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Expression Strategy of Soluble Recombinant Human TGF-β3 in *Escherichia coli*: sfGFP -Fusion Tag

Sema BİLGİN *[1]

Abstract

Transforming growth factor-beta 3 (TGF-β3) is an important cytokine involved in various biological processes. TGF-β3 is used as a scar-reducing antifibrotic agent for acute and chronic wounds and fibrosing disorders. TGF-β3, a valuable therapeutic protein, is produced recombinantly in different expression systems. TGF-β3, produced in the *Escherichia coli* (*E. coli*) expression system, widely used due to its various advantages in recombinant production, is commercially available. However, the main problem encountered in protein expression in *E. coli* cells is the formation of an inclusion body. Various approaches have been developed to solve this problem. The use of a fusion tag is one of the most powerful strategies used to obtain protein in the soluble active form in the *E. coli* expression system. Superfolder GFP (sfGFP) is one of the fusion tags used to increase the solubility of the fusion partner in *E. coli*. In this study, TGF-β3 with sfGFP fusion tag (sfGFP-TGFβ3) was successfully produced in soluble form in *E. coli* BL21 (DE3) in high yield and purity for the first time. Purified protein was identified by western blot and SDS-PAGE. 20 mg of protein with 98% purity was obtained from 1 L of bacterial culture. It was determined that the obtained high purity protein did not have a cytotoxic effect on BJ normal human skin fibroblast cells. The impact of sfGFP-TGFβ3 fusion protein on wound healing was evaluated with *in vitro* scratch wound healing assay. The results showed that the sfGFP-TGFβ3 fusion protein produced in soluble form in the *E. coli* expression system has the potential to support the wound healing process.

Keywords: Escherichia coli, fusion tag, inclusion body, sfGFP, TGF-β3, recombinant protein

1. INTRODUCTION

Transforming growth factor-beta 3 (TGF-β3) is a multifunctional growth factor. TGF-β3, which is involved in cell proliferation and differentiation, is a valuable therapeutic protein used in treating various diseases, especially in wound healing and scar reduction [1, 2]. The mature active form of human TGF-β3 is 13-kDa and does not contain glycosylation or other post-translational modifications [3, 4].

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There are many studies on the use of TGF-β3 as a clinical therapeutic protein due to its antifibrotic effect and scar-free healing properties [5-7]. Research on the mechanisms of action of TGF-β3 in chronic wounds and fibrosis disorders has gained momentum in recent years. For these reasons, research on recombinant production and the high yield of TGF-β3 in soluble form has become the focus of attention.

Human TGF-β3 is produced recombinantly in eukaryotic [2, 4] and prokaryotic [8] expression systems. Eukaryotic expression systems are low-throughput, methodologically complex, and expensive [9]. Bacterial expression systems offer significant advantages, especially in the large-scale production of recombinant proteins, due to rapid growth at low cost and ease of genetic manipulation [10]. Despite its advantages, the *E. coli* expression system has some disadvantages such as lack of cellular mechanisms required for posttranslational modifications of eukaryotic proteins, misfolding encountered in the production of proteins containing complex disulfide bonds, and expression of proteins as insoluble inclusion bodies [11].

Inclusion bodies are a misfolded inactive form of proteins. Converting a protein produced as an inclusion body to the active form is very difficult, and procedures involving isolation, solubilization, and refolding of inclusion bodies result in low yields [12, 13]. Dissolution of inclusion bodies is mainly accomplished with high concentrations of denaturants such as urea, HCl, and guanidine hydrochloride, which disrupt intramolecular interactions [14-17]. Therefore, it is a more effective approach to reduce the inclusion body form and provide soluble expression of the protein, rather than obtaining the protein using the refolding procedure. In this context, various strategies such as various fusion tags, molecular chaperones, low temperature, appropriate promoter, secretion of the protein into the periplasm with ladder tags are used to prevent inclusion body formation in the production of recombinant proteins [18, 19].

