Cellular Engineering Strategies to improve Recombinant Factor VIII Productivity in CHO Cells

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Introduction

Lower recombinant protein productivity significantly impacts the efficiency and cost of therapeutic proteins. Factor VII deficiency, Hemophilia A (FVIII deficiency), and Hemophilia B (FIX deficiency) are treated using recombinant clotting factors, [1,2] which provide safer, virus-free alternatives to plasma-derived products [1]. Clotting factors are recognized as difficult-to-express proteins (with reported titers ranging between 40–200 mg/L), as their production is hindered by multiple bottlenecks in the secretory pathway [1,3]. Over 200,000 people suffer from Hemophilia A globally, with annual therapy costs exceeding $200,000 per individual, highlighting the need for enhanced productivity of FVIII [4].

Factor VIII is a large (~300 kDa), heavily glycosylated protein comprising six domains (A1-A2-B-A3-C1-C2). It undergoes proteolysis in the Golgi Apparatus to form a Heavy Chain (A1-A2-B) and a Light Chain (A3-C1-C2). In the blood plasma, FVIII circulates as a heterodimer bound to von Willebrand Factor (vWF), which stabilizes the protein [5]. The following sections categorize cellular engineering strategies by their location in the secretory pathway (as shown in **Figure 1**).

Methods of Transgene Integration

Methods of Random Integration have several drawbacks, including low integration efficiency and a lack of control over the site in the host cell genome where the gene of interest is integrated. Methods of Semi-Specific Integration, however, have been known to act as efficient alternatives to traditional Random Integration Methods in the production of recombinant proteins [6].

A *PiggyBac*-based transposon system was shown to provide a yield of 3 mg/L of rFVIII in adherent cell cultures of CHO-K1 cells; a yield of 100 mg/L was achieved with the formation of suspension culture and fed-batch cultivation for 14 days. The plasmid construct consisted of the *PiggyBac* transposon, a GFP reporter gene downstream of a strong CMV promoter, and the FVIII gene downstream of a strong EF1-α promoter [7].

Codon Optimization

Codon Optimization resulting in changes in mRNA sequence but not in the final amino acid sequence of the polypeptide results in the stalling of the ribosome during translation at certain sites, thereby allowing the step-wise folding of the nascent polypeptide into its respective domains as it enters the Endoplasmic Reticulum (ER) [8].

Expression of rFVIII in 293T cells and in vivo models of hemophilic mice showed a 29-44-fold increase in the secretion of rFVIII products comprising B-Domain Deletions (BDD) [8]. Another study reported a 7-fold yield of codon-optimized BDD-rFVIII over wild-type rFVIII in 293T cells (as illustrated in **Figure 2**); moreover, codon-optimized BDD-rFVIII was also shown to possess similar kinetic parameters for vWF-binding and similar PTM sites, and glycosylation patterns, as its wild-type counterpart [9].

Protein Folding in the ER

Binding-immunoglobulin Protein (BiP) preferentially binds to nascent, unfolded proteins in the ER, thereby activating UPR, which leads to an expansion in ER volume and increased transcription of chaperones [2]. Activation of the UPR has been shown to induce oxidative stress, and thereby apoptosis in response to misfolding of rFVIII in the ER, which could be relieved by treatment with antioxidants and knockout of downstream UPR targets that activate apoptosis, e.g. CHOP [10].

Mutation of the Phenylalanine residue at the 309th position to a Serine (F309S) in the rFVIII-BiP binding domain was shown to increase activity 2.3 times in comparison to the r-protein obtained without mutagenesis in COS-1 cells (the binding of rFVIII to BiP is shown in **Figure 3**); this was correlated with an increase in the extracellular rFVIII antigen, thus pointing to an increased secretion of the product [11]. Another study reported a

decrease in rFVIII secretion on overexpression of BiP; moreover, rhFVIII secretion was observed to increase by a factor of 2 upon BiP knockdown using shRNA, thus pointing to how downregulation of BiP can release nascent polypeptides that would otherwise bind to BiP in the ER, thereby improving secretion [12].

B-Domain Deletion

A knockout is a genetic technique that inactivates a specific gene to prevent functional protein production. In CHO-based systems, it is used to remove proteases or block competing pathways, thereby improving recombinant protein yield [13].

However, B-domain deletion (BDD) is not a knockout of the host cell but a change to the FVIII gene, where the part of the gene that makes the B-domain is removed. The B-domain is proteolytically cleaved upon activation by thrombin and is not required for procoagulant activity in vitro or in vivo. The heavily glycosylated B-domain with its clustered N-linked sites makes folding inefficient, promotes prolonged BiP (GRP78) binding in the ER, and leads to a reduction in the secreted rFVIII [14].

In early B-domain deletion (BDD) constructs, a 14–amino acid linker containing the furin recognition motif was retained (as shown in **Figure 4**), assuming furin activity was necessary for proper FVIII maturation and activity. Further studies demonstrated that furin inhibition enhanced FVIII-BDD secretion and reduced ER retention. A recent FVIII transgene sequence lacking the furin site (FVIII-ΔF) achieved an approximately three-fold increase in secreted rFVIII [15].

A subsequent study (for production of rFVIII using BHK cell lines) demonstrated that deletion of the furin recognition site resulted in higher efficacy in blood clotting assays and in vivo models (however, this was done at a small scale as a proof of concept) [16]. This advancement represents a next-generation refinement of the BDD strategy, combining improved secretion, stability, and hemostatic activity without an associated increase in immunogenicity.

