An engineered U1 small nuclear RNA rescues splicing-defective coagulation F7 gene expression in mice

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Summary. Background: The ability of the spliceosomal small nuclear RNA U1 (U1snRNA) to rescue pre-mRNA splicing impaired by mutations makes it an attractive therapeutic molecule. Coagulation factor deficiencies due to splicing mutations are relatively frequent and could therefore benefit from this strategy. However, the effects of U1snRNAs in vivo remain unknown. Objectives: To assess the rescue of the F7 c.859+5G>A mutation; RNA splicing; U1 snRNA.

Methods: Mice expressing the human F7 c.859+5G>A mutant were generated following liver-directed expression by plasmid or recombinant adeno-associated viral (AAV) vector administration. The rescue of the splice-site defective pre-mRNA by U1+5a was monitored in liver and plasma through hFVII-specific assays. Results: Injection of plasmids encoding the U1+5a rescued plasma hFVII levels, which increased from undetectable to ~8.5% of those obtained with the wild-type hFVII plasmid control. To assess long-term effects, mice were injected with low and high doses of two AAV vectors encoding the FVII+5A splice site mutant as template to be corrected by U1+5a. This strategy resulted in hFVII plasma levels of 3.9 ± 0.8 or 23.3 ± 5.1 ng mL−1 in a dose-dependent manner, corresponding in patients to circulating FVII levels of ~1–4.5% of normal. Moreover, in both experimental models, we also detected correctly spliced hFVII transcripts and hFVII-positive cells in liver cells. Conclusions: Here we provide the first in vivo proof-of-principle of the rescue of the expression of a splicing-defective F7 mutant by U1snRNAs, thus highlighting their therapeutic potential in coagulation disorders.

Keywords: factor VII deficiency; genetic diseases; mouse; mutation; RNA splicing; U1 snRNA.

Introduction

Protein replacement therapy has significantly improved the quality of life of patients with inherited coagulation factor deficiencies [1]. However, there are still significant practical, technical and economical limitations that support research into alternative therapeutic strategies.

The U1 small nuclear RNA (U1snRNA), the ribonucleic acid component of the U1 ribonucleoprotein that mediates recognition of the donor splice site (5′ss) in the earliest splicing step [2], is emerging as an attractive therapeutic molecule for human diseases caused by splicing mutations, often associated (>15%) with severe forms [3,4]. It has been demonstrated that U1snRNAs with increased complementarity to mutated 5′ss are able to redirect the spliceosome assembly and rescue splicing in various cellular models of human genetic disease [5–10], including coagulation factor deficiencies [11–14]. Recently, we have also demonstrated that modified U1snRNAs, designed to improve the definition of the 5′ss, can also restore splicing impaired by mutations at the acceptor splice site [14]. However, the U1snRNAs strategy has not been explored in vivo, thus preventing the assessment of its potential therapeutic effect.

Mutations at splice sites are relatively frequent in patients with coagulation factor VII (FVII) deficiency (~17%, and 8% at 5′ss) [13,15], as well as among patients with X-linked bleeding diseases such as FIX deficiency.

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 (~14%, and 7% at 5′ss) (http://www.hgmd.cf.ac.uk/ac/index.php and http://www.factorix.org). Notably, the prevalence of these mutations further increases among patients with severe diseases (< 1% of normal factor levels). Here we sought to investigate the in vivo U1snRNA-mediated rescue of gene expression impaired by 5′ss splicing mutations in a model of human FVII deficiency caused by the F7 c.859+5G>A (NM_000131.4) mutation [16,17]. We have previously shown this mutation to be efficiently rescued in vitro by the modified U1snRNA+5a (U1+5a) molecule [11,12]. It is worth noting that intervention at the pre-mRNA level permits the restoration of gene expression while maintaining gene regulation in physiological tissues, and overcomes limitations related to delivery of large genes, such as the F8 gene, via vector-mediated approaches. In these inherited bleeding diseases the approach using U1snRNA can be particularly beneficial because even a modest increase of functional protein levels would result in improvement of the disease phenotype [18].