Fusion tags are proteins or peptides attached to the target protein and help produce proteins in soluble active form. Some of the fusion tags used are glutathione S-transferase (GST) [20], thioredoxin (Trx) [21], maltose-binding protein (MBP) [22], small ubiquitin-related modifier (SUMO) [23], and superfolder green fluorescent protein (sfGFP) [24]. sfGFP is a fluorescent protein with high solubility and stability [25]. sfGFP has a relatively smaller molecular weight than other fusion tags such as MBP and GST but still contributes greatly to the expression in soluble form of the fusion partner [25, 26]. Studies show that sfGFP as a fusion tag increases the stability of the target protein and its solubility [24, 27].

This study, it was aimed to produce human TGF-β3 in soluble form in *E. coli* using the sfGFP fusion tag. In this context, The sfGFP-TGFβ3 fusion protein was produced in *E. coli* BL21(DE3) in soluble form and purified in high yield and purity. The wound healing potential of the sfGFP-TGFβ3 fusion protein in BJ human skin fibroblast cells was analyzed by *in vitro* scratch assay. The results revealed that the sfGFP-TGFβ3 fusion protein had wound healing potential.

2. MATERIALS AND METHODS

2.1. Plasmid Design

DNA sequences of sfGFP (GenBank: ASL68970.1) and human TGF-β3 (UniProtKB - P10600) proteins were optimized for the *E. coli* K12 organism using the JCat codon optimization program (http://www.jcat.de/). The histidine tag (6x-His) and sfGFP tag were added to the 5' end of the TGF-β3 DNA sequence. In addition, the TEV protease recognition site was placed between the 6xHis-sfGFP fusion and TGF-β3 to remove the fusion tag from the target protein when needed (Figure 1). To provide flexibility, a 2-amino acid linker was placed between 6xHis Tag, sfGFP, TEV protease

recognition site, and TGF-β3 to provide flexibility. The DNA sequence was designed by fusing into the pET-22b (+) vector and provided by Biomatik company (Figure 2).

