Bioengineering of differentiated hepatocytes with \textit{human factor IX}-expressing plasmids \textit{in vitro} \\

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\textbf{ABSTRACT} \\

For somatic gene therapy of hemophilia B, hepatocytes as the main cellular host for expression of hFIX are attractive targets. In gene therapy protocols, an efficient expression vector equipped with \textit{cis}-regulatory elements such as introns is required. With this in mind, \textit{hFIX}-expressing plasmids equipped with different combinations of 2 human \textit{\beta}-globin (\textit{hBG}) introns inside the \textit{hFIX}-cDNA and Kozak element were used for bioengineering of HepG2 cells as a model for differentiated hepatocytes and CHO cells a cell line generally used to produce recombinant hFIX (rhFIX).

In HepG2 cells, the highest hFIX secretion level occurred for the intron-less plasmid with 8.5 to 53.8-fold increases, while in CHO cells, the \textit{hBG} intron-I containing plasmid induced highest hFIX secretion level with 2.3 to 14.3-fold increases as compared to other plasmids. The first \textit{hBG} intron appears to be more effective than the second one in both cell lines. The expression level was further increased upon the inclusion of the Kozak element. The highest hFIX activity was obtained from the cells that carrying the intron-less plasmids with 470 mU/ml and 25 mU/ml for HepG2 and CHO cells respectively. Secretion of active hFIX by all constructs was documented except for \textit{hBG} intron-II containing construct in both cell lines. Presence of both \textit{hBG} intron-I and II inside the \textit{hFIX}-cDNA provides properly spliced hFIX transcripts in both cell lines.

In conclusion, the advantages of \textit{hBG} introns as attractive \textit{cis}-regulatory elements to obtain higher expression level of hFIX particularly in CHO cells were demonstrated. Hepatocytes could be effectively bioengineered with the use of plasmid vectors and this strategy may provide a potential \textit{in-vitro} source of functional hepatocytes for \textit{ex-vivo} gene therapy of hemophilias and production of \textit{rhFIX} \textit{in vitro}.

\textbf{Introduction} \\

Human factor IX (hFIX) is a member of vitamin K-dependent blood clotting factors and plays an important role in the middle phase of the blood coagulation.\textsuperscript{1} It circulates as an inactive precursor before activation with either factor Xa and calcium ions or tissue factor/factor VIIa and calcium ions during blood coagulation.\textsuperscript{2}

Hemophilia B is an X-linked bleeding disorder caused by deficiency or absence of FIX.\textsuperscript{1} Replacement therapy with either pooled plasma concentrates or recombinant hFIX (rhFIX) successfully improves patients’ life expectancy.\textsuperscript{3} However, the cost of replacement therapy, risk of blood-borne pathogens transmission, formation of inhibitors, allergic and thrombosis reactions justify the development of gene and cell-based therapies of hemophilias.\textsuperscript{4,5}

Major challenges in gene or cell-based therapies of hemophilias are choice of cell type, low expression level of the coagulation factor and immunological complications after using viral vectors.\textsuperscript{6,7}

For the somatic gene therapy of hemophilia B, different cell types have been evaluated.\textsuperscript{8-15} In our previous study, bone-marrow mesenchymal stem cells were used to transfect with different \textit{hFIX}-expressing plasmids. As these cells are non-hepatocyte cell types, the capacity of cells to produce fully active hFIX was limited at high expression rates.\textsuperscript{15} Hepatocytes as the main cellular host for expression of functional hFIX.
cells induce antigen specific tolerance with successful delivery of synthesized FIX protein into the circulation. Therefore, they are attractive targets for cell-based gene therapy of hemophilias.

In addition to the choice of cell type, an efficient expression vector equipped with suitable regulatory elements might facilitate efforts toward higher expression level of transgene and reduce the immune responses to the transgene product.

Viral vectors have the potential for long-term expression via stable integration into the target cell genome. But there are still different hurdles that need to be overcome. In this regard, unwanted complications provoked by viral gene transfer into cells have been shown. Therefore, to bypass safety concerns associated with viral vectors, non-viral gene transfer systems are attracting increasing interest due to their biosafety issues and ease of handling. Furthermore, Non-viral vectors have the advantage of being less toxic and unable to generate harmful replication-competent-by-products through recombinant events as with viral vectors.

