



Genetic modification of bone-marrow mesenchymal stem cells and hematopoietic cells with human coagulation factor IX-expressing plasmids



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ABSTRACT

Ex-vivo gene therapy of hemophilias requires suitable bioreactors for secretion of hFIX into the circulation and stem cells hold great potentials in this regard. Viral vectors are widely manipulated and used to transfer hFIX gene into stem cells. However, little attention has been paid to the manipulation of hFIX transgene itself. Concurrently, the efficacy of such a therapeutic approach depends on determination of which vectors give maximal transgene expression. With this in mind, TF-1 (primary hematopoietic lineage) and rat-bone marrow mesenchymal stem cells (BMSCs) were transfected with five hFIX-expressing plasmids containing different combinations of two human β -globin (*hBG*) introns inside the hFIX-cDNA and Kozak element and hFIX expression was evaluated by different methods.

In BMSCs and TF-1 cells, the highest hFIX level was obtained from the intron-less and *hBG* intron-I,II containing plasmids respectively. The highest hFIX activity was obtained from the cells that carrying the *hBG* intron-I,II containing plasmids. BMSCs were able to produce higher hFIX by 1.4 to 4.7-fold increase with activity by 2.4 to 4.4-fold increase compared to TF-1 cells transfected with the same constructs. BMSCs and TF-1 cells could be effectively bioengineered without the use of viral vectors and hFIX minigene containing *hBG* introns could represent a particular interest in stem cell-based gene therapy of hemophilias.

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1. Introduction

Hemophilia B is an X-linked bleeding disorder results from the absence or low level of FIX in plasma [1]. This genetic disease causes

high mortality in patients and its treatment requires frequent infusion of normal hFIX, produced either from human plasma or recombinant expression systems [1].

The high cost of replacement therapy, risk of blood-borne pathogens transmission, formation of inhibitors, allergic and thrombosis reactions, caused attention to other treatments, including gene and cell-based therapy of hemophilias [2–4].

Two types of strategies have been considered so far for treatment of hemophilias. A first strategy, consist of removing cells from the patients, modifying the cells *ex vivo* and injection of genetically modified cells expressing the therapeutic protein to the patients

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[4]. The second strategy, involved a direct injection of a vector containing the transgene in target tissues or in the circulation [5].

Several potential target cells were shown to be able to produce an active hFIX such as hepatocytes [6], myoblasts [7], keratinocytes [8,9], endothelial cells [10], bone-marrow stromal cells [11] and hematopoietic stem cells (HSCs) [12].

Among examined cells, hepatocytes are the main cellular host for expression of functional hFIX. These cells induce antigen-specific tolerance with successful delivery of synthesized FIX protein into the circulation [13]. Thus, they are attractive candidate for cell-based gene therapy approaches. Unfortunately, the isolation of sufficient transplantable hepatocytes depends on and restricted by different conditions and viral liver infections in hemophiliacs. Therefore, attention to other suitable bioreactors for production and delivery of hFIX into the circulation is essential. HSCs and BMSCs are attractive target cells in this regard. HSCs have capacity for self-renewal with immediate access to the blood circulation by virtue of their progeny representing all blood cell types, induction of antigen-specific tolerance, ability to trans-differentiate into non-hematopoietic cells such as hepatocytes and relative ease of transfusing *ex vivo*-generated stem cell-derived cells [12,14,15]. BMSCs have several unique properties which make them ideally suited for cell-based therapy of hemophiliacs. These include; relative ease of isolation from various tissues, the ability to self-renew and differentiate into multiple lineages, the ability to be extensively expanded in culture without loss of differentiation capacity and lower immunogenicity in transplant procedure with anti-inflammatory properties [16,17].

Despite the improvement of recombinant viral vectors, little attention has been paid to the bioengineering of transgene itself and generation of genetically modified cells using plasmid vectors. In this respect, introduction of first intron of hFIX into *hFIX*-cDNA increased hFIX production compared to the intron-less cDNA [18]. In our previous study, *hBG* intron-II and *hBG* intron-I,II were introduced at their corresponding positions inside the *hFIX*-cDNA and increased hFIX production by 5 to 25-fold increase in comparison with the intron-less cDNA in Chinese hamster ovary (CHO) cells [19].

Human β -globin gene expressed at high level in hematopoietic cells and experimental studies showed that the *hBG* introns play critical roles in the expression of the corresponding gene [20]. The enhancer-like activities of the *hBG* introns on the expressions of different transgenes have also been shown [21–23]. With this in mind, we assumed that *hBG* introns would improve the hFIX expression in hematopoietic and mesenchymal expression systems.

To examine this assumption, in our bioengineering strategy, five *hFIX*-expressing plasmids with different combinations of two *hBG* introns inside *hFIX*-cDNA and Kozak element were constructed and introduced into the TF-1 cells as a model for primary hematopoietic cells and BMSCs.

