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Prokaryotic Expression and Purification of Recombinant Human Leukemia Inhibitory Factor; Analysis of the Ability to Maintain Pluripotency in Embryonic Stem Cells

Behrouz Taheri [1], Mohammad-Hossein Modarressi [2, 3], Mahmoud Hashemitabar [4], Mohammad Miryounesi [5] and Elahe Motevaseli [2, 6, *]

[1]Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran
[2]Department of Molecular Medicine, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran
[3]Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[4]Department of Anatomical Sciences, Cellular and Molecular Research Center, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[5]Genomic Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[6]Food Microbiology Research Center, Tehran University of Medical Sciences, Tehran, Iran

[*]*Corresponding author*: Ph.D, Department of Molecular Medicine, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, 14167-53955, Poursina Ave., Keshavarz Blvd., Tehran, Iran. Tel/Fax: +98-2143052000, Email: e_motevaseli@tums.ac.ir

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Abstract

Background: Leukemia Inhibitory Factor (LIF) is largely used in stem cell researches for the maintenance of Embryonic Stem Cells (ESCs) in a pluripotent state. However, the relatively high costs of LIF is a potential limit of such researches.
Objectives: The aim of this study was prokaryotic expression and purification of the recombinant hexahistidine-tagged human LIF (His6-hLIF) fusion protein and assessment of its ability to maintain a pluripotent or undifferentiated state of ESCs.
Methods: Encoding the DNA sequence of mature hLIF was codon optimized for expression in *Escherichia coli* and chemically synthesized and cloned in the expression vector pET- 28a (+). Immobilized Metal Affinity Chromatography (IMAC) was performed to purify the recombinant His6-hLIF. Then, His6-hLIF was tested for its ability to maintain mESC by comparison with commercial LIF as a control.
Results: The yield for the recombinant His6-hLIF was assessed to be approximately 1.7 mg from one liter of culture. There were no statistically significant differences in expression of two pluripotency gene markers, oct-4 and Nanog, between mESCs treated with His6-hLIF and those with commercial hLIF ($P = 0.09$ and $P = 0.13$, respectively). Besides, morphological characteristics (round cellular morphology) were similar between them.
Conclusions: Collectively, the findings showed that the ability of the recombinant His6-hLIF protein in maintaining pluripotent state of ESCs was comparable to commercial hLIF, providing evidence that the presence of the N-terminal hexahistidin tag does not influence biological activities of hLIF.

Keywords: Embryonic Stem Cell, Leukemia Inhibitory Factor, Pluripotency, Recombinant Protein

1. Background

Embryonic Stem Cells (ESCs) have the potential to treat diseases resulting from cell death or dysfunction of specific cell groups. Thus, research efforts have focused on the therapeutic potential of stem cells for the treatment of diabetes, heart failure, and neurodegenerative disorders (1, 2). One of the critical aspects of studies on ESCs is the maintenance of a pluripotent or undifferentiated state of ESCs in culture medium. One of the essential components to maintain pluripotency of ESCs is Leukemia Inhibitory Factor (LIF), which represents the main cost of the culture media (3, 4). Human LIF (hLIF), a member of the Interleukin-6 cytokine family, is secreted in the form of a glycoprotein. However, this cytokine can retain biological activity in its deglycosylated form, which is an approximately a 20-kDa protein, containing 180 amino acids (5). Besides its biomedical research utilization, recent studies present strong evidence indicating potential therapeutic applications of hLIF for neurodegenerative injury and female infertility (6-8). Together, various potential applications and a high price of hLIF indicate the necessity of establishment of simple and cost-effective methods for hLIF production.

2. Objectives

The present experimental study aimed at expressing recombinant fusion hLIF (His6-hLIF) in *E. coli*, and assessing its activity in maintenance of an undifferentiated state of ESCs.

3. Methods

3.1. Construction of the pET-28a(+)/hLIF Expression Vector

The amino acid sequence of mature human LIF was taken from the NCBI sequence database (Accession No. NP 002300). Encoding DNA sequence of hLIF was codon optimized for expression in *E. coli* (9) and chemically synthesized and cloned in the pET- 28a (+) expression vector, using *NdeI* and *XhoI* restriction enzymes (Generay Biotech Co., Ltd). This vector adds a hexahistidine (His6) tag to N-terminus of the recombinant protein to facilitate purification of the expressed protein using metal affinity chromatography.