Introns as cis-regulatory elements have potential to improve gene expression in a broad range of organisms. Introns and their removal by spliceosomes regulate the expression of genes in different levels, including transcription, polyadenylation, nuclear mRNA export, translational efficiency and mRNA decay.

Kurachi and colleagues showed that the hFIX intron-I has potential to improve hFIX gene expression in-vitro. Improvements of transgene expression by introduction of the hBG introns in a number of expression systems have also been shown.

In most of studies, the introns have been introduced in the upstream of cDNAs with rather ignoring the potentials of introducing the introns inside the transgene open reading frame. Introduction of introns into coding regions may have substantial effects over their insertion into the untranslated regions. It is reasonable to assume that introns function more effectively in locations similar to their natural positions. This approach would create a gene structure reminiscent of typical mammalian genes to provide a near-natural substrate for gene expression.

In our bioengineering strategy, 5 hFIX-expressing plasmids equipped with different combinations of 2 hBG introns inside the hFIX-cDNA and Kozak element (Fig. 1) were used for expression analysis in HepG2 and CHO cells. HepG2 cells have maintained several features of differentiated hepatocytes and do not express hFIX. Thus, these cells are attractive model for evaluation of hFIX expression in-vitro.

Our main goals in the present study were to establish a non-viral gene transfer system in differentiated hepatocytes to evaluate the potential of these cells for expression of functional hFIX from recombinant hFIX-expressing plasmids. Besides, various aspects of the functions of heterologous hBG introns on expression of the hFIX in HepG2 cells as well as in CHO cells, a cell line generally used to produce rhFIX were investigated.

**Comparison of the functions of hBG introns on expression of hFIX in HepG2 and CHO cells**

Gene therapy has provided an alternative approach for treatment of hemophilia B. Concurrently, the efficacy of
such a therapeutic approach depends on determination of which vectors give maximal transgene expression. With this in mind, 5 recombinant hFIX-expressing plasmids (Fig. 1) were used for transient expression analysis in HepG2 and CHO cells.

As the data indicate, successful expressions of the hFIX were obtained from each of the constructs in the examined cell lines. In HepG2 cells, the highest expression levels of the hFIX occurred for the cells containing p.KhFIX and p.hFIX-I constructs respectively. Concomitantly, the lowest expression level of the hFIX occurred for the cells containing the p.hFIX-I,II construct (Fig. 2A). When the cell numbers on the second day of post-transfection were adjusted, the hFIX expression level of the cells containing the intron-less construct was 2690 ng/ml per 10^7 cells with 18.5 to 53.2-fold increases when compared with those of the cells containing other constructs (Table 1).

In CHO cells, the level of the hFIX protein was significantly higher in the presence of the hBG intron-I containing construct (Fig. 2A). On the second day of post-transfection, the hFIX expression level of the CHO cells containing the hBG intron-I construct was 114 ng/ml per 10^7 cells with 2.7 to 14.3-fold increases in hFIX levels when compared with those of the cells transfected with other constructs. The lowest expression level of the hFIX occurred for the minigene that carries the hBG intron-II (Table 1).

To compare the expression rate of 5 plasmids in 2 cell lines, evaluation of trasfection efficiency in both cell lines is required. To this end, optimization of different parameters such as transfection reagent, plasmid

![Figure 2](Image)
FIX protein levels in supernatants produced by different vectors were calculated using ELISA and normalized by the cell numbers. To compare expression levels of hFIX by different vectors, hFIX protein level produced by a vector divided to the hFIX protein levels produced by other vectors. All experiments were repeated twice using duplicate assays and the results were presented as the mean. Asterisk indicates the significant difference (P < 0.05) between 2 different vectors for expression of the hFIX from the same cell line using analysis of variance.

DNA concentration and duration of exposure of cells to transfection reagent: plasmid DNA complexes are required to determine the best parameters and conditions. Based on our experiments, under the same conditions, equal transfection efficiencies were obtained for both cell lines. Our data showed that, mixture of 6 μl transfection reagent with 2 μg plasmid DNA induced highest expression of eGFP in both cell lines 48 hours post-transfection and resulted in equal transfection efficiencies for both cell lines (data not shown). In this context, under the same conditions, HepG2 cells were able to secret higher hFIX levels by 0.6 to 112.2-fold increases compared to CHO cells transfected with the same constructs.

