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REVIEW ARTICLE

RNA-based therapeutic approaches for coagulation factor deficiencies

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Summary. Substitutive therapy has significantly ameliorated the quality of life of patients with coagulation factor deficiencies. However, there are some limitations that support research towards alternative therapeutic approaches. Here we focus on the rescue of coagulation factor biosynthesis by targeting the RNA processing and translation, which would permit restoration of the altered gene expression while maintaining the gene regulation in the physiological tissues. The essential prerequisite of the three reported RNA-based correction approaches (i–iii), which rely on mutation types and are applicable even to large size mRNAs, is the presence in cells of the precursor (pre-mRNA) or mature mRNA forms. (i) In the *F7* gene, modification of the small nuclear RNA U1 (U1 snRNA), the key component of the spliceosomal U1 ribonucleoprotein, re-directs correct usage of a mutated exon–intron junction, triggering synthesis of correct mRNA and secretion of functional factor (F)VII. (ii) Spliceosome-mediated RNA trans-splicing (SMaRT) between mutated and engineered pre-mRNAs produces normal FVIII mRNA and secretion of functional protein. (iii) Aminoglycoside drugs induce ribosome readthrough and suppress premature translation termination caused by nonsense mutations in FVII, VIII and IX. The rescued expression levels ranged from very low (aminoglycosides) to moderate (U1 snRNA and SMaRT), which could result in amelioration of the disease phenotypes. These findings prompt further studies aimed at demonstrating the clinical translatability of RNA-based strategies, which might open new avenues in the treatment of coagulation factor deficiencies.

Keywords: coagulation factor deficiencies, modified U1 snRNA, ribosome readthrough, RNA-based correction approaches.

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Introduction

The inherited deficiency of procoagulant factors is associated with bleeding diathesis in patients, whose clinical manifestations are related to the clotting factor involved and the reduction extent of its plasma levels.

Deficiencies of factors (F)VIII (hemophilia A) or IX (Hemophilia B), which are caused by mutations in the X-linked *F8* and *F9* genes, respectively, represents 95%–97% of all inherited hemorrhagic coagulation factor disorders [1]. The other rare deficiencies are transmitted as autosomal recessive traits with a prevalence ranging from approximately 1 in 2 million for prothrombin and FXIII deficiency to 1 in 500 000 for FVII deficiency [2].

Current treatment for hemophiliacs is based on the intravenous administration of the missing proteins (replacement therapy), either plasma derived or produced by recombinant DNA technology [3], in response to bleeding episodes or prophylactically [4].

Although protein replacement has significantly increased the quality of life and prolonged the life expectancy of patients suffering from coagulation factor disorders, the cost and short half-life of these proteins impose limitations on this therapy that motivated research towards alternative therapeutic approaches.

As even a tiny increase in coagulation factor levels would result in a significant amelioration of the clinical phenotype, coagulation factor deficiencies represent preferred models to investigate innovative therapeutic approaches in a quantitative manner, by virtue of functional and protein assays in plasma.

Enormous efforts have made with regards to substitutive gene therapy that consists of viral or non-viral mediated delivery of a copy of the defective gene (or better of the coding DNA sequence) into the patient's cells, thus triggering stable endogenous expression of the missing protein [5,6].

Another area of research has been focused on the correction of the gene expression of the mutated clotting factor gene by modulation of the messenger RNA (mRNA) processing and translation, which has been successfully explored for the treatment of other human genetic disorders [7–9]. Notably, RNA targeting would permit restoration of gene expression

while maintaining the gene promoter regulation in the cells belonging to the physiological tissue. Moreover, it has the potential to circumvent some limitation owing to the large size of certain human disease genes, and could be also effective in addressing dominant-negative disease forms.

An essential prerequisite for an RNA-based therapy is the presence of the transcribed RNA either in its precursor (pre-mRNA) or mature form (mRNA), the targets of the therapeutic compounds. Depending on the mutation type, three approaches can be foreseen (Table 1). Whereas *trans*-splicing can theoretically act on several types of mutations, the strategy aimed at inducing ribosome readthrough over stop codon or at restoring splicing represent interventions for disease forms caused by nonsense substitutions or mutations affecting pre-mRNA processing, respectively.

The different strategies for splicing correction include the usage of small nuclear RNAs and antisense oligonucleotides. In general, antisense oligonucleotides bind to target sequences on pre-mRNA and can either directly mask splicing regulatory elements or recruit additional binding sites for positive splicing factors. The usage of antisense oligonucleotides for splicing correction has been illustrated in previous excellent reviews [10–13] and will not be described here.