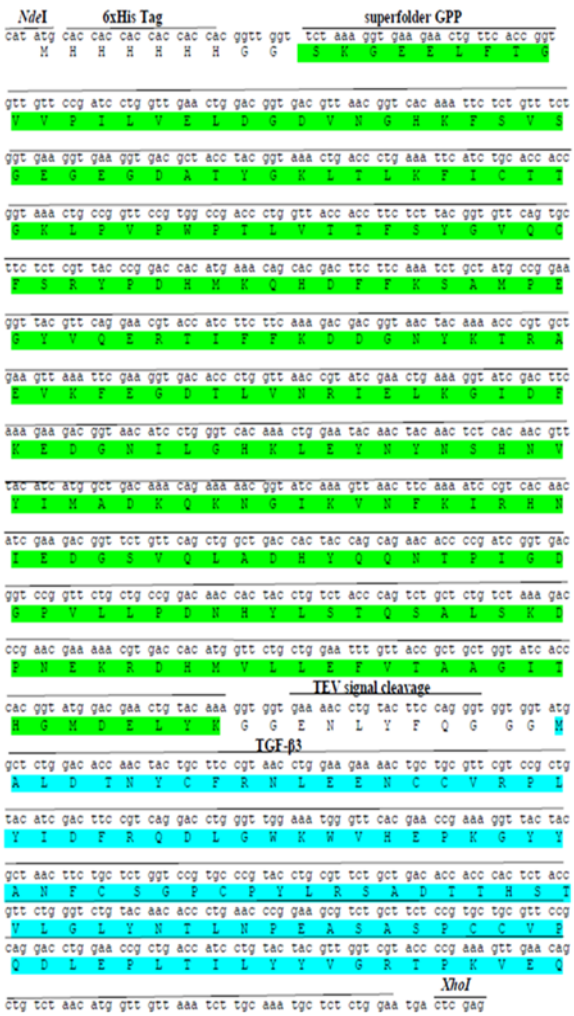

Figure 1 Optimized DNA and amino acid sequence of the sfGFP-TGFβ3 protein

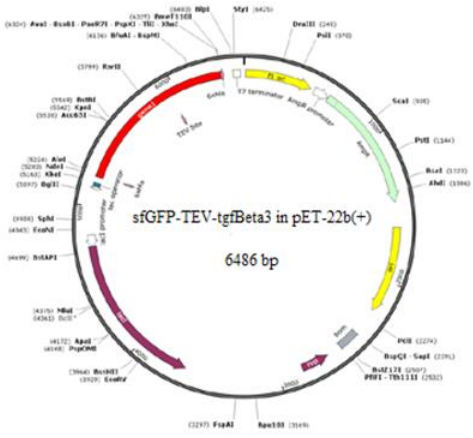

Figure 2 The construct map used for expression of sfGFP-TGFβ3 protein

2.2. Protein Expression

The expression vector was transformed into competent *E.coli* BL21 (DE3) cells by heat shock. Transformants were grown overnight in a selective Luria–Bertani (LB) agar plate containing 100 μg/mL ampicillin. A single colony was inoculated 4 mL of selective LB medium (containing 100 μg/mL ampicillin), incubated overnight at 37 °C in a shaking incubator at 240 rpm. The overnight starter culture was transferred into 600 mL of selective LB medium at a ratio of 1:100. When the culture absorbance at 600 nm reached 0.6, cells were induced for protein production by adding isopropyl-1-thio-β-galactopyranoside (IPTG) at a final concentration of 1 mM. The culture was incubated at 37 °C and 240 rpm for 3 h. The cells were then harvested by centrifugation at 8 000 rpm for 10 minutes. The supernatant was removed and the pellet was stored at -20°C until used in the purification step.

2.3. Protein Purification

Frozen cell pellets were resuspended by adding lysis buffer (25 mM Tris-HCl and 300 mM NaCl and pH: 7.8) 100 mM phenylmethanesulfonylfluoride (PMSF), 100 mM benzamidine, RNAse (20 μg/mL), DNAse (20 μg/mL) and lysozyme. The cells were lysed with a sonicator in an ice/water bath. The cell lysate was centrifuged at 30000 rpm for 1 hour. The sfGFP-TGFβ3 fusion protein was purified using Ni-NTA column to the protocol detailed in our previous studies [28, 29]. The purified sfGFP-TGFβ3 fusion protein was dialyzed overnight against 20 mM Tris-HCl buffer (pH 7.8) containing 100 mM NaCl at 4°C. Protein concentration was determined by UV absorption at 280 nm.

2.4. SDS–PAGE and Western Blot Analysis

The expression of the sfGFP-TGFβ3 fusion protein was qualitatively assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western

Blotting by using Anti-His antibody (GeneTex-GTX115045).

The purified proteins were visualized using 12% acrylamide/bis-acrylamide gels. The gels were stained with Coomassie Brilliant G250.