The possible accumulation of the expressed hFIX inside the recombinant cells was investigated by measurement of the hFIX antigenicities of the cellular lysate of the cell pools of different transfectedants. As the data indicate, highest amount of the hFIX was accumulated inside the genetically modified HepG2 and CHO cells when the cells transfected with the intron-less and hBG intron-I containing constructs (Fig. 2B, Table 2).

The secretion efficiency of a particular protein is defined as the ratio between the secreted fraction and its total amount. Based on our data, the secretion efficiency of the hFIX obtained from expression of different constructs in HepG2 and CHO cells varied between 45–96% and 50–91% respectively (Table 3).

| Recombinant plasmid | Fold (hFIX level in supernatant) | Fold (hFIX level within the recombinant cells) |
|---------------------|---------------------------------|-----------------------------------------------|
| p.KhFIX vs.         | p.KhFIX-I 18.5 ± 2.4 | p.KhFIX-I 0.9 ± 1.1 |
| p.KhFIX-II vs.      | p.KhFIX-II 29.2 ± 3  | p.KhFIX-II 1.1 ± 1.6 |
| p.KhFIX-I vs.       | p.KhFIX-I,II 46.4 ± 0.6 | p.KhFIX-I,II 1.3 ± 1.4 |
| p.KhFIX-II vs.      | p.KhFIX-I,II 53.2 ± 0.6 | p.KhFIX-I,II 1.1 ± 1.4 |
| p.KhFIX-I,U vs.     | p.KhFIX-I 1.6 ± 14.3 | p.KhFIX-I 3.2 ± 1.2 |
| p.KhFIX-II,U vs.    | p.KhFIX-I 2.5 ± 2.7 | p.KhFIX-I 2.3 ± 1.4 |
| p.KhFIX-I,II U vs.  | p.KhFIX-I 2.9 ± 2.3 | p.KhFIX-I 2.5 ± 1.4 |
| p.KhFIX-I,II,U vs.  | p.KhFIX-I,II 1.6 ± 0.2 | p.KhFIX-I,II 1.4 ± 0.2 |
| p.KhFIX-U vs.       | p.KhFIX-U 1.8 ± 0.2 | p.KhFIX-U 2.1 ± 0.7 |
| p.KhFIX-U vs.       | p.KhFIX-U 1.8 ± 0.2 | p.KhFIX-U 3.4 ± 0.9 |
| p.KhFIX-I,II,U vs.  | p.KhFIX-I,II 1.2 ± 1.1 | p.KhFIX-I,II 1.4 ± 1.2 |

Table 2. Comparison of the recombinant plasmids for expression of the hFIX within the genetically modified cells after 2 d of cultivation.

Table 3. Secretion efficiency of hFIX in genetically modified cells on the second day of post-transfection.

| Recombinant plasmid | Secretion efficiency of hFIX protein (%) |
|---------------------|-----------------------------------------|
| p.KhFIX             | 96 ± 2.1 ± 1.1 ± 2.2  |
| p.KhFIX-I           | 57 ± 5.7 ± 0.7  |
| p.KhFIX-II          | 48 ± 7.1 ± 0.7  |
| p.KhFIX-U           | 55 ± 0.7 ± 0.7  |
| p.KhFIX-U           | 45 ± 7.7 ± 0.7  |
| Secretion efficiency| 45–96% ± 0.7 ± 0.7 |

The results represent the mean ± SD from 2 independent experiments with duplicate assays. Asterisks indicate samples that are significantly different (P < 0.05) compared to other samples from the same cell line, using analysis of variance.
behavior in the context of the heterologous intron function and a possible effect of the host cell type on intronic fate and functions.

**Conclusions**

This is the first report to show the role of the hBG introns inside the hFIX-cDNA in the context of plasmid vectors on the expression of hFIX in differentiated hepatic cell line and CHO cells. In HepG2 cells, the highest expression levels of the hFIX obtained from the intron-less and hBG intron-I containing constructs respectively. While, in CHO cells, the highest expression levels of the hFIX obtained from the hBG intron-I and Intron-I, II containing constructs respectively. Our results demonstrate the advantages of hBG introns as attractive cis-regulatory elements to obtain higher expression level of hFIX particularly in CHO cells and suggest that the present non-viral gene delivery system may provide a potential in-vitro source of functional hepatocytes for ex-vivo gene therapy of hemophilies and production of rhFIX.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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