Materials and methods

Generation of vectors

Plasmids expressing human FVII (hFVII) wild-type (pFVIIwt) or harbouring the c.859+5G>A splicing mutation (pFVII+5A) were generated by cloning the wild-type or the mutated hFVII splicing-competent cassette [12] into the pAAV-hAAT backbone [19] using the ClaI and XhoI sites (Fig. 1). The hAAT promoter contains the human α1-antitrypsin (hAAT) promoter with four copies of the human ApoE enhancer and a synthetic intron [20,21]. Plasmid pU1wt harbouring the wild-type U1snRNA cassette (U1wt) and pU1+5a were created by cloning the corresponding cassette [11] and its own promoter into a promoterless pAAV backbone through a BamHI cloning site. The U1+5a differs from the U1wt at positions +4 and +5 and contains a C and a U, respectively.

Recombinant adeno-associated viral (AAV) vectors of serotype 2 (AAV2-FVII+5A) and serotype 8 (AAV8-U1+5a) were produced as previously described [22].

Animal procedures

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at The Children’s Hospital of Philadelphia. Eight-week-old male C57BL/6 mice were used for in vivo delivery of plasmids by hydrodynamic injection of DNA (2.5 mL phosphate buffer saline-PBS) or AAV vectors (0.2 mL in PBS/5% sorbitol) by tail vein injection. While plasmid DNA vectors were co-injected, the AAV vectors have been administrated in a sequential fashion, with the AAV8-U1+5a injected 2 weeks after the AAV2-FVII+5a. Blood samples were collected from the retro-orbital plexus into 3.8% sodium citrate.

Measurement of hFVII antigen, hFVII mRNA splicing profiles and liver enzymes

Human FVII antigen levels in mouse plasma were evaluated by a hFVII enzyme-linked immunosorbent assay (ELISA; Diagnostica Stago Inc., Asnieres-sur-Seine, France). To validate and optimize the assay, a standard
curve was created by adding known amounts of human pooled normal plasma to mouse plasma. The sensitivity threshold of the assay in our hands was 0.25 ng mL\(^{-1}\) of hFVII. For the assay, mouse plasma samples were diluted 1 : 10 and 1 : 20.

To detect hFVII transcripts, total RNA was isolated from random sections of mouse liver using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s protocol. Two micrograms of total mouse liver RNA were retro-transcribed using SuperScript\textsuperscript{TM} III Reverse Transcriptase (Invitrogen) with random primers and subsequently PCR amplified \cite{23} with hFVII-specific primers (h6F [5\textsuperscript{′} TAGAGAAAGAAATGCCACGCAA CC-3\textsuperscript{′}], h7F [5\textsuperscript{′} GTCTGGTTGTTGGGAATGGAAGCT CA-3\textsuperscript{′}], h8R [5\textsuperscript{′} GTGCACAAATGAGAACGCAACGAA CC-3\textsuperscript{′}]). The PCRs were carried out in a total volume of 25 \(\mu\)L under the following conditions: preheating, 95 °C for 2 min followed by 40 cycles of 95 °C for 30 s, 60 °C for 20 s and 72 °C for 30 s.

Amplicons were resolved on a denaturing capillary electrophoresis system (Experion; BioRad Laboratories, Hercules, CA, USA).

Measurement of alanine aminotransferase (ALT) activity levels in plasma has been conducted through the commercially available kit from Teco Diagnostics (Anaheim, CA, USA).

Statistical analysis

Statistical differences among levels were evaluated by a Student’s \(t\)-test with a \(P < 0.05\) considered significant.

Results

We used two vector systems (viral and non-viral) for the assessment of the effect of U1+5a in rescuing the defective splice site \(F7\) mutant harbouring the c.859+5G>A mutation (FVII+5A) as template (Fig. 1). Our model consists of the hepatocyte-restricted expression of the template pre-mRNA by using liver-specific promoter/enhancer elements. The availability of species-specific immunologic and nucleic acid amplification assays allows the evaluation of the hFVII expression without confounding effects with the endogenous murine FVII of the C57BL/6 mice (Fig. 2A, untreated cohort of mice exhibited undetectable levels in our hFVII ELISA). A series of previous attempts to use a liver-specific promoter for U1 expression were unsuccessful (D. Balestra and M. Pinotti, unpublished observations). Thus, we used the U1snRNA endogenous promoter in the experiments reported here.