Our main goals in the present study were to evaluate the potential of nonviral FIX gene transfer into hematopoietic and mesenchymal stem cells for expression, storage and secretion of biologically active hFIX from the *hBG* introns containing constructs *in vitro*.

2. Materials and methods

2.1. Bacterial strain, cells, plasmids and primers

DH5 α strain of *Escherichia coli* (Stratagene, La Jolla, CA, USA) was used as the bacterial host for various cloning and sub-cloning steps. TF-1 cell-line as a model for primary hematopoietic cells was obtained from the National Cell Bank of Iran (NCBI; Pasture Institute of Iran). BMSCs were isolated from the tibias and the femora of rats

and used as expression host. Plasmid pET26-hFIX was used for cloning and sub-cloning steps as well as source of the *hFIX*-cDNA. Plasmid pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) was used for construction of the *hFIX*-expressing plasmids. The primers used for polymerase chain reactions (PCRs) were synthesized by MWG Biotech (Ebersberg, Germany).

2.2. Media, enzymes, chemicals, antibodies and kits

Luria-Bertani medium was used for bacterial growth in which either ampicillin (100 μ g/ml) or Kanamycin (30 μ g/ml) was added when required, to maintain selection pressure. RPMI-1640, fetal bovine serum (FBS), bovine serum albumin (BSA) and L-glutamine were obtained from Gibco-BRL Life Technology (Karlsruhe, Germany). Penicillin G and streptomycin were purchased from Sigma-Aldrich (Munich, Germany). All the enzymes used for molecular techniques in addition to kits for PCR product purification, plasmid isolation, RNA preparation, X-tremeGENE HP transfection reagent and protease inhibitors were purchased from Roche (Mannheim, Germany). Interleukin-3 (IL-3) was purchased from R & D systems (Minneapolis, USA). The kit for the cloning of PCR products (InsT/Aclone) was obtained from Fermentas (Burlington, Ontario, Canada). The enzyme-linked immunosorbent assay (ELISA) kit for the measurement of the hFIX antigen (Asserachrom, hFIX:Ag) and FIX-deficient plasma were purchased from Diagnostica Stago (Asnieres sur Seine, France). One-step reverse transcription-PCR (RT-PCR) kit (Accupower RT/PCR PreMix) was purchased from Bioneer (Alameda, USA). Monoclonal antibodies against antigens CD90, CD44 and CD45 were purchased from eBioscience (San Diego, USA). Monoclonal antibody against CD29 was purchased from Abcam, USA.

2.3. DNA manipulation techniques

DNA manipulation techniques were carried out based on standard protocols. *Cis*-regulatory elements used to construction of *hFIX* expression cassettes are shown in Table 1.

2.4. Construction of *hFIX* expressing plasmids

The first plasmid, p.KhFIX carrying an intron-less *hFIX*-cDNA was used both as the parental *hFIX*-expressing plasmid for the construction of *hBG* intron-containing plasmids and as a control *hFIX*-expressing plasmid. The four other constructed plasmids, namely p.KhFIX-I (carry *hBG* intron-I), p.hFIX-II (carry *hBG* intron-II), p.KhFIX-I, II and p.hFIX-I, II (carry *hBG* intron-I, II) represent the second generations of the p.KhFIX plasmid (Table 1).

Detailed construction of the intron-less and *hBG* intron-containing plasmids were described previously [19].

2.5. Verification of the recombinant plasmids

Verification of various plasmids constructed during cloning and sub-cloning steps was done by restriction digestion followed by nucleotide sequence analysis of both strands of the inserted fragments in the *hFIX*-expressing plasmids; using ABI 373A automated sequencer (MWG-Germany). All expression cassettes preserved the consensus splice donor, acceptor and lariat branch point elements at the 5' and 3' end of introns.

2.6. Ethics statements

Prior to study, all procedures were approved by our institution's animal welfare regulatory committee.

Table 1
Various cis-regulatory elements used for the construction of hFIX expression cassettes in plasmid pcDNA3.1.

Construct	Enhancer/promoter (CMV IE)	Kozak element	hBG introns (in their identical positions inside the hFIX-cDNA)	Bovine growth hormone poly adenylation signal sequence
p.KhFIX	+	+	–	+
p.KhFIX-I	+	+	Intron-I (130 bp)	+
p.hFIX-II	+	–	Intron-II (850 bp)	+
p.KhFIX-I, II	+	+	Intron-I&II (130 bp and 850 bp)	+
p.hFIX-I, II	+	–	Intron-I&II (130 bp and 850 bp)	+

CMV IE: CMV immediate early enhancer/promoter. hBG intron-I located between exons 1 and 2 of the hFIX gene. hBG intron-II located between exons 2 and 3 of the hFIX gene. hBG intron-I&II located between exons 1 and 2 and between exons 2 and 3 of the hFIX gene respectively. Verification of various recombinant pcDNA3 based-vectors was done by nucleotide sequence analysis.