The obtained protein samples were also analyzed by western blotting using His tag antibody in addition to SDS-PAGE. Initially, protein samples were run in 12% SDS-PAGE. Each well of the gel was loaded with an equal amount of protein. The gels, run at 200V for 60 minutes, were taken and transferred to PVDF membranes. Transfer to PVDF membranes was performed semi-dry with BioRad Trans-Blot Turbo. Bjerrum Schafer-Nielsen buffer (48 mM Tris, 39 mM Glycine, pH 9.2, and 20% methanol) was used in the transfer process. After the PVDF membrane was incubated in methanol for 1 min, it was taken into the transfer buffer, and gel was added to the same buffer and left for 15 min. Filter papers used for transfer were also wetted with transfer buffer before processing. The prepared transfer sandwich was placed in the device, and the transfer was carried out at 25 V, 1.3 mA, and 10 minutes. Membranes were incubated in 5% skimmed milk powder prepared in TBST (TBST: (20mM Tris (pH: 7.5), 150mM NaCl, %0.1 Tween20) for 1 hour at room temperature, and the blocking process was performed. Blocked membranes were incubated overnight at +4ºC with the primary antibody 6xHisTag antibody (GeneTex-GTX115045, (1:5000)). The primary antibodies were removed, the membranes were washed 5 times with TBST for 5 minutes, and the secondary antibody was incubated with Goat anti-rabbit IgG H&L (Abcam-ab205718, 1:10000) for 1 hour at room temperature. After 1 hour, the membranes were rewashed with TBST. Then, the membranes were taken to a separate place for chemiluminescence imaging, and chemiluminescence (ECL) substrate was added to them and kept in the dark for 5 minutes. At the end of 5 minutes, antibody-specific protein bands on the membranes placed between acetate films were visualized with the ChemiDoc ™ imaging system (Bio-Rad).

2.5. *In Vitro* Cytotoxicity

The cytotoxicity of the purified recombinant sfGFP-TGFβ3 fusion protein against BJ cells (ATCC CRL-2522 normal human skin fibroblast cells) was assayed by MTT analysis. Commercial TGF-β3 (SRP3171-10UG) was used as a positive control. Cultivated cell lines were seeded in 96 well culture dishes in triplicate at a concentration of 5×104 cells/mL. Cells were incubated for 24 hours in a humidified incubator at 37 °C containing 5% CO_2. After 24 hours of incubation, the cells were treated with purified recombinant sfGFP-TGFβ3 fusion protein and commercial TGF-β3 at different concentrations (150-2.34 ng/mL). Cells were incubated with these components for 24 and 48 hours. At the end of these periods, a viability test was performed with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide). For the MTT test, the used medium was withdrawn from the plate, and Eagle's Minimum Essential Medium (EMEM Eagle 10% FBS) containing 10% MTT (5 mg/mL) was added onto the cells. Cells were incubated in the dark at 37ºC in a 5% CO_2 incubator for 3 hours. After incubation, the medium containing MTT was removed from the wells. DMSO was added to dissolve the formed formazan crystals. Then absorbance values at 570 nm wavelength were recorded using a Microplate Reader. The cell viability was calculated as the percentage of untreated cells.

2.6. *In Vitro* Scratch Wound Healing Assay

In this study, the effect of the produced recombinant fusion protein on wound healing was performed using the in vitro scratch wound healing assay by applying the protocol given in previous studies [30]. BJ cells were seeded (5×10^4 cells/well) in 6-well plates in the EMEM Eagle (containing %10 FBS) culture medium for wound healing analysis. The cells were treated with 2ng/mL and 10

ng/mL sfGFP-TGFβ3 fusion protein and positive control TGF-β3. At various time intervals (0, 24, 48 hours), images were captured by microscope (Olympus CKX41) after compound administration.

2.7. Statistical Analyses

All experiments were repeated three times. All values were expressed using GraphPad Prism 9 Statistical Software. Two-Way ANOVA analysed experimental resultsanalyzed. P value of less than 0.05 ($P<0.05$) was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Expression, Purification, and Characterization of Recombinant Human TGF-β3