Two forms of spliceosomal small nuclear RNAs (U1 and U7 snRNA) have been used so far to modulate splicing. These molecules were exploited in human disease models other than coagulation factor deficiencies as antisense molecules to inhibit splicing, for example by masking the disease-causing mutation and inducing exon skipping and/or by interfering with the spliceosome formation [11,13].

On the other hand, U1 snRNA is becoming an attractive molecule for its specific capacity to correct splicing mutations at the donor splice site [14–21].

In the present review, we will focus on the principle of the different approaches, their application and future perspective in the treatment of inherited coagulation factor disorders. Particular attention will be given to novel approaches with modified U1 snRNA to restore normal splicing in donor splice site defects, and with drug-mediated ribosome readthrough over nonsense mutations.

Table 1 Summary of correction strategies targeting pre-messenger RNA(mRNA) processing and mRNA translation

RNA-based therapeutic strategies in coagulation factor deficiencies		
Strategy	Target molecule	Mutation type
Splicing correction (U1 snRNAs. oligos, drugs)	pre-mRNA	Splicing defects
Trans-splicing	pre-mRNA	Mutations not impairing pre-mRNA levels
Ribosome readthrough (drugs)	mRNA	Nonsense mutations*

*Nonsense mutations associated with major nonsense-mediated mRNA degradation may not respond to treatment.

Rescue of coagulation factor expression by splicing modulation

The splicing process and the splicing pathology

The splicing process Exonic sequences located on primary transcripts (pre-mRNAs) must be precisely identified and joined together to form the mature mRNA along with the removal of the usually larger intronic sequences. In the past, this process has revealed a large complexity, rendering it particularly susceptible to derangements caused by human mutations [22–24]. Pre-mRNA splicing requires the correct identification of several *cis*-acting elements in an ordered fashion [25,26]. The core elements are moderately conserved and consist of the classic or canonical splice sites (the 5′ and 3′ splice sites named also donor and acceptor sites), the polypyrimidine tract and the branch site (Fig. 1). However, the nascent pre-mRNA transcript contains several potential cryptic donor (5′ss) and acceptor (3′ss) splice sites, which are never used. To correctly identify the correct splice site, the splicing machinery requires the assistance of additional splicing regulatory elements. According to their position and functional effect, these elements are classified as exonic or intronic, and enhancer or silencer, depending on their effect on splicing [25,26].

Several previous studies have demonstrated that these regulatory elements play important roles in modulating RNA splicing in normal and pathological conditions. A classic example is represented by purine-rich Exonic Splicing Enhancers (ESE). A mutation in this element may induce, independently of its effect on the amino acid code, a pathological skipping of the exon from the mRNA. In general, the splice sites and the additional splicing regulatory elements are recognized by several *trans*-acting factors that form a network of interactions on the exon and contribute to its definition. As a result, the final outcome of a splicing decision

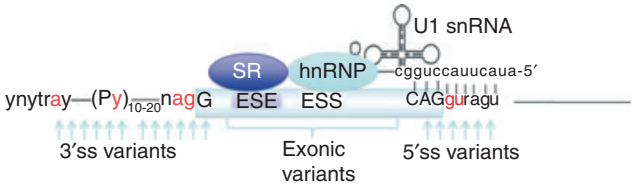

Fig. 1. Regulatory elements in pre-messenger RNA (mRNA) splicing. Schematic representation of the major splicing regulatory elements on pre-mRNA along with corresponding *trans*-acting factors and the genomic variants that affect splicing. The canonical splicing signals (in red) are composed of the branch site, the polypyrimidine tract (Py), the acceptor (3′ss) and the donor (5′ss) splice sites. In the first step of splicing, the 5′ tail of U1 snRNA recognizes the consensus 5′ss mainly by base pair complementarity. The boxed exon contains regulatory sequences, Exonic Splicing Enhancer (ESE) and Silencer (ESS), which contribute positively or negatively to exon recognition through interaction with SR proteins and hnRNPs (heterogeneous RiboNuclearParticles), respectively. Genomic variants (arrows) at canonical splicing signals, in the exonic or intronic (not shown) splicing regulatory sequences can affect splicing.

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depends on the equilibrium between multiple positive and negative interactions.

The splicing pathology As a result of the complexity of the process, splicing defects can originate from mutations at the splice sites or at the splicing regulatory elements located either in the exon or in the intron (Fig. 1). A thorough investigation of the composition of splicing regulatory elements is not only important to improve our knowledge of the basic splicing mechanisms, but it provides potential targets for splicing correction. A paradigmatic example is offered by Spinal Muscular Athrophy (SMA), in which an intronic splicing silencer (ISS) in the *SMN2* gene can be specifically targeted with an oligonucleotide [11,27]. This results in improved inclusion of SMN2 exon 7, which compensates for the lack of the paralogue *SMN1* gene, and ameliorated phenotype in a mouse model of SMA [28].