2.7. Culture and expansion of TF-1 cells

TF-1 cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 5 ng/ml recombinant human IL-3, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in humidified air with 5% CO₂. After TF-1 cells were grown to 80% to 90% confluency in flask, the cells were passaged and used for transfection.

2.8. Isolation, culture and expansion of rat BMSCs

Bone-marrow was aspirated from the tibias and the femora of female Wistar rats (6–8 weeks old) by insertion a 21-gauge needle into the shaft of the bone and flushing it with DMEM medium supplemented with penicillin and streptomycin. After centrifugation and re-suspension, the isolated cells were seeded in 25-cm² culture dishes in medium containing low glucose DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin and kept in a humidified CO₂ incubator. The cells were allowed to adhere for 3 h, and non-adherent cells were washed out with medium changes. Thereafter, the medium was changed every 24 h during 21 days of culture. After removing the suspension cells, adherent BMSCs were grown to 80 to 90% confluency in flask and then passaged. The isolated BMSCs from third or fourth passage were used for flowcytometry and transfection.

2.9. Flowcytometric analysis of culture-expanded BMSCs

To characterize the cell markers of culture-expanded cells, monolayer adherent cells (Passages 3 or 4) were trypsinized and stained with monoclonal antibodies specific for BMSCs as follows: anti-CD90-phycoerythrin (PE), anti-CD44-fluorescein isothiocyanate (FITC), anti-CD29-FITC and anti-CD45-FITC. Anti-IgG-PE and anti-IgG-FITC were used as isotype negative controls. Flowcytometric analyses were performed using a PAS flowcytometry (Partec GmbH, Germany).

2.10. Transfection efficiency in TF-1 cells and BMSCs

One day before transfection, the cells were placed in six-well plates at a density of 3×10^5 cells in 2 ml of medium. Various concentrations of pEGFP-N1 plasmid vector (0.5, 1 and 2 µg) carrying eGFP as a reporter gene were mixed with 3 or 6 µl of transfection reagent and used for transient transfection of the prepared cells. Six hours after transfection, conditioned media were replaced by fresh media and 24 h to 72 h post-transfection, the number of GFP positive cells was counted by hemocytometer using inverted fluorescence microscope. Transfection efficiency (TE) was calculated according to the following formula: TE (%) = (number of GFP positive cells/total cell count) × 100.

2.11. Immunoassay of the hFIX antigen in cultured media

The hFIX antigen in supernatant of genetically modified cells was assayed by the sandwich ELISA using a microplate, coated with a specific anti-hFIX antibody. The hFIX bound to the first antibody was revealed by using a second mouse anti-hFIX monoclonal antibody, labeled with horseradish peroxidase that binds to another antigenic determinant of the hFIX. The enzymatic activity was then demonstrated by its oxidative action on the substrate tetramethyl benzidine in the presence of urea-hydrogen peroxide. The reaction was then stopped by the addition of sulfuric acid and the resulting color was measured at 450 nm. The cultured medium collected from non-transfected cells was used as negative control. A standard curve was provided from 2-fold serial dilutions of normal human pooled plasma, containing 5 µg/ml of the FIX. Subsequently, concentrations of the hFIX in the cultured media were calculated based on the standard curve and normalised by the cell numbers and stated in ng/ml per 10⁶ cells.

2.12. Analysis of intracellular accumulation of the hFIX

Genetically modified TF-1 cells and BMSCs were lysed in ice cold lysis buffer containing; 100 mM KCl, 2 mM MgCl₂, 10 mM HEPES pH 7.5, 0.5% Triton X100 and protease inhibitors for 10 min. The cell lysate was centrifuged at 12,000 g for 15 min at 4 °C and the supernatant was used for analysis. Concentration of the hFIX in the lysate was measured by ELISA method as described by the manufacturer and normalised by the cell numbers and stated in ng/ml per 10⁶ cells.

2.13. Measurement of hFIX biological activity

Biological activity of the expressed hFIX was examined using immuno-depleted plasma for FIX and activated partial thromboplastin (APTT) reagent (cephalite), according to the instructions provided by the manufacturer. A standard curve was constructed by making different serial dilutions of normal citrated pool plasma (1:10, 1:20, 1:40, 1:80 and 1:160) in Owren-Koller buffer and plotting the log clotting time against the log plasma FIX activity. The conditioned cultured media were then used for determining the activity of the expressed hFIX, based on the standard curve. 100 µl of culture medium was added to 100 µl of FIX-deficient plasma and 100 µl of APTT reagent (cephalite). After 3 min pre-incubation at 37 °C, clotting was initiated by addition of 100 µl of 25 mM/L prewarmed CaCl₂ and the clotting time was measured. By definition, the normal concentration of hFIX in human plasma is equal to 100% activity or one unit. In the next stage, the hFIX-specific activity was calculated by dividing the clotting activity by the antigen level and stated in U/mg.