TGF-β3 has been the subject of many studies as an anti-scarring agent due to its antifibrotic and scar-free healing effects. In the literature, many studies aim to elucidate the effect mechanism of TGF-β3 on wound healing. Therefore, studies on recombinant production of TGF-β3 inactive form in high yield and purity for use in these studies are still of interest. TGF-β3 has been produced using a variety of eukaryotic and prokaryotic expression systems. Each system has several advantages and disadvantages. The *E. coli* expression system has important advantages, enabling rapid, high yield recombinant protein production at low cost [18]. The active mature form of TGF-β3 is not a glycosylated protein. Therefore, using the *E. coli* expression system in its recombinant production is appropriate considering the above-mentioned advantages. However, TGF-β3 can be produced as an inclusion body in the *E.coli* expression system, and the mature TGF-β3 homodimer is known to have a highly folded structure with four intramolecular and one intermolecular disulfide bonds [31]. Therefore, additional protocols must be applied to obtain the protein active form. This both increases the production cost and reduces efficiency. This study, TGF-β3 was produced in *E. coli* with high purity and yield in soluble form with the sfGFP fusion tag. The sfGFP fusion tag is one of the effective strategies used to produce proteins in soluble form in *E. coli*. It is known that when sfGFP is used as a fusion tag, it increases the stability of the target protein and its solubility. In addition, GFP and its derivatives are fluorescent proteins used as photosensitizers in photodynamic therapy, which is one of the alternative new approaches used in wound healing. [32-34]. In this respect, it is thought that sfGFP may increase the effect of TGF-β3 on wound healing when combined with photodynamic therapy. Therefore, the production of TGF-β3 infusion with sfGFP is an important strategy not only in terms of protein solubility but also in terms of increasing wound healing activity. As assessed by SDS-PAGE, the sfGFP tagged recombinant human TGF-β3 was successfully expressed in soluble form in *E. coli* (Fig. 3).

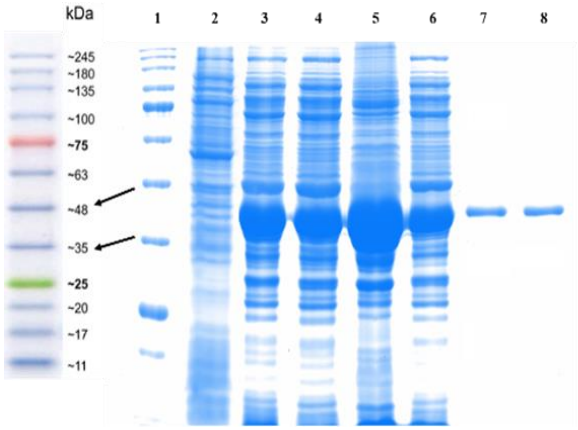

Figure 3 Purification of recombinant sfGFP-TGFβ3 was confirmed with SDS-PAGE (%12). 1. Marker (GoldBio BLUEstain™ Protein ladder) 2. Bacterial cell lysates before IPTG addition, 3. Bacterial cell lysates after IPTG addition 4. Collected pellet after centrifugation of the lysate 5. Collected supernatant after centrifugation of the lysate, 5. Flow-through (after Ni-NTA Agarose column), 7-8. Elutions

The molecular weight and molar absorption coefficient of the sfGFP-TEV-TGFβ3 fusion protein were, respectively, calculated as 41876.26 Da and 43945 $M^{-1}cm^{-1}$ using "ExPASy ProtParam tool." Subsequently, the total yield of purified recombinant protein was determined as 4.04 mg/mL by measuring

absorbance at 280 nm on a UV spectrophotometer. SDS-PAGE analysis's experimentally determined molecular weight of the recombinant protein is very close to the calculated molecular weight. 20 mg of protein was obtained from 1 L of bacterial culture, and this protein was of high purity (98%). In addition, the purified protein was analyzed by western blotting using an anti-His antibody. As expected, blots of the purified protein around 41 kDa were observed (Figure 4). This result was also correlated with the protein bands identified in the SDS-PAGE analysis.