Rescue of human FVII expression by transient expression of \(U1+5a\)

Hydrodynamic injection of two different doses of plasmid pFVIIwt (1 or 2 \(\mu\)g \(\text{g}^{-1}\) of mouse body weight), used as a positive control, resulted in a dose-dependent increase of hFVII antigen levels in mouse plasma, reaching ~2 \(\mu\)g mL\(^{-1}\) at 48 h post-injection. These findings were further supported by data on hFVII mRNA and protein in the liver. Specifically, mice injected with pFVIIwt displayed only the normally spliced transcripts (first column, Fig. 2B) and immunohistochemical analysis of their livers revealed frequent hFVII-positive cells (Fig. 2C).

Injection of pFVII+5A alone at both doses tested (1 or 2 \(\mu\)g \(\text{g}^{-1}\) of mouse body weight) showed no detectable hFVII antigen levels (Fig. 2A), as expected. Analysis of hFVII mRNA in the liver showed that the recipient mice generated almost exclusively the aberrant mRNA form, having the intronic 37 bp repeat inclusion (Fig. 2B), with minimally detectable levels of correct transcripts (mean ± standard deviation, 2 ± 1.5% of total hFVII transcripts; 95% CI, –0.7 to 6.5). Furthermore, we could not detect any hFVII expressing liver cells by immunofluorescence staining (Fig. 2C). These findings suggest that the splicing change in the hFVII mutant template prevents the hFVII biosynthesis and secretion in mice, thus mimicking findings in humans homozygous for the \(F7\) c.859+5G>A mutation.

To evaluate the U1-mediated rescue of hFVII expression impaired by the c.859+5G>A mutation, we injected
the pFVII+5A plasmid at either 1 or 2 μg g⁻¹ of mouse body weight with a corresponding molar excess (1.5 fold) of pU1+5a (Fig. 1). Forty-eight hours post-injection, a significant increase (P < 0.05) of circulating hFVII levels was detected (50.6 ± 16.0 ng mL⁻¹, 95% CI 9–91, or 178 ± 126 ng mL⁻¹, 95% CI –21 to 379, for each plasmid amount, respectively, Fig. 2A). These values correspond to 5% and 8.5% of the expression levels of pFVIIwt at low and high dose cohorts, respectively (Fig. 2A). Moreover, co-expression of the U1+5a resulted in rescue of hFVII splicing (26 ± 10% of total transcripts, 95% CI –0.04 to 52) in the liver (Fig. 2B, last column). Corroborating these findings, immunohistochemical analysis of livers from mice co-injected with pFVII+5A and pU1+5a showed an appreciable number of hFVII-positive staining cells that were absent in livers of mice injected with pFVII+5A alone (Fig. 2C). Injection of pU1+5a alone in C57Bl/6 showed no effect on circulating hFVII, as expected (Fig. 2A).

To confirm the specificity of the U1+5a towards the defective splice site, we injected mice with the pFVIIwt (2 μg g⁻¹ of mouse body weight) and a molar excess of pU1+5a (Fig. 2A). This resulted in circulating hFVII levels of 1780 ± 391 ng mL⁻¹ (95% CI 767–2713), comparable (P = 0.31) to those measured in mice injected with pFVIIwt alone (2146 ± 466 ng mL⁻¹, 95% CI 988–3304). Furthermore, we assessed the ability of the U1wt to rescue the hFVII+5A mutant (Fig. 2A, inset). Injection of the high dose of pFVII+5A (2 μg g⁻¹) with a molar excess of pU1wt produced minimal hFVII levels in mouse plasma (13 ± 8 ng mL⁻¹, 95% CI 10–22), which was significantly lower (P < 0.05) compared with what we observed using pU1+5a (178 ± 126 ng mL⁻¹). Furthermore, injection of pU1wt together with pFVIIwt resulted...
in similar hFVII levels compared with mice receiving only pFVIIwt (2584 ± 728, CI 1680–3488, vs. 2146 ± 466 ng mL⁻¹, respectively, P = 0.4). Thus, the U1wt is not effective in rescuing the splice defect in vivo, in accordance with data obtained in cellular models [11,12].

Collectively, these data suggest that the rescue of the defective splice site mutation in the F7 gene by transient expression of U1+5a is feasible and specific towards the mutant template, thus providing the first evidence of the efficacy of this strategy in vivo.

