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Expression and Secretion of the Human Erythropoietin Using an Optimized *cbh1* Promoter and the Native CBH I Signal Sequence in the Industrial Fungus *Trichoderma reesei*

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Abstract The human erythropoietin (HuEPO) structural gene was fused with the secretion signal of the *Trichoderma reesei* cellobiohydrolase I and controlled by a newly optimized *cbh1* promoter in an integrated expression vector pTrCBH-EPO. The recombinant HuEPO construct was transformed into two different *T. reesei* strains, a protease-deficient strain RutC-30 M3, and a glycosylation-modified strain T108. After lactose induction, the heterologous rHuEPO was found to be stably expressed in the selected transformants T47 (derived from RutC-30 M3) and T112 (derived from T108), which were shown to have high genetic stability. Secretion of erythropoietin in these transformants was further confirmed by SDS-PAGE and western blot analysis. Moreover, the secreted rHuEPO from T112 had an apparent molecular weight of 32 kDa, which was higher than from T47 (28 kDa) and similar to that of mammals (more than 30 kDa). These results demonstrate the potential of using industrial filamentous fungi for the production of human-derived erythropoietin.

Keywords Erythropoietin · Heterologous expression · *Trichoderma reesei* · Glycosylation · *cbh1* promoter

Introduction

Human erythropoietin (HuEPO) is a glycoprotein hormone responsible for stimulating the production of red blood cells and hence has significant pharmacological values in anemia treatment [1, 2]. Native HuEPO is primarily synthesized in the adult kidney and consists of 166 amino acids, containing three potential *N*-glycosylation and one *O*-glycosylation sites. It is synthesized by the kidney and, generally, exists in plasma at low concentrations. Due to the very limited amounts of EPO from native sources and its significant value for clinical

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application, recombinant HuEPO (rHuEPO) has attracted constant attention since its encoding gene was cloned from human kidney [1].

At present, nearly all the rHuEPO available in the market is produced by Chinese hamster ovary cells [1, 2]. There have been a few attempts to produce rHuEPO in animal bioreactors exhibiting a glycosylation pattern closer to the natural HuEPO, but most of mammalian-derived rHuEPO is produced with low efficiency and high cost [3, 4]. Several groups have investigated the expression of rHuEPO in bacterial hosts, such as *Escherichia coli* and *Bacillus brevis* and the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris* [2, 4, 5]. However, recombinant proteins expressed in prokaryotes lack sugar chains while they are hyperglycosylated in yeasts. It is well known that many filamentous fungi have the ability to produce large amounts of extracellular proteins and become more promising cell factories for heterologous protein production [6]. So far, the potential for production of rHuEPO by filamentous fungi has not been investigated.

Trichoderma reesei (anamorph of *Hypocrea jecorina*) is an industrially important filamentous fungus that has been well developed for the production of different hydrolyzing enzymes (e.g., 40 g/L of cellobiohydrolase I, CBH I) and heterologous proteins because of its excellent secretion capacity and the accessible fermentation conditions [7]. Especially, as a multicellular eukaryote, it employs similar translational and posttranslational modification mechanisms to those in mammalian systems [8, 9]. Therefore, *T. reesei* is considered to be a suitable host for the production of high-value mammalian proteins, which are difficult to be expressed in bacteria or produced in animal cell cultures at very low levels.

In our previous studies, a set of optimized cellobiohydrolase I gene (*cbh1*) promoters was constructed to improve the efficiency of heterologous gene expression [10] and a glycosylation-modified *T. reesei* strain T108 was obtained by introducing the human *N*-acetylglucosaminyl transferase I gene into the fungal glycosylation pathway with the aim of synthesis of mammalian-like *N*-glycan (our unpublished data). Here, based on these studies, we explored the possibility of heterologous expression and secretion of rHuEPO in the industrial fungus *T. reesei*.

Materials and Methods

Strains and Growth Conditions

The hosts for heterologous expression of rHuEPO used in this study were *T. reesei* RutC-30 M3, a protease-deficient strain that has only 26% extracellular protease activities of the parent strain RutC-30 [11], and *T. reesei* T108, a glycosylation-modified strain, which was obtained by transforming the human *N*-acetylglucosaminyl transferase I gene into RutC-30 M3. Media and culture conditions for fungal growth were performed as described before [12]. *E. coli* strain DH5α was used as a recipient for transformations during plasmid construction and for plasmid propagation and storage.