2.14. Reverse transcription-PCR

Total RNA, isolated from 5 to 10×10^6 BMSCs, was used as the template to perform RT-PCR using a one-step RT-PCR kit, to generate a first cDNA strand according to the manufacturer's instructions. The generated fragments were subsequently used as templates for the PCR-amplification of double stranded cDNA corresponding to a preselected region of the *hFIX* coding sequence, using oligonucleotides hKozF9-F: 5'-GGATCCGCCACCATG-CAGCGCGTGAACATGAT-3' and hFIXE4-R: 5'-CCTTGCAACTGCCGC-CATTAAAC-3' as forward and reverse primers, respectively with the following program: after an initial denaturation step at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 62 °C for 1 min, extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min were performed. For internal control, the generated cDNAs were used as templates for PCR amplification of a section of the *GAPDH* coding sequence, using oligonucleotides GAPDH-F: 5'-CAAGGTCATCCATGACAACCTTTG-3' and GAPDH-R: 5'-GTCCACCACCTGTTGCTGTAG-3' as forward and reverse primers, respectively. The PCR products were visualized on 1% agarose gels after ethidium bromide staining.

2.15. Statistical analyses

All expression analyses were repeated twice using duplicate assays and the results were presented as the Mean \pm SD. Analysis of variance followed by a Tukey post-hoc test was performed to evaluate differences among the constructs. $P < 0.05$ was considered statistically significant. All statistical analyses were carried out with SPSS version 15 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Morphological and phenotypic characterization of BMSCs isolated from rat bone-marrow

On the basis of adhesion properties of BMSCs to the culture flasks, BMSCs were isolated from other bone-marrow cells. On days 7 and 14, after the third passage, the culture-expanded cells become uniform morphologically and the cells with fibroblast-like morphology were observed (Fig. 1A, B). Phenotypic characterization confirmed that the cells are BMSCs (Fig. 1C). The results obtained from flowcytometry showed that the isolated cells were positive for the markers CD44 (59.2%), CD90 (86.4%) and CD29 (89.5%) while negative for the hematopoietic marker CD45. These results show that the isolated cells have the basic properties of the BMSCs.

3.2. Transfection efficiency

Non-viral gene transfer into BMSCs and hematopoietic cells has been restricted by poor transfection efficiency because of stem cells are regarded as cells that are notoriously difficult to transfect. Therefore, optimization of different parameters such as transfection reagent, plasmid DNA concentration and duration of exposure of cells to transfection reagent: plasmid DNA complexes are required to determine the best parameters and conditions. Our data showed that, mixture of 6 μ l transfection reagent with 2 μ g plasmid DNA induced highest expression of eGFP in transfected cells (7% of total cell count in TF-1 and BMSCs cells) 48 h post-transfection (Table 2).

3.3. Effect of hBG introns on secretion of hFIX from genetically modified TF-1 cells and BMSCs

Based on ELISA results, genetically modified TF-1 cells and BMSCs were capable of producing hFIX *in vitro*. When the cell numbers on the second day of post-transfection were adjusted, TF-1 cells secreted highest level of the hFIX (53 ng/ml/ 10^6 cells) into the culture medium from *hBG* intron-I, II containing plasmid. In BMSCs, the highest level of the hFIX (223 ng/ml/ 10^6 cells) was obtained from the intron-less construct. In both of the genetically modified TF-1 cells and BMSCs, the first *hBG* intron appeared to be more effective than the second one (Fig. 2A, B). Under the same conditions, BMSCs secret significantly higher hFIX protein levels by 5, 3.8, 3.4, 1.4 and 2.8-fold increase for p.KhFIX, p.KhFIX-I, p.hFIX-II, p.KhFIX-I, II and p.hFIX-I, II respectively compared to the TF-1 cells transfected with the same constructs (Fig. 3).

3.4. Intracellular accumulation of the expressed hFIX

The possible accumulation of the expressed hFIX inside the genetically modified TF-1 cells and BMSCs was investigated by measurement of the hFIX antigenicity of the cellular lysate of the cell pools of different transfectants on the second day of post-transfection. As the data indicate, the relative amounts of intracellular hFIX contents of the examined cell-pools more or less follow those of their extracellular hFIXs. In both of the TF-1 cells and BMSCs cells, the highest amount of intracellular hFIX were obtained from the *hBG* intron-I, II containing plasmid and intron-less construct respectively (Fig. 2A, B).

Evaluation of recombinant plasmids for total expression of hFIX (extracellular + intracellular) revealed that p.KhFIX-I, II and p.KhFIX have highest impact on the hFIX protein expression from the TF-1 cells and BMSCs respectively (Fig. 3, Table 3).

3.5. Secretion efficiency of the hFIX

The secretion efficiency of a particular protein is defined as the ratio between the secreted fraction and its total amount [24]. Based on our data, the secretion efficiency of the hFIX obtained from expression of different constructs in TF-1 cells and BMSCs varied between 45–52% and 52–58% respectively (Table 4).