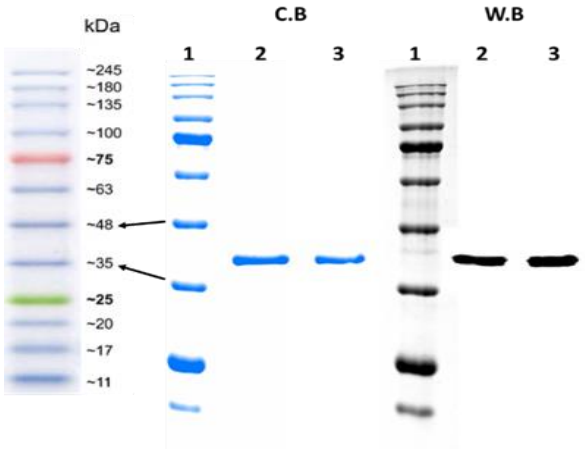

Figure 4 SDS-PAGE (C.B.) and Western Blot (W.B) Analysis of purified recombinant sfGFP-TGFβ3 1) Marker (GoldBio BLUEstain™ Protein ladder) 2) Purified His Tagged sfGFP-TGFβ3 fusion protein

3.2. Cytotoxicity

The cytotoxicity of purified recombinant human sfGFP-TGFβ3 was tested by MTT assay against BJ cells. Commercial TGF-β3 was used as a positive control. Recombinantly produced sfGFP-tagged TGFβ-3 did not have a cytotoxic effect on healthy human skin fibroblast BJ cells in the tested concentration range (150 ng/mL-2.34 ng/mL) (Figure 5). When the tested concentrations were compared to their effects on cell proliferation, no significant difference was found between them.

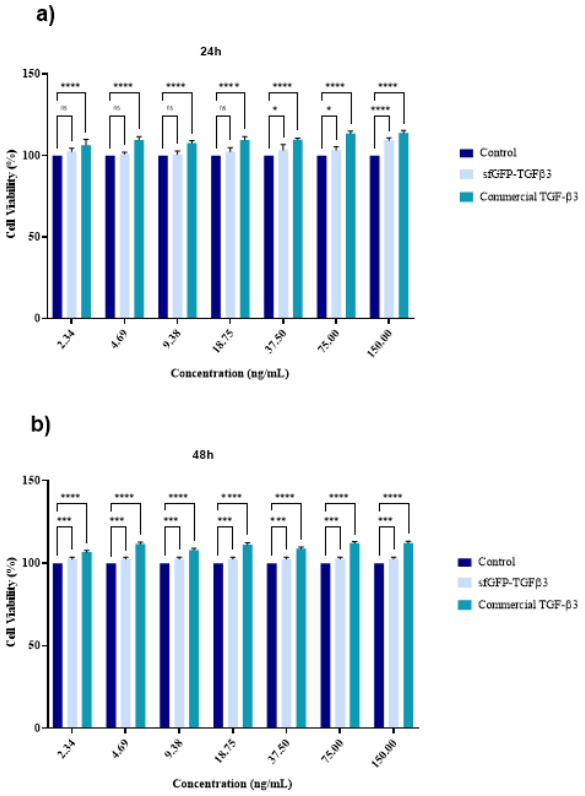

Figure 5 The effect of recombinant sfGFP-TGFβ3 and commercial TGF-β3 on the viability of BJ normal human skin fibroblast cells. Viability was measured by the MTT assay after 24h (a), 48h (b). Significant differences between cells are indicated by *p < 0.05, ***p < 0.0001, ****p < 0.00001

3.3. *In Vitro* Scratch Wound Healing Assay

The produced recombinant sfGFP-TGFβ3 was not cytotoxic in the tested concentration range. Based on the cytotoxicity results and studies examining the effects of recombinant TGF-β3 on wound healing, *in vitro* scratch wound healing assay was performed to investigate the effects of 2 ng/mL and 10 ng/mL protein on the migration of BJ cells [35, 36] (Figure 6).