Construction of the Expression Vector pTrCBH-EPO

The *T. reesei cbh1* signal sequence (51 bp) was amplified from the genome of *T. reesei* using the primers CSP-F and CSP-R (Table 1). An *Xho*I restriction site was added to the 5′ end of the *cbh1* signal sequence during amplication. Using the primers EPO-F-CR and EPO-R (Table 1), the human kidney EPO cDNA without native signal sequence (501 bp) was amplified from the plasmid pGEM-T-EPO (provided by Prof. Qingsheng Qi, Shandong University). The

Table 1 Oligonucleotide primers used in this study

Primer	Sequence
CSP-F[a]	5′-GCGCTCGAGATGTATCGGAAGTTGGCC-3′
CSP-R	5′-AGCACGAGCTGTGGCCAA-3′
EPO-F-CR[b]	5′-*GGCCACAGCTCGTGCT*GCCCCACCACGCCTCATC-3′
EPO-F	5′-GCCCCACCACGCCTCATC-3′
EPO-R	5′-TCATCTGTCCCCTGTCCTGC-3′
TCT-F-ER[c]	5′-*AGGACAGGGGACAGATGA*AGCTCCGTGGCGAAAGCCTG-3′
TCT-R[d]	5′-GCGGAATTCTCTGCCGTCAATCCTCGA-3′

[a] *Xho*I restriction site is underlined

[b] The first 16 nucleotides shown in italics are complementary to the reversed sequence of primer CSP-R

[c] The first 18 nucleotides shown in italics are complementary to the reversed sequence of primer EPO-R

[d] *Eco*RI restriction site is underlined

primer EPO-F-CR was designed such that an addition of 16 bases from 3′ end of *cbh1* signal sequence was present in the 5′ end of PCR product of EPO cDNA for the following fusion PCR amplification. Then the *cbh1* signal sequence and the mature human EPO cDNA were fused by using primers CSP-F and EPO-R with the two amplified fragments as the template. The *T. reesei cbh1* terminator (790 bp) was also directly amplified from *T. reesei* genomic DNA using a pair of specific primers TCT-F-ER and TCT-R (Table 1). The PCR product of *cbh1* terminator contained an additional 14-bp sequence of the 3′ end of *epo* gene and an *Eco*RI restriction site at its 3′ end. The 1,342-bp rHuEPO fusion cassette consisting of *cbh1* signal sequence, mature *epo* gene and *cbh1* terminator was obtained by PCR using primers CSP-F and TCT-R based on the above amplified fragments as the template. Then, the PCR product of rHuEPO fusion cassette was digested with *Xho*I/*Eco*RI double restriction enzymes and ligated into *Xho*I/*Eco*RI-digested plasmid pDelta-p4C containing the modified *T. reesei cbh1* promoter, which has four copies of a 200-bp region including the CCAAT box and the Ace2 binding sites [10], resulting in the EPO expression vector pTrCBH-EPO (Fig. 1). Finally, the 2.7-kb rHuEPO expression cassette consisting of *cbh1* promoter, *cbh1* signal sequence, EPO cDNA, and *cbh1* terminator was confirmed by DNA sequencing.

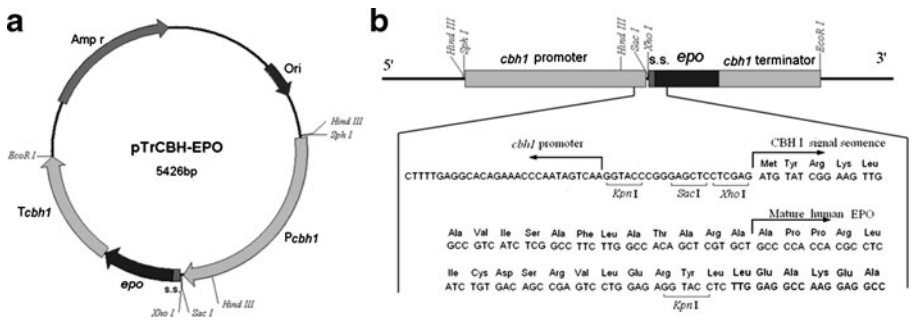

Fig. 1 Genetic constructions for rHuEPO expression in *T. reesei*. **a** Plasmid diagram of pTrCBH-EPO. **b** Schematic representation of the rHuEPO expression cassette and the fusion region of the *cbh1* promoter and CBH I signal sequence with the mature human EPO. *Pcbh1* promoter of *T. reesei cbh1* gene, *S.S.* CBH I signal sequence, *epo* human erythropoietin gene, *Tcbh1* terminator of *T. reesei cbh1* gene, *Ori* plasmid replication origin, *Amp r* ampicillin resistance