Sustained expression of FVII by AAV-U15a

To investigate whether U1+5a-mediated correction of the hFVII splicing mutation could be sustained over time we used a strategy based on AAV vectors. The FVII+5A and the U1+5a expression cassettes (Fig. 1) were packaged into AAV serotype 2 (AAV2-FVII+5A) and serotype 8 (AAV8-U1 + 5a), respectively. These serotypes were chosen because of their tropism for the liver following peripheral intravascular delivery. In a preliminary study, AAV2-FVII+5A was delivered at doses of 1.2 × 10¹² vector genomes (vg) per mouse and during the 5-week period, no expression of circulating hFVII was detected (Fig. 3A). In the rescue mode, at day 0, mice (n = 4) received AAV2-FVII+5A at doses of 1.2 × 10¹² or 6.0 × 10¹² vg per mouse and, 2 weeks later, the AAV8-U1+5a was administrated at a fixed dose of 1.2 × 10¹¹ vg per mouse (Fig. 3A). The initial injection of AAV2-FVII+5A resulted in no increased levels of circulating FVII for the first 2 weeks, as expected (Fig. 3A). However, upon injection of AAV8-U1+5a, there was a progressive increase in plasma hFVII antigen levels in a dose-dependent manner (P < 0.02), reaching 5.5 ± 0.7 ng mL⁻¹ (95% CI 3.7–7.2) and 8.7 ± 1.9 ng mL⁻¹ (95% CI 6.9–10.4) during the 6-week follow-up (Fig. 3A). Conversely, using a fixed dose of the AAV2-FVII+5A (1.2 × 10¹² vg per mouse, template) we increased the AAV8-U1+5a dose by 5-fold (6.0 × 10¹¹ vg per mouse). The U1+5a-mediated correction effect was directly related to the AAV8-U1+5a dose, as measured by circulating hFVII levels of 3.9 ± 0.8 ng mL⁻¹ (95% CI 1.8–5.8) with 1.2 × 10¹¹ vg per mouse or 23.3 ± 5.1 ng mL⁻¹ (95% CI 10.6–35.9) with 6 × 10¹¹ vg per mouse at 2 weeks post-injection, but with the highest AAV8-U1+5a dose the mice did not survive beyond this time-point (see below). These findings were further supported by the detection of the correctly spliced hFVII transcripts (4 ± 0.5% or 16 ± 3% of the total transcripts; Fig. 3B) and of hFVII-positive staining cells in the liver (Fig. 3C). Altogether these findings showed that there is a dose-related effect on the rescue of the hFVII splice site mutant by increasing the expression of the therapeutic gene (U1+5a) or of the template (FVII+5A). In humans, the range of FVII levels in plasma is 350–500 ng mL⁻¹. Therefore, if the sustained hFVII levels obtained in mice (8.7 ± 1.9 ng mL⁻¹) are translated into F7 c.859+5G>A homozygous patients, it would achieve the ~2% of normal, which could ameliorate the severe bleeding phenotype [18].

Over-expression of U1+5a is associated with hepatocellular toxicity

In the experimental design we choose AAV8 for the expression of U1+5a because of the superior liver transduction efficiency compared with AAV2 [24]; thus a more robust correction of the defective splice site could be achieved. We carried out a pilot dose-response experiment using AAV8-U1+5a vector at doses ranging from 1.2 × 10¹¹ to 2.4 × 10¹² vg per mouse (n = 2–4 per dose) while keeping the AAV2-FVII+5a dose of 1.2 × 10¹² vg per mouse. A Kaplan–Meier survival curve demonstrated premature mortality in mice injected with AAV8-U1+5a doses of 6.0 × 10¹¹ vg per mouse compared with the lower dose cohort, but at the highest doses all mice died by day 20 (Fig. 4A). There was a direct correlation between dose and liver toxicity as determined by increased levels of ALT over time in mice injected with 6.0 × 10¹¹ vg per mouse or higher, whereas in the low-dose cohort the ALT levels remained similar to the baseline values (Fig. 4B). Notably, the mice injected with 1.2 or 6.0 × 10¹² vg per mouse of AAV2-FVII+5A and 1.2 × 10¹¹ AAV8-U1+5a, resulting in sustained levels of hFVII (Fig. 3A), exhibited no change in the levels of ALT. Unfortunately, in mice injected with the two highest AAV8-U1+5a doses, due to liver failure, no detectable hFVII levels were observed (data not shown). Thus, these data support our choice of the two low doses of U1+5a for the rescue experiments (Fig. 3A). These studies showed that a dose of AAV8-U1+5a of 1.2 × 10¹¹ vg per mouse was the safest because it was not associated with increased mortality or liver toxicity, thus allowing long-term correction of the defective splice site and sustained expression of hFVII (Fig. 3A).