3.2. Expression and Purification of His6-hLIF

For disulfide bond formation, the *E. coli* strain Rosetta-gami™ 2 (DE3) pLacI competent cells were used as the expression host system. Protein expression and purification were performed as previously described (10, 11). Briefly, the selected clones on a Luria-Bertani (LB) agar plate with kanamycin (Roche, Germany) were grown overnight in LB (Merck, Germany), containing 25 μg/mL kanamycin at 37°C. After overnight incubation, cultures were diluted at 1:100 in LB broth, in the presence of the antibiotic, and grown at 37°C until an OD 600 of 0.6. To examine optimal expression conditions, two concentrations of Isopropyl β-D-1-Thiogalactopyranoside (IPTG) (Merck, Germany) (0.5 mM and 1 mM) and two different expression temperatures (15°C and 25°C), for 6 hours with shaking (200 rpm), were tested. Following centrifugation of the induced bacteria at 5000 $\times$ g for 15 minutes, the cell pellets were frozen at -70°C until the purification step. Recombinant His6-hLIF was purified by the Ni-NTA Fast Start Kit (Qiagen), according to manufacturer's protocol, in which a gradient of two concentrations of 25 mM/L and 250 mM/L imidazole was applied to wash and elute the recombinant protein, respectively. For removing imidazole from protein solution, dialysis overnight against phosphate buffered saline (pH 7.4) was performed. The protein concentration was determined by the Bradford method with bovine serum albumin as the standard. After purification, the His6-hLIF protein solution was filtered, using 0.2 μm filters and stored at -20°C, until analysis. Expression of the hLIF was evaluated by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (12%) and western blotting using monoclonal anti-hLIF antibody (Santa Cruz Biotechnology, Inc.).

3.3. Evaluation of Biological Activity of His6-hLIF: The Ability to Maintain Pluripotency of mESCs

In order to analyze the biological activity of recombinant His6-hLIF, its ability to maintain pluripotency of mESCs was compared with commercial hLIF (Merck, Germany). For this purpose, cell morphology, and expression of pluripotency markers were investigated. The mESCs were cultured in the presence of 10 ng/mL of purified His6-hLIF, which is comparable to 1000 IU/mL of commercial hLIF (12).

3.3.1. ESC Culture

The culture of mESCs was performed as described previously (12). BrieflDepartment of Molecular Medicine, School of Advanced Technologies, the mouse Embryonic Stem Cell line (mESCs) C57BL/6 (Pasteur Institute, Iran) was cultured in six-well plates coated with 0.1% gelatin (Cinnaclone, Iran) without feeder cells in ES media, DMEM (Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA), containing 0.1 mM β-mercaptoethanol (Merck, Germany), 200 mM l-glutamine (Sigma, Germany), minimum essential amino acid (Invitrogen, USA), penicillin/streptomycin (Invitrogen, USA), and hLIF. Following protease treatment with 0.025% trypsin, the cell suspensions were passed every three days with a density of 2×10^4 cells/mL.

3.3.2. RT-qPCR

The relative expression of the gene markers for the undifferentiated state of ESCs (Nanog and oct4) was assessed using real-time quantitative Reverse-Transcription Polymerase Chain Reaction (RT-qPCR), as previously described (12). RNA extraction was performed using the high pure RNA isolation kit (Roche, Germany). Reverse transcription was done using the cDNA Synthesis Kit (Thermo Scientific, USA). The synthesized cDNA was used to carry out quantitative PCR using SYBR Premix Ex Taq II (Takara Bio, Inc. Japan) by the ABI Step One TM System (Applied Biosystems, USA), which had been calibrated for optical and thermal accuracy. Forward primer Nanog-F (5'-AGGGTCTGCTACTGAGATGCTCTG-3') and reverse primer Nanog-R (5'-CAACCACTGGTTTTTCTGCCACCG-3') pertaining to the Nanog gene and forward primer Oct4-F (5'-ATCCTGGGGGTTCTATTTGG-3'), and reverse primer Oct4-R (5'-CTGGTTCGCTTTCTCTTTCG-3') pertaining to the oct4 gene were used in the RT-qPCR analysis, as previously described (12). Expression of the β-actingene was used as the endogenous control to normalize nanog and oct-4 expression in RT-qPCR analysis, using a forward primer

β-actin-F (5'-GGACTTCGAGCAAGAGATGG-3'), and reverse primer β-actin-R (5'-AGCACTGTGTTGGCGTACAG-3'), as mentioned previously.