Transformation of *T. reesei* RutC-30 M3 and T108

Transformation of the *T. reesei* strains was performed according to the standard transformation method [12]. Plasmid pAN7-1, which contains the hygromycin resistance gene [13], was co-transformed with pTrCBH-EPO in order to allow selection of transformants that were able to grow on minimal medium containing hygromycin B (150 μg/L). Transformants were collected after 5 days of growth and then submitted to a second and a third round of selection by transferring the mycelium to fresh minimal medium plates containing hygromycin B. Finally, transformants were sporulated on potato dextrose agar plates and stored at 4 °C for further research.

Molecular Analysis of Transformants

Total genomic DNA from fungal strains was prepared as described previously [12]. Then the genomic DNA was used as the template for molecular analysis. PCR detection of the *epo* gene was done using primers EPO-F and EPO-R, which defined a 501-bp *epo* gene. The primers CSP-F and EPO-R were used to amplify the fusion gene (561 bp) formed by the *cbh1* signal sequence and the EPO cDNA from the genomes of transformants. For further dot blot analysis of transformants, the PCR-amplified 501-bp-long fragment of the *epo* gene was used to detect the rHuEPO gene integrated into the fungal genomes. Probes were labeled with the nonradioactive digoxigenin (DIG) method and hybridization procedure carried out according to recommendations in the accompanying manual (Roche Diagnostics).

Reverse Transcription PCR Assay

The *T. reesei* strains were cultivated in shaker flasks in minimal medium for 2 days to obtain sufficient mycelia. The mycelia were then transferred to the lactose medium (minimal medium containing 2% lactose as a source of carbon) which induced expression of rHuEPO under the control of the optimized *cbh1* promoter. Total RNA of fungal strains was extracted using freeze-dried mycelia cultivated in induction medium as previously described [10]. The RT-PCR amplification of the rHuEPO gene was carried out using the TaKaRa RNA PCR kit (AMV) ver 3.0 (TaKaRa, Dalian, China) as described by the manufacturer. One microgram of total RNA was used as the template to produce single-stranded cDNA. The cDNA synthesis was primed by the reverse primer EPO-R. Then the cDNA was used directly for further PCR using the pair of primers EPO-F and EPO-R. Amplification products and their sizes were determined by electrophoresis on 0.8% agarose gels.

SDS-PAGE and Western Blot Analysis

The culture supernatants of different fungal strains were collected in lactose-inducing conditions as described above and analyzed for protein content by SDS-PAGE. Total protein content was resuspended in SDS treatment buffer (62 mM Tris–HC1, pH 6.8, containing 10% glycerol, 2% SDS, and 1% β-mercaptoethanol). Then the suspension was boiled for 5 min, and an aliquot of total protein (10 μg) was loaded onto a 12% SDS–polyacrylamide gel. Total protein concentration of samples precipitated by ammonium sulfate was determined using the Bradford assay (Bio-Rad, Hercules, CA) with bovine serum albumin as a protein standard. After electrophoresis, resolved proteins were visualized by staining

with Coomassie brilliant blue. The expression level of rHuEPO was estimated by scanning the SDS-PAGE map with Gene Tool (Version 3.00.22, SynGene, Frederick, MD, USA). For western blot analysis, the protein samples were separated by 12% (*v*/*v*) SDS-PAGE and subsequently transferred to a PVDF membrane (Hi-bond Amersham Biosciences, USA). For the detection of rHuEPO, the membrane was incubated with mouse anti-rHuEPO polyclonal antibody (Shanghai Shenggong Co. Ltd., China) for 1 h and then with HRP-conjugated goat anti-mouse IgG1 (Sigma) for 30 min according to the supplier's recommendations. Finally, the band was revealed by the peroxidase activity on 4-chloro-1-naphthol (Sigma) as the chromogenic substrate.