Role of U1 snRNP in splicing regulation A key molecule involved in the initial discrimination between exons and introns is the U1 small nuclear RiboNuclearParticle (U1 snRNP) [29]. In common with other snRNPs, it contains a small RNA (165bp for U1 snRNA) complexed with several proteins. The 5′ end of the U1 snRNA interacts by complementarity with the donor splice site, whose sequence is moderately conserved. In fact, its consensus motif consists of a nine-nucleotide sequence, CAG/GURAGU where R is a purine. However, with the exclusion of the nearly obligate GU dinucleotide, the other positions can tolerate some substitutions. About 40%, 22% and 5% of normal donor splice sites contain, respectively, two, three or four mismatches towards the U1 snRNA [30]. As a consequence, in some cases the effect of mutations flanking the canonical GU site may be difficult to predict without an analysis of the mature mRNA produced. Furthermore, additional splicing regulatory sequences are involved in the recognition, and can compensate for the defective complementarity between the U1 snRNA and the donor splice site.

Splicing correction using U1 snRNA

Splicing defects caused by mutations at the donor splice site consensus can be corrected in target cells with a molecular approach that re-directs the endogenous spliceosome machinery to the proper splicing junction of the mutant mRNA, thus rescuing exon definition and gene expression.

Donor splice site substitutions represent approximately 8% of all different mutations found to be associated with human inherited diseases [23,31–33]. In coagulation factor genes, mutations affecting the donor splice sequences (GU dinucleotide or the nearby positions) occur at a similar rate (7% in *F7*; 8% in *F9*; 3% in *F8*) (http://www.hgmd.cf.ac.uk/ac/index.php). These mutations, reducing the complementarity of the U1 snRNA to the donor splice site, can result in exon skipping, intron retention or activation of cryptic splice sites. *In silico* analysis through a number of computer-assisted tools available on the web helps the prediction of their disease-causing effect. However, for many mutations, in particular those nearby the invariant GU dinucleotide, it can be difficult to clearly predict their effect on splicing based only on the DNA sequence.

For example, the intron 7 donor splice site of *F7* gene, where a cluster of mutations has been mapped [34], displays a very low score, and significantly deviates from the consensus sequence (Fig. 2).

As donor splice site mutations disrupt the complementarity of the donor site with the endogenous U1 snRNA, restoring the complementarity through engineered modification of the U1 snRNA represents a valuable approach. For this purpose, the normal 5′ end sequence of the U1 snRNA is substituted with a