Discussion

Gene replacement and the correction of disease-causing mutations are the most attractive strategies for the treatment of genetic diseases. Currently, clinical studies are focused on gene replacement of the mutated gene. RNA-targeted strategies have been also developed for human diseases. To date, modified U1snRNAs have been successfully exploited in vivo to mask splicing regulatory elements and induce skipping of defective exons [25]. However, this strategy is not suitable for the correction of the most common inherited bleeding disorders. U1snRNA-based therapy, by redirecting the spliceosome assembly to the mutated splicing junction and restoring proper exon inclusion, represents a correction strategy able to produce full-length functional proteins. There are several advantages of such an approach for bleeding disorders: (i) there is a relatively high frequency of splice site

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mutations [3,4,26,27], (ii) correction is independent of the size of the target gene, while (iii) maintaining the endogenous regulatory elements for gene expression. Moreover, the corrected protein product can be measured in plasma over time. However, the efficacy of the U1snRNA approach in vivo for inherited bleeding disorders as well as for other human genetic diseases has not been demonstrated. Based on our encouraging results from the rescue of FVII expression impaired by a donor splice site mutation in cellular models [11,12], we evaluated the U1snRNA efficacy in mice in a quantitative manner.

Homozygosity for the c.859+5G>A mutation in the F7 gene [16,17] is associated with clinically severe FVII deficiency. It represents a prototypical example of a point mutation affecting the 5′ss and inducing aberrant splicing, and we have previously demonstrated its effective correction by the modified U1snRNA U1+5a in cellular models [11,12]. Specifically, the in vitro studies indicated that the presence of the modified 5′ tail of the U1+5a with increased complementarity to the mutated F7 5′ss is fundamental for the correction effect [11]. The absence of mouse models of FVII deficiency for splicing mutations, and the perinatal lethal phenotype of F7 knock-out mice [28], prompted us to generate a novel in vivo model by hepatocyte-restricted expression of the mutated hFVII splicing-competent cassette harboring the F7 c.859+5G>A mutation (FVII+5A, template). The rationale behind this strategy is the possibility of evaluating the hFVII transcripts and the hFVII protein levels in mice by exploiting hFVII-specific assays. For the delivery of the F7 mutant expression cassette we chose two vector systems, non-viral vector (plasmid DNA) and transduction by AAV vectors, to evaluate transient and prolonged hFVII expression, respectively. Although these vectors do not integrate into the hepatocyte genome, they guarantee the liver-specific transcription of the hFVII pre-RNA template (FVII+5A),
the key target of the proposed U1snRNA-mediated correction strategy. Expression of the mutated cassette by both delivery systems did not result in detectable circulating hFVII levels in mouse plasma, thus mirroring the coagulation phenotype observed in the F7 c.859+5G>A homozygotes [16,17]. Moreover, investigation of hFVII mRNA in mouse liver revealed aberrantly spliced forms due to the usage of the cryptic 5′ss in the intron, and trace levels of the correct form, thus recapitulating the pathological mechanisms dissected by studies in cellular models [11]. Altogether these data provided us with a mouse model for the in vivo evaluation of U1+5a efficacy at different levels, from the mRNA to the protein in liver and into the circulation.

In the transient model using plasmid DNA, the co-expression of the U1+5a was associated with the detection of correctly-spliced transcripts in mouse liver, thus indicating the ability of U1+5a to redirect usage of the mutated 5′ss in vivo by the spliceosome machinery. This observation was paralleled by the synthesis of hFVII, as indicated by hFVII-positive hepatocytes, and most importantly, by the increase of circulating hFVII levels, the final goal of the U1+5a-mediated correction strategy. In addition, in this model we demonstrated that U1+5a is specific for the splice site mutant, because no increase in hFVII levels was obtained by replacing the template with a hFVII-wt expressing plasmid. Moreover, over-expression of the U1wt had very modest effects on the FVII+5A mutant, further indicating the necessity of the engineered U1snRNA 5′ tail to mediate a significant correction effect in vivo.