3.6. Biological activity of hFIX

Based on the data obtained from the one-stage clotting assays, successful secretions of biologically active hFIX by all recombinant constructs were documented except for *hBG* intron-I and *hBG* intron-II containing constructs in TF-1 cells. The highest hFIX activity was obtained from TF-1 cells (8.6 mU/ml/ 10^6 cells) and BMSCs (24.7 mU/ml/ 10^6 cells) that carrying the *hBG* intron-I, II containing plasmids (Table 5). BMSCs were able to secrete significantly higher biologically active hFIX by 2.5, 4 and 2.4-fold increase for p.KhFIX, p.KhFIX-I, II and p.hFIX-I, II respectively compared to the TF-1 cells transfected with the same constructs.

Evaluation of the hFIX specific activities revealed that the cells that carrying *hBG* intron-I and intron-II containing vector secrete hFIX with the highest specific activity compared to other vectors (Table 5).

3.7. Reverse transcription-PCR analysis

Proper intron-splicing of a transcript precursor is essential for a gene to pass through its translation stage which results in a successful expression of the corresponding gene. With this aim, genetically modified TF-1 cells and BMSCs which carry intron-

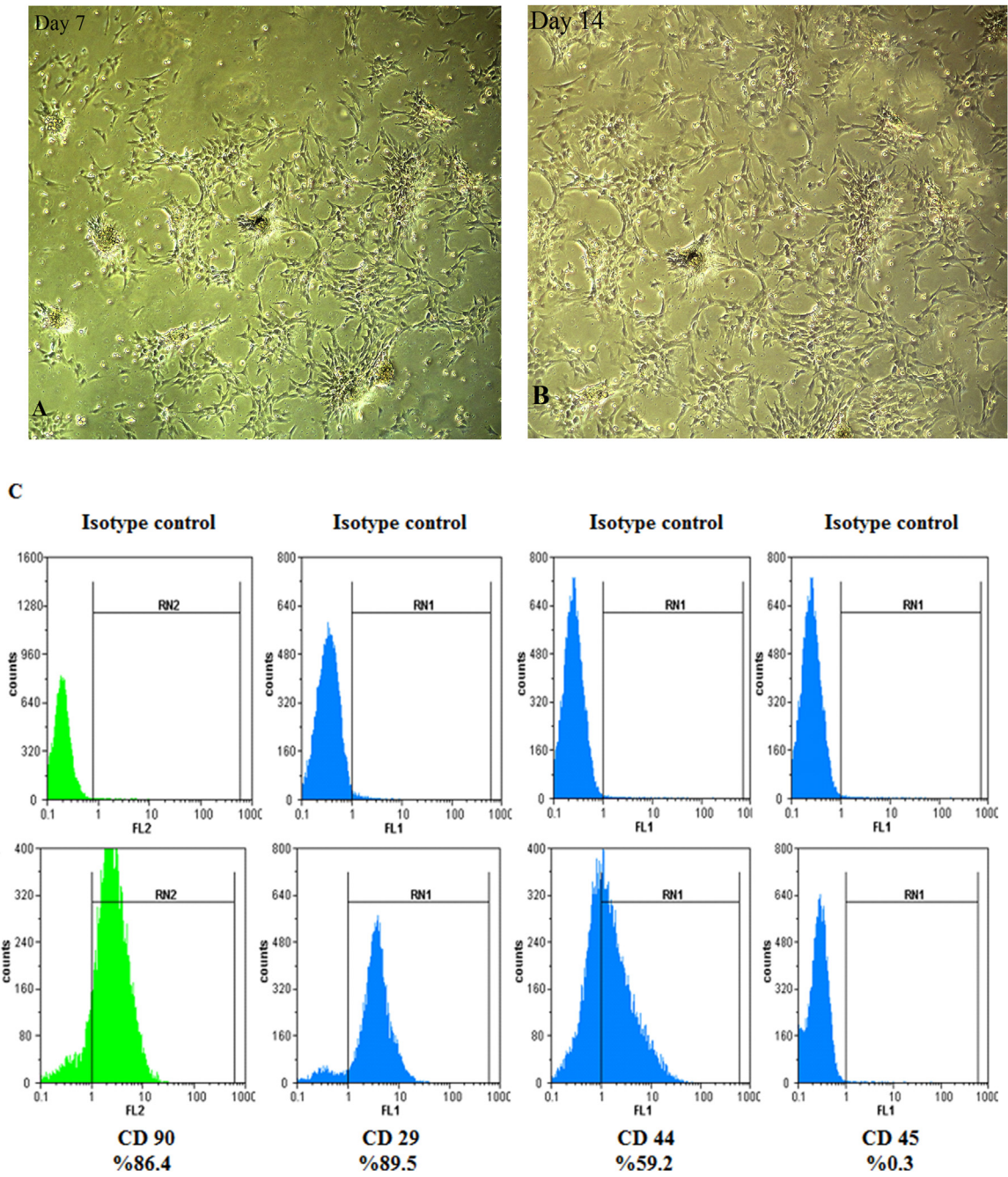


Fig. 1. Morphological and phenotypic characterization of BMSCs. Morphology of the culture-expanded cells on days 7 (A) and 14 (B), with magnification: 10×. (C): Immunophenotypic characterization of rat-BMSCs.