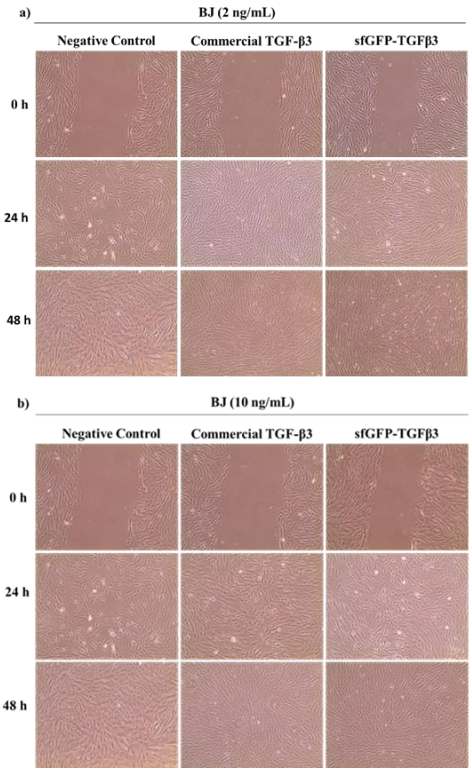

Figure 6 The effects of recombinant sfGFP-TGFβ3 and commercial TGF-β3 on BJ cells migration ability were tested by wound healing assay. BJ cells were treated with 2 ng/mL (a), 10 ng/mL (b) concentrations of recombinant sfGFP-TGFβ3 and commercial TGF-β3 allowed to migrate into the scratched area for 24–48 h. Images from the wound-healing assay of BJ cells treated with these compounds (4× magnification)

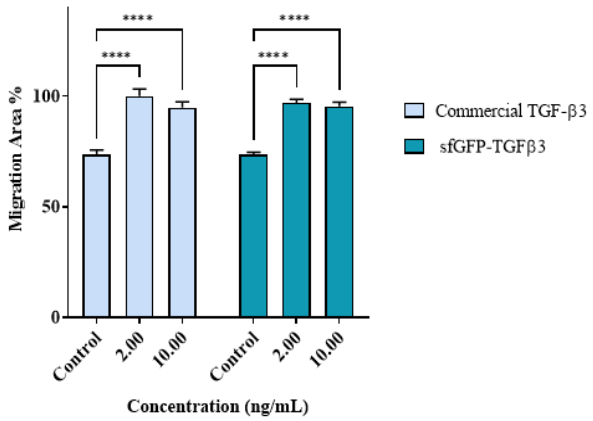

Figure 7 The effects of recombinant sfGFP-TGFβ3 and commercial TGF-β3 on BJ cells migration ability were tested by wound healing assay. BJ cells were treated with 2 ng/mL,10 ng/mL concentrations of recombinant sfGFP-TGFβ3 and commercial TGF-β3 allowed to migrate into the scratched area for 24h. Quantification of wound area in control and compounds treated BJ cells **** p<0,000001

According to the analysis results, a 73.7% closure occurred in the negative control at the 24th hour. In the wound area where 2 ng/mL and 10 ng/mL concentrations of commercial TGF-β3 and sfGFP-tagged TGF-β3 were applied, 100%, 95.27%, 97.13%, 95.38% closure was achieved, respectively (Figure7). At the 48th hour, 100% closure was achieved in all experimental groups.

4. CONCLUSION

In this study, the use of protein fusion tags, one of the most common strategies used to increase the solubility of recombinantly expressed proteins in *E. coli*, was preferred. sfGFP has been used as a fusion tag because it is relatively small (26781.21 da), facilitates the follow-up of production and purification processes as a colored fluorescent protein, and has an important area of use in photodynamic therapy. As a result of the study, human TGF-β3, a therapeutic protein, could be highly pure in soluble form with the sfGFP fusion tag. It is thought that the effect of the obtained sfGFP-TGFβ3 fusion protein on the wound healing process with the photodynamic therapy approach is worth investigating.

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Authors' Contribution
Sema Bilgin formed the research idea and designed the experiments, performed the experiments, analyzed the data, write the manuscript.

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No conflict of interest or common interest has been declared by the author.

The Declaration of Ethics Committee Approval The author declares that this document does not require an ethics committee approval or any special permission.

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