Results

Construction of Human Erythropoietin Expression Plasmid

As described in the "Materials and Methods" section, the 51-bp *cbh1* signal sequence, 501-bp EPO cDNA, and 790-bp *cbh1* terminator were cloned sequentially into plasmid pDelta-p4C, which contained the modified *cbh1* promoter for improved heterologous gene expression [10], to generate pTrCBH-EPO (Fig. 1a). In this construct, the mature part of the human erythropoietin was linked to the CBH I signal peptide for extracellular secretion (Fig. 1b). And between the modified *cbh1* promoter and signal sequence, the multiple restriction sites were maintained (Fig. 1b).

Generation of Recombinant *T. reesei* Strains

The resultant pTrCBH-EPO plasmid was co-transformed with pAN7-1 into *T. reesei* strains RutC-30 M3 and T108, respectively. Transformants were selected and spore purified on plates containing hygromycin B. Among the transformants, two colonies, T47 and T112, originating from RutC-30 M3 and T108, respectively, were found to have high genetic stability and express rHuEPO and were chosen for further analysis. Firstly, insertion of the expression cassette into the *T. reesei* chromosomes was confirmed by specifically amplifying the *epo* gene with primers EPO-F/EPO-R and the fusion region consisting of *cbh1* signal sequence and *epo* gene with primers CSP-F/EPO-R. Single bands of approximately 500 and 560 bp, which corresponded to the *epo* gene and the fusion region, were observed in the transformants T47 and T112, respectively (Fig. 2a). Then, the genomic DNA of the transformants was further analyzed by dot blot assay using a DIG-labeled probe specific for the target *epo* gene (Fig. 2b). Clear hybridization signals were observed in both of the transformants T47 and T112, suggesting that integration of the expression cassette had occurred in the genomes.

Expression of Human Erythropoietin in *T. reesei* Detected with RT-PCR

Total RNA from the *T. reesei* transformants T47 and T112 cultivated in induction medium was extracted, cDNA was synthesized, and RT-PCR was performed using the specific primers for the *epo* gene to detect the levels of rHuEPO mRNA. As shown in Fig. 2c, transformants T47 and T112 both gave a 500-bp RT-PCR product corresponding to the *epo* gene while no product was present in the parental strains RutC-30 M3 and T108. This result confirmed that the recombinant EPO expression cassette was successfully transcribed in *T. reesei* transformants.

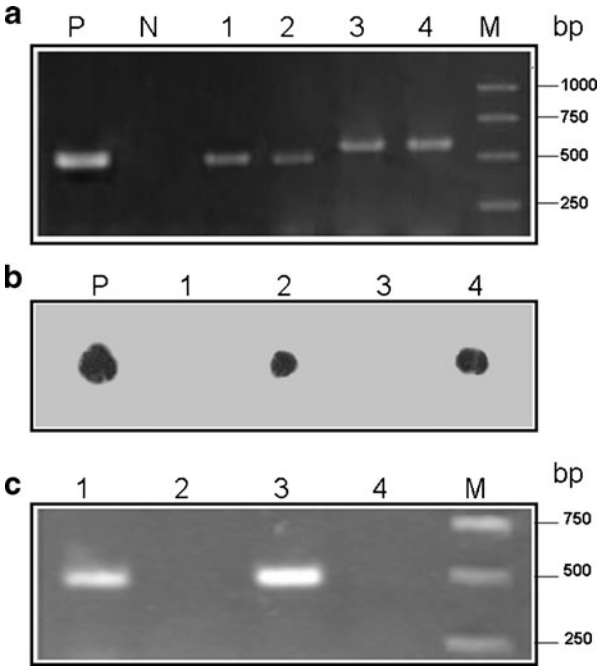

Fig. 2 Molecular analysis of the *epo* gene integrated into the fungal genomes. **a** Polymerase chain reaction assay of the *T. reesei* transformants. *Lanes 1, 2* amplification of a 501-bp fragment of the *epo* gene from transformants T47 and T112, respectively. *Lanes 3, 4* Amplification of a 561-bp fragment of the fusion of *cbh1* signal sequence and *epo* gene from transformants T47 and T112, respectively. *Lane P* positive control with vector pTrCBH-EPO for amplification of the *epo* gene. *Lane N* negative control with wild-type *T. reesei* genomic DNA. *Lane M* DNA molecular weight markers. **b** Dot blot hybridization with a DIG-labeled *epo* gene probe. *P* positive control with vector pTrCBH-EPO. *1–4* show the hybridization signals from genomic DNA of *T. reesei* RutC-30 M3, T47, T108, and T112, respectively. **c** Reverse transcription polymerase chain reaction analysis of EPO mRNA in transformants. *Lane M* DNA molecular weight markers. *Lane 1* transformant T47. *Lane 2 T. reesei* RutC-30 M3. *Lane 3* transformant T112. *Lane 4 T. reesei* T108