Table 2

Transfection efficiency in BMSCs and TF-1 cells at different time points.

Plasmid (ng/ml)	Post transfection (time)											
	24 h TF-1		24 h BMSCs		48 h TF-1		48 h BMSCs		72 h TF-1		72 h BMSCs	
	3 µl	6 µl	3 µl	6 µl	3 µl	6 µl	3 µl	6 µl	3 µl	6 µl	3 µl	6 µl
0.5	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	3.3%	3.3%	3.3%	3.3%	0	0	0	0
2	0	3.3%	0	3.5%	3.3%	7%	3.5%	7%	0	0	0	0

Cell viability following each transfection was >90%. Transfection efficiency (TE) was calculated according to the following formula: TE (%) = (number of GFP positive cells/total cell count) × 100. The results represent the mean transfection efficiency of two independent experiments.

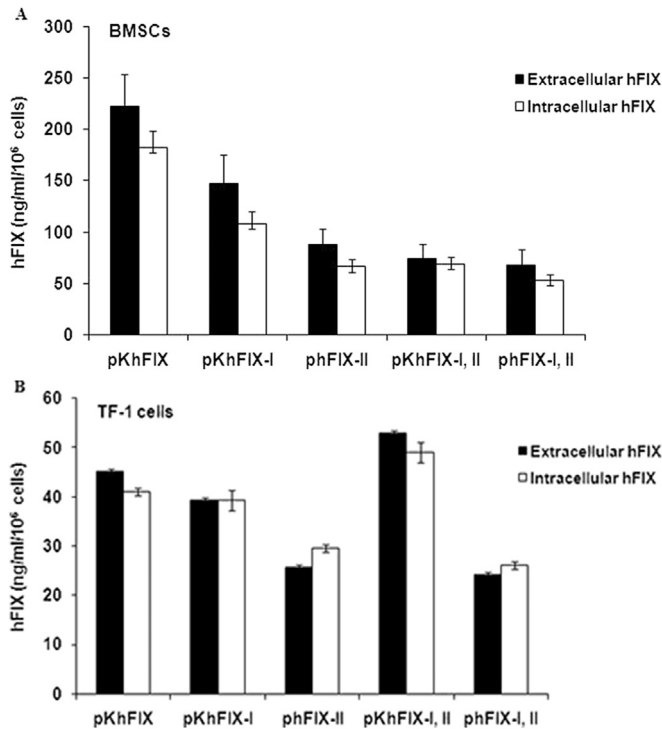


Fig. 2. Evaluation of the hFIX using ELISA method. On the second day of post-transfection, the supernatants and lysates of cells (3×10^5 cells) transfected with different vectors were collected and prepared for hFIX expression analysis. Concentration of the hFIX in the supernatants and lysates were normalized by the cell numbers and stated in ng/ml/per 10^6 cells. The results represent the mean hFIX level \pm SD from two independent experiments with duplicate assays. Detectable level of hFIX was not identified in the cultured media and lysate of non-transfected cells (data not shown).

containing plasmids were examined by performing RT-PCR on their corresponding mRNA extracts. RT-PCR analysis showed the expression of mature hFIX mRNAs except for *hBG* intron-I and intron-II containing construct in TF-1 cells on the second day of post-transfection, while BMSCs were able to splice properly *hBG* intron-I or intron-II from the hFIX pre-mRNAs. Interestingly, presence of the *hBG* intron-I,II inside the hFIX-cDNA provides properly spliced hFIX transcripts in genetically modified TF-1 cells (Fig. 4).

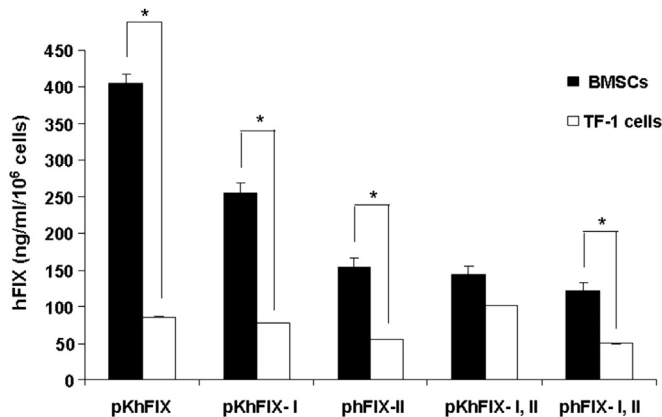


Fig. 3. Comparison of BMSCs and TF-1 cells for total expression of hFIX (Intracellular + extracellular) from the same recombinant plasmids on the second day of post-transfection. The results represent the mean hFIX level \pm SD from two independent experiments. *P < 0.05.