Co-expression of von Willebrand Factor

The vWF ensures that once FVIII chains are cleaved and leave the cell, they associate into a stable complex immediately after secretion [14]. In serum-free cultures, most secreted rFVIII binds to phosphatidylserine (PS) on cell membranes, reducing soluble yield (90% of the expressed rFVIII was membrane-bound) [17]. Co-expression of vWF with FVIII in CHO (10A1C6) cells demonstrated that vWF and rFVIII form a stable complex in the extracellular medium, eliminating the need for exogenous vWF and protecting FVIII from degradation. This stabilization prevents loss of FVIII activity in the culture medium, including sequestration by phosphatidylserine (PS) in the membrane. A key bottleneck is FVIII retention in the ER due to inefficient folding and prolonged BiP/GRP78 association, limiting the secreted rFVIII [18].

Ranking of Cellular Engineering Strategies

The cellular engineering strategies discussed above are ranked below solely on their individual effects on rFVIII yield and activity. Each technique has been evaluated independently, without accounting for possible combinatorial or multiplexed applications, to ensure the ranking reflects only the intrinsic merit of each approach.

I) While most of the studies reviewed here relied on methods of Random Integration during transfection, a single study reported a FVIII yield of 100 mg/L after fed-batch cultivation for 14 days upon **semi-specific integration** using a **Transposon-based construct** [7]. This approach can help ensure the integration of the gene of interest in constitutively active regions of chromatin in host cells, thereby addressing multiple issues arising from Random Integration.

II) **Codon optimization** was shown to increase r-protein secretion in several transgenes containing varying BDD modifications [9], thus establishing codon optimization as a promising method of improving protein folding, reducing protein aggregation in the ER and fast-tracking transit of the protein through the secretory pathway.

III) **B-Domain Deletion** in CHO 10A1C6 cells led to a ~5 to 10 times increase in the secreted rFVIII [14]. Additionally, the deletion of the furin binding sites in BHK cells to produce FVIII-ΔF led to a 3-fold increase in the yield of FVIII as compared to the secreted FVIII-BDD [15].

IV) A **point mutation** in the **A1 domain** of the rFVIII protein has been shown to increase protein secretion by reducing the affinity of BiP for the r-protein [11]. **Bypassing BiP**

interaction by a point mutation, thus, can be considered as a potent solution that preserves overall protein function, reduces protein aggregation and downregulates the activation of downstream UPR targets that are known to induce apoptosis.

V) **Co-expression of vWF** for increasing the stability of secreted rFVIII shows a higher fraction of secreted rFVIII into the extracellular space. The drawback is that misfolded proteins tend to aggregate in the ER in spite of a two to threefold increase in BiP expression compared with the original rFVIII-expressing cells, leading to a large fraction of rFVIII aggregating in the ER [18].

However, if we are to consider the potential of a method to increase rFVIII productivity when combined with other strategies, co-expression of vWF will yield the highest secretion levels, provided protein aggregation in the ER due to BiP binding is dealt with. We propose that combining vWF co-expression with the A1 domain point mutation effectively addresses this limitation. Furthermore, to increase efficiency of integration and transcription, we propose the use of semi-targeted integration using a *PiggyBac* transposon-based construct.

Experimental Protocol

We aim to compare the production of rFVIII by CHO 10A1C6 (a stable FVIII expressor) and a modified CHO-K1 cell line to produce 2 recombinant products (vWF and FVIII) in a serum free media without the addition of exogenous vWF.

I) Cell Line Development

1. Plasmid Construction

The plasmid can be constructed so as to allow expression of rFVIII, vWF and the *PiggyBac* transposase under the influence of suitable promoters, along with specific antibiotic resistance genes, as established in [6]. The transposon and the exact plasmid construct have been described in (**Figure 5**).

2. Transfection of the Host Cells

Integration of the Plasmid into the host cells can be performed using standard techniques such as electroporation or the calcium phosphate technique.

3. Cell line development

The CHO-K1 cell line that does not possess modifications from the wild type can be transfected with the plasmid described above to co-express vWF with rFVIII possessing the desired A1 domain point mutation. The CHO-10A1C6 cell line that expresses stable, active r-FVIII can be used as the control.

4. Establishment of stable cell lines

Clones selected based on drug screening are expanded from 96-well plates or T-flasks (adherent cell cultures), adapted to serum-free media, and developed into suspension cultures upon treatment with Trypsin. These cultures enable rFVIII and rFVIII-vWF production studies under batch and fed-batch modes.

II) Evaluating Product Parameters

1. Detection of rFVIII (using Western Blot)

Proteins of interest can be isolated via His-tag affinity chromatography or detected using standard Western blot protocols.

2. Quantification of secreted r-protein (using ELISA)

Concentration of rFVIII (expressed by CHO-A10C6 cells) and rFVIII-vWF (secreted by transformed CHO-K1 cells) can be measured using ELISA with the help of antibodies directed against conserved domains of rFVIII in both products.

3. rFVIII activity assay

Several commercial kits help measure the activity of secreted rFVIII. The coagulation property of rFVIII can be measured using standardized protocols or as per the manufacturers' instructions.

III) Cell growth and Specific Productivity

Suspension cultures of CHO-K1 or CHO-A10C6 cells can be used for batch or fed-batch cultivation to determine specific productivity of the cell lines for their respective products under specific cultivation conditions. Daily measurements can be carried out to determine viable cell density and product concentration, and plot their variation with time. Specific productivity can then be determined based on the obtained bioreactor culture data.

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