3.3.3. Immunofluorescent Staining

Immunofluorescent staining was performed with a specific antibody for surface marker SSEA-1 to confirm pluripotent state. After fixation in 4% paraformaldehyde (Merck, Germany) for 20 minutes, cells were permeabilized using 0.2% triton X-100 (Merck, Germany) for 30 minutes and blocked in 10% goat serum in Phosphate-Buffered Saline (PBS) for 60 minutes. Incubation of cells with primary antibody anti-SSEA-1 (1:50, Santa Cruz Biotechnology) was done at 37°C for one hour, and were then washed three times, and incubated with FITC-conjugated secondary antibody IgG (1:200, Sigma-Aldrich, Germany), as appropriate, at 37°C for one hour.

3.4. Statistical Analysis

Differences in the expression levels of pluripotency gene markers between ESCs treated with recombinant His6-hLIF protein and those with commercial hLIF were statistically analyzed using the relative expression software tool-RG© version three. The Pairwise Fixed Reallocation Randomization Test© was applied by this software, in which a P value of < 0.05 was considered statistically significant.

4. Results

4.1. Expression and Purification of the Recombinant His6-hLIF Protein

Mature hLIF (aa 23 - 202), biologically active form, results from precursor hLIF (aa 1 - 202) after the removal of signal peptide (aa 1 - 22). The pET-28a (+)/hLIF vector was constructed to express His6-hLIF fusion protein. There was no significant difference between the concentrations of eluted His6-hLIF (soluble expression), induced with two different concentrations of IPTG, whereas soluble expression at 15°C was higher than at 25°C (data not shown). The yield for His6-hLIF at 15°C was assessed to be approximately 1.7 mg His6-hLIF from one liter of culture. The SDS-PAGE analysis showed a single protein band corresponding to the expected sizes of the His6-hLIF (about 20 kDa) (Figure 1A). After western blot analysis using anti-hLIF monoclonal antibody, both purified His6-hLIF fusion protein and commercial hLIF were observed as single bands on the membrane (Figure 1B).

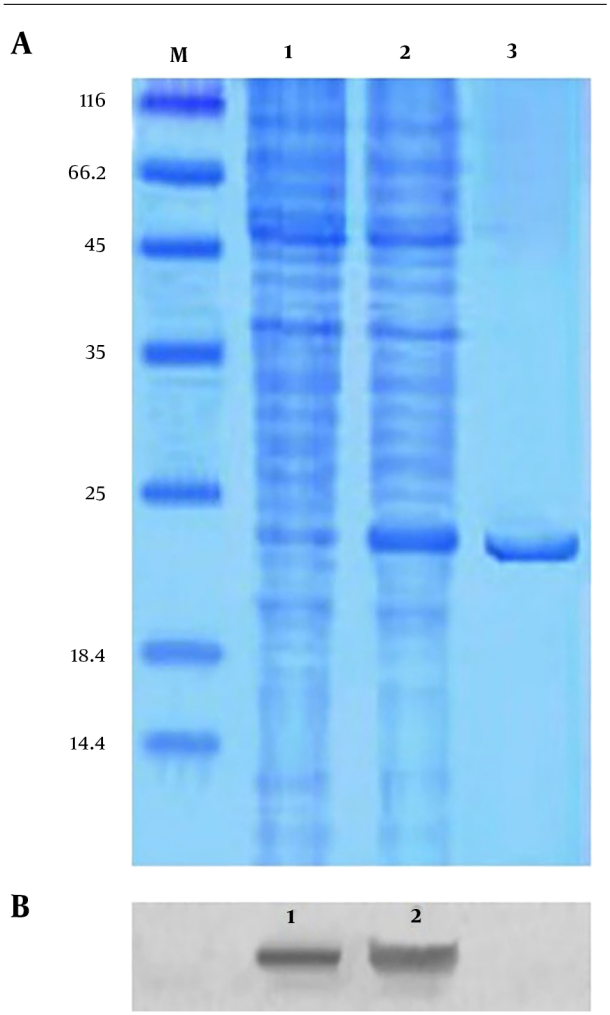

Figure 1. Analysis of His6-hLIF expression. A, The SDS-PAGE analysis showed a single protein band corresponding to the expected sizes of hLIF (about 20 KDa); lane M: molecular mass markers, lane 1: uninduced supernatant, lane 2: induced supernatant, and lane 3: purified His6-hLIF. B, Western blot analysis. B: Western blot analysis carried out using monoclonal anti-hLIF showed a band corresponded to hLIF size (20 KDa); lane 1: His6-hLIF, lane 2: commercial hLIF.