Table 3
Evaluation of recombinant plasmids for total expression of hFIX.

Plasmid		Fold hFIX (total expression)	
		TF-1	BMSCs
p.KhFIX vs.	p.KhFIX-I	1.1	1.6*
	p.hFIX-II	1.6*	2.6*
	p.KhFIX-I,II	0.9	2.8*
p.KhFIX-I vs.	p.hFIX-I,II	1.7*	3.3*
	p.hFIX-II	1.4	1.7*
	p.KhFIX-I,II	0.8	1.8*
p.hFIX-II vs.	p.hFIX-I,II	1.6*	2.1*
	p.KhFIX-I,II	0.6	1.1
	p.hFIX-I,II	1.1	2.1*
p.KhFIX-I,II vs.	p.hFIX-I,II	2*	1.2

Total hFIX protein levels produced by different vectors were calculated using ELISA method and normalised 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 difference between two different constructs for expression of the hFIX from the same cell line ($p < 0.05$).

4. Discussion

HSCs and BMSCs are attractive target cells for *ex-vivo* gene therapy of hemophilia B, due to their several unique properties and their potentials for systemic release of hFIX [12,14–16].

Viral vectors are widely used and adapted to hFIX gene transfer into BMSCs and HSCs [11,12,14,25–27]. However, use of non-viral hFIX gene transfer systems to target BMSCs and HSCs has so far been very limited.

Unwanted complications provoked by viral gene transfer into stem cells have been recently 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 [28].

Stem cells are regarded as cells that are notoriously difficult to transfect [28]. In this context, we showed that, with optimization of different parameters, the generation of genetically modified BMSCs and TF-1 cells with hFIX-expressing plasmids is feasible and these cells have appropriate potential for hFIX expression and secretion *in vitro*.

To evaluate hFIX gene transfer to HSCs, high density of cell numbers is required *in vitro*. As bone-marrow contains very low population of HSCs, TF-1 cells represent an attractive model for primary hematopoietic cells in this regard. These cells have the phenotype of normal HSCs and express several markers such as CD34, CD33 and c-Kit and differentiate into erythroid or megakaryocytic lineages upon appropriate stimulation [29].

In our study, in spite of low transfection efficiency, the result obtained from the ELISA method (in the case of highest expression

Table 4
Secretion efficiency of hFIX in genetically modified cells on the second day of post-transfection.

Plasmid	Secretion efficiency of hFIX protein (%)	
	TF-1	BMSCs
p.KhFIX	52 \pm 1.1	55 \pm 2.1
p.KhFIX-I	48 \pm 0.7	58 \pm 2.8
p.hFIX-II	49 \pm 0.7	57 \pm 0.7
p.KhFIX-I,II	52 \pm 0.7	52 \pm 1.4
p.hFIX-I,II	45 \pm 1.4	56 \pm 1.4
Secretion efficiency	45–52%	52–58%

The results represent the mean \pm SD from two independent experiments with duplicate assays.

Table 5
Clotting and specific activities of hFIX in the supernatant of TF-1 cells and BMSCs transfected with different vectors on the second day of post-transfection.

Plasmids	TF-1		BMSCs	
	Clotting activity (mU/ml/10 ⁶ cells)	Specific activity (U/mg)	Clotting activity (mU/ml/10 ⁶ cells)	Specific activity (U/mg)
p.KhFIX	^b 8.1 ± 0.38	^c 180.5 ± 7.7	^b 20.2 ± 0.35	^{a,c} 91.3 ± 1
p.KhFIX-I	^{a,b} ND	^{a,c} ND	^b 22.7 ± 0.42	^c 154.7 ± 3.4
p.hFIX-II	^{a,b} ND	^{a,c} ND	^{a,b} 7.3 ± 0.25	^{a,c} 82.2 ± 3.2
p.KhFIX-I,II	^b 8.6 ± 0.64	182.5 ± 21.9	^{a,b} 13.7 ± 0.25	199.2 ± 1.7
p.hFIX-I,II	^{a,b} 5.6 ± 0.7	^c 232.4 ± 29.7	^b 24.7 ± 0.42	^{a,c} 334.1 ± 5.5

The results represent the mean ± SD from two independent experiments with duplicate assays. "a" symbol indicates the difference compared to other samples from the same cell line ($p < 0.05$). "b" and "c" symbols indicate the difference in clotting activity and specific activity of sample compared to other sample from the different cell line transfected with the same construct respectively ($p < 0.05$). ND: not detect. Specific activity of plasma-derived hFIX is 200 U/mg.

level of hFIX in BMSCs) was comparable to those obtained by other groups who used retroviral vectors to direct the expression of hFIX *in vitro* [11,30]. Therefore, optimization of hFIX-cDNA with aim of enhancement of FIX expression could be an attractive way rather than increasing transfection efficiency in plasmid based systems.