Secretion of Recombinant Human Erythropoietin in Different *T. reesei* Strains

The culture supernatants after lactose induction were concentrated and subjected to SDS-PAGE. The transformants were found to secrete rHuEPO with a maximum expression level at 48 h (Fig. 3). Arrows indicated the secreted rHuEPO in the fungal cultures. The rHuEPO with the putative molecular mass 28 kDa in transformant T47 was smaller than that secreted in T112 (32 kDa, Fig. 3). The expression level of secreted rHuEPO reached approximately 97 and 46 mg/L in the culture supernatants of T47 and T112, respectively. The secretion of the rHuEPO protein was further confirmed by western blot using the anti-EPO antibody. As shown in Fig. 4, the rHuEPO proteins from T47 and T112 exhibited a single band at approximately 28 and 32 kDa, respectively, indicating that the sizes of these heterologous proteins shown in SDS-PAGE are correct. *N*-glycosidase F treatment reduced the molecular mass of rHuEPO to about 20 kDa in both transformants T47 and T112 (data not shown), indicating that the lower and higher rHuEPO bands might be glycosylated in different ways by their parental *T. reesei* strains. Moreover, no specific band was observed in the SDS-PAGE or western blot of the parental strains or the noninduced strains. Therefore, these results suggested that the rHuEPO were well expressed and secreted in *T. reesei* strains.

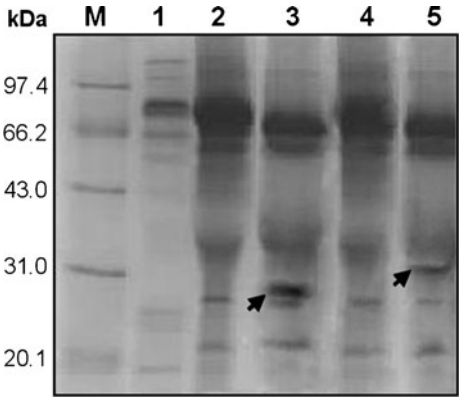

Fig. 3 Analysis of the expressed rHuEPO in extracellular culture supernatants of *T. reesei* strains T47and T112 by SDS-PAGE. *Lane M* protein molecular weight markers indicated in kilodaltons. *Lane 1* extracellular proteins of T47 without lactose induction. *Lane 2* extracellular proteins of lactose-induced RutC-30 M3. *Lane 3* extracellular proteins of lactose-induced T47. *Lane 4* extracellular proteins of lactose-induced T108. *Lane 5* extracellular proteins of lactose-induced T112. The bands corresponding to the rHuEPO are marked with *arrowheads*

Discussion

Filamentous fungi have the capacity to secrete large amounts of extracellular proteins and become the attractive hosts for production of foreign proteins such as pharmaceutical glycoproteins. There is an outstanding example showing that a humanized Fab′ fragment can be produced heterologously up to 1.2 g/L in *Aspergillus niger* [14], but most cases rarely exceed tens of milligrams per liter for production of foreign proteins [6]. Strategies to enhance production of heterologous gene products have been proposed and carried out successfully to construct novel recombinant fungal producers. Promoter copy number studies with *glaA* implied an activator limitation effect, and then introduction of additional activator binding sites into the different promoters to improve expression levels confirmed this finding [15]. The newly optimized multicopy *cbh1* promoter, which was adopted for use in this study, has been shown to improve the expression level of the heterologous gene