4.2. Biological Activity of His6-hLIF in the Maintenance of mESCs in a Pluripotent State

The recombinant His6-hLIF was examined for its ability to maintain an undifferentiated state of mESCs and compared to commercial hLIF, as the control. After five passages, morphological characteristics of mESCs (round cellular morphology) treated with His6-hLIF were similar to those with commercial hLIF. Non-round cellular morphology with elongated spikes appeared in the absence of LIF (Figure 2A). Statistical analysis indicated that there were no significant differences in expression of two pluripotency gene markers, oct-4 and Nanog, between mESCs supplemented with His6-hLIF and commercial hLIF (P = 0.09

and P = 0.13, respectively). The expression levels of these two markers were reduced to < 10% by the removal of LIF from the mESCs culture (Figure 2B). As shown in Figure 2C, immunofluorescent staining with specific antibody for surface marker SSEA-1 confirmed the pluripotent state of mESCs treated with His6-hLIF and commercial hLIF. Collectively, these results indicated that the activity of recombinant His6-hLIF for maintaining pluripotency of mESCs was comparable with commercial hLIF.

5. Discussion

Various potential applications and the high price of hLIF indicate the necessity of establishment of simple and cost-effective methods for hLIF production. To date, however, low expression yield for hLIF has limited large-scale clinical applications of this cytokine. The expression yield for recombinant hLIF in several studies was reported to be less than 2 mg/L (5, 13, 14) and in others higher than 2 mg/L (15, 16). The major drawback resulting in low purification yields were inclusion body formation. Previous studies revealed that increasing solubility of inclusion bodies using the refolding techniques limits the recovery rate of recombinant LIF (2, 4). Several strategies have been used to optimize the yield of recombinant hLIF. Codon optimization for expression in *E. coli* can improve the yield of hLIF, as reported previously (17). In the current study, encoding the DNA sequence of this protein was codon optimized for expression in *E. coli*. Furthermore, *E. coli* strains harboring the *trxB/gor* mutations offered the potential to form correct disulfide bonds in the cytoplasm, and consequently enhanced the solubility of the recombinant proteins (2). In the present work, *E. coli* strain Rosetta-gami (DE3) pLacI, which is the *trxB/gor* mutant, was used as the expression host system. Besides, in agreement with other studies, the findings demonstrated that the soluble expression of His6-hLIF could be improved when proteins are expressed at low temperature (4, 5). Inconsistently, another study revealed that the induction temperatures had no significant effect on the solubility of His6-hLIF (12).

The findings of the current study showed that the ability of a recombinant His6-hLIF fusion protein in the maintenance of mESCs in a pluripotent state was comparable to commercial hLIF, indicating that the presence of the N-terminal His6 tag does not affect the biological activities of hLIF. In agreement with the researcher's observations, a previous study showed that the N-terminal His6 tag has no significant effects on the ability of hLIF in the maintenance of pluripotency in mESCs (12). These findings may provide evidences that the N-terminal segment of LIF does not interfere with its binding to the receptor. Consistently, a previous study documented that the N-terminal part of LIF is flexible and far from the receptor-binding site (18). Accordingly, given that the His6-tag has no effect on the activity of LIF, there is no need to cleave His6 tag using expensive protease, and consequently there is no need for an additional chromatography step to remove the protease and the His6-tag, suggesting a potential advantage for the N-terminal His6 tag system in production of the recombinant His6-hLIF protein. This advantage can lead to an easy, simple, and cost- effective production of active His6-hLIF for biomedical research applications. The N-terminal His6-tag system might be useful in generation of other growth factors and cytokines after confirming that the His6 tag has no effect on the functionality. However, considering the possible stimulation of immune responses, the recombinant His6-tagged fusion proteins may not be suitable for *in vivo* medical applications, representing a potential disadvantage for the N-terminal His6 tag system. Furthermore, one of the critical factors for *in vivo* medical application is the endotoxin content of the recombinant proteins. As a limitation of the current study, the endotoxin level of His6-hLIF was not assessed. There have been several conflicting reports on the endotoxin content of His6 tag in the recombinant His6-hLIF fusion protein (3, 19). Collectively, the mentioned limitations can limit the application of His6-hLIF to biomedical researches.

5.1. Conclusion

The present study revealed that the biological activities of the recombinant His6-hLIF fusion protein are comparable to commercial hLIF, indicating that the presence of the N-terminal His6 tag does not affect functional characterizations of hLIF. Thus, the active His6-hLIF fusion protein could be easily prepared from soluble *E. coli* extracts by a single purification step.

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Footnotes

Authors' Contribution: Mohammad-Hossein Modarressi and Elahe Motevaseli designed and supervised this project. Behrouz Taheri, Mahmoud Hashemitabar and Mohammad Miryounesi contributed to data analysis and laboratory operations. Behrouz Taheri wrote the manuscript. All authors approved the manuscript.