Based on data, expression of hFIX in TF-1 cells generated a pool of intracellular hFIX, suggesting that upon appropriate stimulation *in vivo*, HSCs-derived megakaryocytes or erythroid cells could effectively deliver hFIX at the site of vascular injury or into the circulation.

There are differences in amount of extracellular and intracellular hFIX expressed by different vectors. The obtained results could be partly attributed to the differences in *cis*-regulatory elements applied in each vector which result in differences in transcriptional, translational and secretion efficiencies of hFIX.

Our data showed that, the highest hFIX specific activity was obtained from the cells that carrying hFIX minigene containing both of the hBG introns. It is possible that, the cooperative functions between the two human β -globin introns have positive effects on post-transcriptional modifications which result in higher specific activity of secreted hFIX in the cells that carrying hFIX minigene containing both of the hBG introns. Further experiments are required to highlight this issue.

We also found that the bioengineered BMSCs secrete significantly higher hFIX protein than TF-1 cells transfected with the same constructs. Higher potential of BMSCs for expression of hFIX may arise from the active role of BMSCs in bone-marrow through release of bioactive molecules to support hematopoiesis.

APTT method is a functional assay used to evaluate activity of hFIX protein and therefore provides invaluable information about functional activity than that obtained by ELISA method which only measures the level of protein and not functions. With this in mind,

interestingly, in spite of highest level of hFIX obtained from the intron-less construct in BMSCs, the hBG intron-I,II containing plasmid induce highest active hFIX production *in vitro*. This likely reflects inefficiency of required post-translational modifications at the higher level of hFIX protein synthesis and underscores the point that, if the hFIX transgene is expressed in BMSCs, there is an upper limit to the amount of functional hFIX as a vitamin K-dependent clotting factor. Our result is in agreement with previous study revealed that the capacity of bone-marrow stromal cells to produce fully active FIX may be limited at high expression rates [31]. In this regard, it has been shown that in non-hepatocyte cell types, the cell's capacity to execute critical post-translational modifications (eg, γ -carboxylation) can be saturated resulting in production of FIX protein with reduced specific activity [32,33]. With this in mind, in our study, it is possible that at high expression level of hFIX in BMSCs, the γ -carboxylase enzyme has been saturated resulting in production of FIX protein with reduced specific activity.

Based on RT-PCR analyses, mature hFIX mRNAs were generated from the hBG intron containing plasmids in BMSCs, while TF-1 cells express mature hFIX mRNAs from the hBG intron-I,II containing plasmids and intron-less constructs. These results demonstrate that why we couldn't detect any biologically active hFIX protein in the supernatant of TF-1 cells carrying hBG intron-I or hBG intron-II containing constructs.

Considering the importance of the sequences surrounding the intron as well as intron identity, the chimerical structure generated by the introduction of hBG intron-I or intron-II, inside the hFIX-cDNA might not favor efficient splicing. Surprisingly, the presence of two hBG introns together inside the hFIX-cDNA spliced properly in TF-1 cells and BMSCs, suggest the cooperative functions of two hBG introns for generation of mature hFIX mRNAs.

Short size of the hBG introns and their cooperative functions with non-lineage specificity are main advantages of these *cis*-regulatory elements which can be tested within the context of different viral vectors.

The current study is the first report to show the ability of BMSCs and primary hematopoietic cell line to express hFIX from the hBG introns containing plasmids and demonstrated that, these cells could be effectively bioengineered using hFIX-expressing plasmids and possess the post-translational modification mechanisms required to produce biologically active FIX. Given the positive effects of hBG introns on the expression of functional hFIX, the hFIX-expressing plasmid containing both of the hBG introns represents a particular interest in stem cell-based gene therapy of hemophilia B.

Conflict of interest

The authors declare there is no conflict of interest.

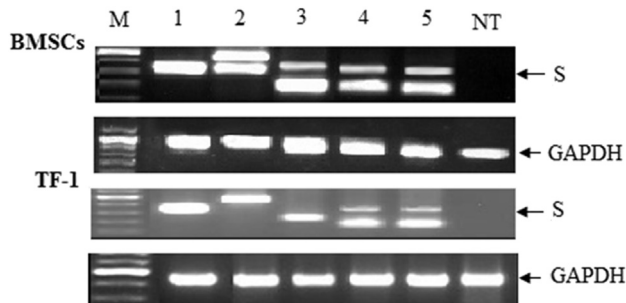


Fig. 4. Detection of hFIX mRNA using RT-PCR. Lane 1: p.KhFIX, Lane 2: p.KhFIX-I, Lane 3: p.hFIX-II, Lane 4: p.KhFIX-I, II and Line 5: p.hFIX-I, II. No detectable product of proper splicing was evidenced from the hBG intron-I and hBG intron-II containing plasmids in TF-1 cells. NT: None-transfected cells, S: Spliced product (mature hFIX), M: DNA size marker.

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