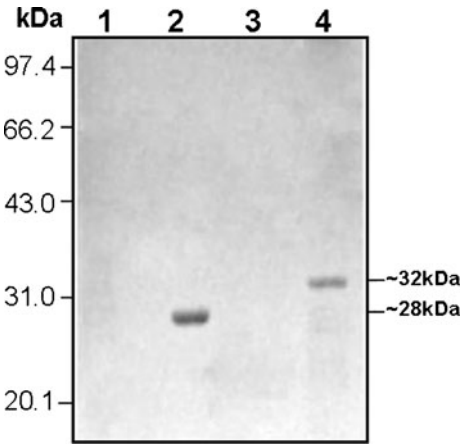

Fig. 4 Western blot analysis of rHuEPO using a mouse monoclonal antibody as primary antibody and HRP-conjugated goat anti-mouse as secondary antibody. *Lanes 1, 2* extracellular proteins of lactose-induced RutC-30 M3 and T47. *Lanes 3, 4* extracellular proteins of lactose-induced T108 and T112

(*gus* gene) [10]. Recently, we also used this promoter to successfully overexpress β-glucosidase and improved the β-glucosidase yield and total cellulase activity in *T. reesei* [16]. Protein fusions with a naturally well-secreted protein have been used as a suitable strategy to overcome the bottlenecks in the secretory pathway, but subsequent protease cleavage to release the desired protein was not always efficient possibly due to inaccessibility caused by the secondary structure [14].

In this study, the natural CBH I secretion signal sequence, not the whole protein, was adopted to be fused with HuEPO and allowed the resulting rHuEPO to efficiently secrete to the culture medium (Figs. 3 and 4). Proteolytic activity was known to considerably reduce the yield of the particular heterologous protein expressed, so it is often necessary to match the protease loss of function strain to the protein of interest [15]. Here, rHuEPO produced in the protease-deficient strain *T. reesei* T108 was found to keep the high level in the culture supernatants for more than 5 days (data not shown). The several strategies used in this study to improve heterologous HuEPO expression in *T. reesei* resulted in the production of relatively higher amounts of rHuEPO (46 to 97 mg/L) than those produced in nonmammalian hosts such as *Drosophila* S2 cells (18 mg/L) and humanized *P. pastoris* (20 mg/L) [4, 17]. The results suggest that using filamentous fungi as the HuEPO expression hosts combined with further optimization of expression system, much higher yields of recombinant HuEPO could be obtained.

Most filamentous fungi often synthesize short-chain *N*-glycans and *O*-glycans, resembling the mammalian high-mannose type [Man(6–9)GlcNAc2] rather than the hyperglycosylated yeast structures [8, 9]. In *Trichoderma*, the glycosylation of proteins has the *N*-glycans with high-mannose-type structures of limited size, in which the mammalian type core glycan (Man5GlcNAc2) is extended with mannose and/or glucose units [8]. Human EPO is a 166-amino acid glycoprotein containing three complex-type *N*-glycans and one mucin-type *O*-glycan, which comprise about 40% of the total mass of HuEPO [1]. The acidic form of EPO migrates in the range of 32~39 kDa on SDS-PAGE dependent upon the extent of such glycolysation [3].

In this study, recombinant HuEPO produced by two *T. reesei* strains T47 and T112, when analyzed by SDS-PAGE and western blotting, showed an apparent molecular mass at approximately 28 and 32 kDa, respectively (Figs. 3 and 4). Based on gel mobility data, the glycan on the *Trichoderma*-derived recombinant HuEPO in T112 was larger than that produced in T47, presumably as the result of glycosylation. It is speculated that the rHuEPO from T112 may be modified with larger *N*-glycan due to the transfer of GlcNAc residues to the glycans by the foreign *N*-acetylglucosaminyltransferase I. Our current results do not confirm the glycosylation of rHuEPO produced in *T. reesei*, and further studies are required to determine whether *T. reesei* are able to correctly process heterologous glycoproteins, particularly rHuEPO. In this regard, the use of genetic engineering strategy to produce humanized glycoproteins, as recently reported in *P. pastoris*, seems to be a powerful tool [4]. Indeed, some efforts in modifying the glycosylation pathway in *T. reesei* have demonstrated the potential of producing humanized complex *N*-glycans by protein *N*-glycan-engineering strategy [18]. The physiological characteristics of rHuEPO produced in this study are still being examined, whereas we did not find any serious obstacles to heterologously express this human glycoprotein by *T. reesei*. Moreover, it should be possible to produce other pharmaceutical gene products using *T. reesei* as a particular host. In summary, this study demonstrates the potential of *T. reesei* as an alternative platform for extracellular human-derived glycoprotein overexpression, providing clues to the large-scale production of pharmaceutical gene products at reduced cost in filamentous fungal systems.

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