRNA. pET-3c vector as an efficient vector had been successfully achieved relative proteins in Escherichia coli [28, 29]. Fortunately, we also acquired high-level productive SUMO-IGF-1 through pET-3c vector. Because of the rare codons (AUA, AGG, AGA, CUA, CCC, and GGA) present in SUMO, we chose the Rosetta (DE3) strain of *E. coli* for a more efficient expression system. Liu et al. had previously demonstrated that the Rosetta (DE3) strain was more effective than the BL21(DE3) strain for the expression of SUMO-FGF23[27]. In this study, we have shown that soluble SUMO-IGF-1 comprised over 25% of all cellular protein at optimal expression conditions (0.5 mM IPTG induction for 4 h at 37°C) in Rosetta (DE3) cells.

Recently, SUMO has become a commonly used N-terminal fusion partner to enhance functional protein production in prokaryotic expression systems because it can significantly improve protein stability and solubility[30]. Then it could be directly used for DEAE Sepharose FF column or Ni-NTA affinity column to purification after dissolved in crude extract. And its purity reach higher that added DEAE Sepharose FF column before used Ni-NTA affinity column to purification. Compared with inclusion bodies, its purification didn't need the difficult process of denaturation and renaturation. Thus it was minimized the operation and time of purification. Simultaneously, its biological activity was maintained at a high degree. Furthermore, the SUMO moiety can be conveniently removed by SUMO protease 1 with remarkable fidelity and efficiency [15, 17]. If the target protein is fused directly to the C-terminus of SUMO, it will be released with the desired N-terminal amino acid sequence when cleaved by SUMO protease 1[18, 31].

## CONCLUSION

In summary, rIGF-1 was successfully expressed in *E. coli* Rosetta (DE3) as a SUMO-IGF-1 fusion protein. The fusion protein was produced as a water-soluble type with a high yield. The biological activity of purified IGF-1 was verified by stimulating the proliferation of NIH-3T3 cells in a dose-dependent manner comparable to commercial IGF-1. This suggests that this expression system may be a more efficient and economical alternative to produce bioactive IGF-1 that can be used in both clinical and research applications.

**Acknowledgments**
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