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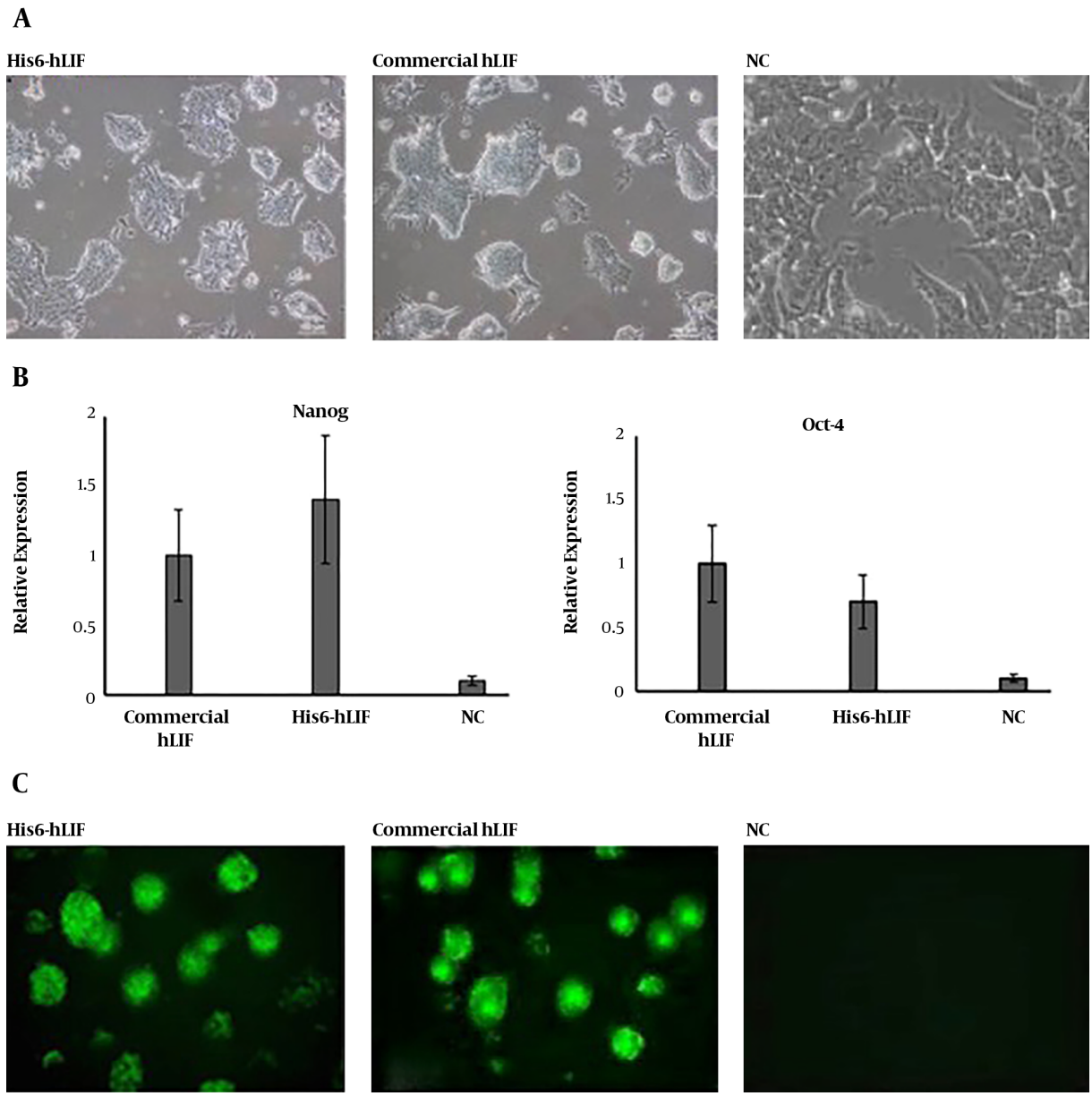

Figure 2. Biological activity. A, mESCs exhibited similar cellular morphology in the presence of His6-hLIF and commercial hLIF but a different morphology was appeared in the absence of hLIF (negative control (NC)). B, The relative expressions of two stem cell markers oct-4 and nanog in mESCs treated by His6-hLIF, commercial hLIF and no LIF (NC) were determined using RT-qPCR; the expression values of commercial hLIF is 1. C, Immunocytochemistry with anti-SSEA-1 confirmed the undifferentiated state of mESCs supplemented with His6-hLIF and commercial hLIF.

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