In the context of AAV vectors, the rescue was prolonged over time, as indicated by the low but appreciable levels of plasma hFVII up to 6 weeks post-injection of the lowest AAV8-U1+5a dose. Clearly, prior to utilizing U1snRNA in a clinical application, further improvements will be necessary to enhance the correction efficacy. Towards that goal, our data show that increasing the template for the U1+5a (higher AAV2-FVII+5A dose) led to increased hFVII plasma levels. In our model, the use of AAV2 for the expression of the template is typically restricted to only 20–30% of hepatocytes. Thus, it is anticipated that in FVII-deficient patients, where all liver cells express the mutated pre-mRNA, targeting by U1+5a will likely result in a more robust increase of hFVII levels. It is worth noting that even this seemingly minimal increase in FVII levels could be associated with an improved phenotype, as seen in hemophilia patients on prophylaxis with protein replacement (aimed at levels > 1% of normal) as well as from emerging data from an early clinical trial on AAV liver gene transfer in subjects with severe hemophilia B [29].

A rational strategy to enhance the extent of rescue of the splicing-defective hFVII allele is to increase the expression of U1+5a. However, our data show that there is a dose-dependent effect that limits the safety of this proposal. As the dose of the AAV8 encoding U1+5a increased by 5-fold the dose associated with long-term expression, it resulted in a significantly increased hFVII expression at 2 weeks but did not reach plateau levels due to liver toxicity. AAV8-U1+5a administered at even higher doses was associated with premature mortality. Early studies on RNA interference technology also encountered liver toxicity upon high doses of AAV8 expressing short hairpin RNA (shRNA) [30]. Remarkably, these findings prompted the development of shRNA/microRNA technologies that are well tolerated.

Clearly, the hepatocellular toxicity that we observed from U1+5a over-expression will need to be sufficiently and extensively addressed prior to a clinical application using our approach. Current ongoing studies will attempt to define the underlying mechanism(s) by which U1+5a induced liver toxicity at high AAV8 doses. It must be pointed out that the U1+5a targets the donor splice site sequence of F7 intron 7. The mammalian donor donor splice site sequences, apart from the invariant dinucleotide +1G and +2T, can significantly differ from the consensus sequence at the other positions, particularly in alternatively spliced exons. Thus, it is possible that the U1+5a, differing from the normal U1snRNA at the two non-conserved positions +4 and +5, interacts directly with other 5′ss and elicits negative effects on the fine regulation of critical alterna-

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tively spliced exons. Similar results have been reported in spinal muscular atrophy [31], where a U1snRNAs complementary to the 5′ss of SMN2 exon7 showed some degree of cellular toxicity.

Towards the goal of overcoming the off-target effects and potential toxicity of U1snRNA, we have recently developed Exon Specific U1snRNA (ExSpU1), targeting non-conserved intronic sequences downstream of the 5′ss and still able to restore exon definition impaired by different mutations in cellular models of hemophilia B [14]. This approach was not feasible for the F7 c.859+5G>A mutation due to the presence in the F7 IVS7 of six highly conserved 37 bp repeated sequences [32], which prevents the identification of unique targets for ExSpU1. Further optimization on the AAV8-U1+5a vector such as modification in the promoter and vector serotype combined with the use of ExSpU1 in a mouse model expressing the target pre-mRNA in the majority of (all) hepatocytes will allow the full evaluation of the efficacy as well as the safety of the U1 snRNA-based correction strategy.

In conclusion, we have provided the first proof-of-concept that exploiting the U1snRNA pathway results in splice site correction and increased circulating levels of a therapeutic protein in a mouse model. Therefore, these data support further studies exploiting U1snRNAs as novel therapeutic strategies for coagulation factor and other genetic disorders.

Addendum

D. Balestra and A. Faella performed the experiments in the animal models and evaluated the expression of hFVII in mice; N. Cavallari created the plasmid constructs; P. Margaritis designed experiments in animals and wrote the manuscript; F. Pagani analyzed data and revised the manuscript; M. Pinotti, V. R. Arruda and F. Bernardi conceived the study and designed research, analyzed and interpreted data and wrote the manuscript.

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Disclosure of Conflict of Interest

M. Pinotti, F. Pagani and F. Bernardi are founders of the start-up company Raresplice. The remaining authors declare no competing financial interests.

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