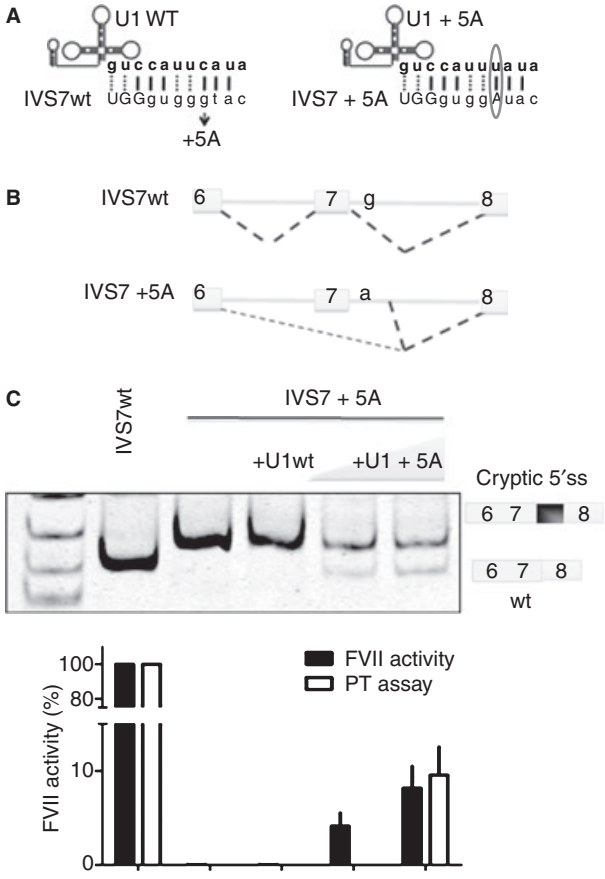

Fig. 2. U1 snRNA-mediated rescue of coagulation factor (F)VII expression impaired by the IVS7 + 5G/A mutation. (A) Schematic representation of the U1 snRNA with its 5′ tail paired to the 5′ donor splice site of intron 7 (IVS7) of the *F7* gene (exonic and intronic sequences are shown in upper and lower case letters, respectively). The normal (left) and mutated (right) IVS7 sequences targeted by the wild-type and modified U1 snRNA (U1 + 5A) are reported. The circle indicates the rescued complementarity at the mutated nucleotide position. (B) Schematic representation of the splicing events (dotted lines) occurring in normal (upper panel) and mutated (lower panel) conditions. (C) Rescue of FVII mRNA processing (upper panel) and of FVII function (lower panel) by the modified U1 + 5A in co-transfection experiments. The figure has been adapted from data previously reported by Pinotti *et al.* [14,15].

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sequence complementary to the target mutation (Fig. 2A), and the modified U1 snRNA gene is tested in appropriate splicing assays.

This approach has been originally employed to demonstrate the role of U1 snRNA in donor splice site recognition in model systems, and recently studied for the rescue of splicing defects in coagulation disorders [14,15].

The 9726 + 5G/A substitution in the *F7* intron 7 (IVS7 + 5G/A) reduces the complementarity with the normal U1 snRNA (Fig. 2A), activates an intronic downstream cryptic donor splice site and induces exon skipping (Fig. 2B). The resulting transcripts do not encode for functional proteins.

Co-expression of a modified U1 snRNA with increased complementarity to the defective donor splice site, named U1 + 5A (Fig. 2A), induced a significant rescue of the splicing defect with synthesis of the normal transcript (Fig. 2C). Interestingly, the splicing correction resulted in the synthesis of functional FVII molecules, whose coagulant activity levels reached 9% of those of the normal construct (Fig. 2C), an extent that would be theoretically sufficient to correct the coagulation defect *in vivo*.

The modified U1 snRNA approach has also been used for some other diseases caused by donor splice site mutations either to correct splicing defects or to detail mechanisms of aberrant splicing [16–21]. It is worth noting that mutations at the invariant GU site are generally not rescued, consistent with the critical importance of this dinucleotide in splicing. Differently, splicing rescue is possible for several substitutions nearby the GU, which account for approximately 40% of all donor splice site mutations. Among them, there are position and sequence preferences influenced by the gene context that could render some variants more susceptible to the rescue effect of U1 snRNAs.

The use of modified U1 snRNA represents a complementary strategy to classic gene therapy with which it shares the delivery issue as well as the potential capability of guaranteeing long-term correction of the genetic defect.

For those genes, like *F8*, in which the large size of the coding region limits their package into some viral vectors such as those derived from the Adeno-associated virus (AAV) [35], modified U1 snRNA might represent a valid option.

The U1 snRNA gene used for splicing rescue includes promoter and regulatory sequences in < 1 Kb and can be easily inserted, even in multiple copies, in AAV vectors, which are now actively studied for gene therapy of coagulation factor disorders [35]. In addition, the U1 snRNA approach, acting on the pre-mRNA, has the advantage of maintaining the expression regulation of the targeted gene in the normal chromosomal context. Future studies in appropriate animal models will be necessary to prove the efficacy *in vivo*.

In common with other rescue strategies based on targeting RNA by complementarity (i.e. oligonucleotides), modified U1 snRNAs have to deal with potential off-target effects that might affect splicing of other genes. This could be dangerous for modified U1 snRNAs that have only one base change from the natural U1 snRNA, and thus might activate normally silent cryptic donor splice sites and induce aberrant splicing in other genes. However, the design of novel Exon-Specific U1 snRNAs (ExSpeU1) complementary to non-conserved sequences downstream of mutant donor splice sites are expected to reduce this type of interaction (F. Pagani and M. Pinotti, unpublished data). ExSpeU1, reminiscent of originally described shifted U1 snRNAs [36], would reduce off-targets and, by binding downstream of the donor splice site, correct different splicing mutations. In this case, a single ExSpeU1 rescues multiple splicing defects that affect a single exon.

The potential therapeutic effect of modified U1 snRNAs on pre-mRNA relies also on the gene context and on the targeting sequence. For example, in Duchenne Muscular Dystrophy (DMD) modified U1 snRNAs that bind extensively to exonic sequences and splice sites induce skipping of an exon that contains a disease-causing stop codon [37]. The resulting mature mRNA devoid of the defective exon is in frame and codes for a functionally active protein. On the other hand, binding of modified U1 snRNA to 3′UTR can be used to silence genes by interfering with polyadenylation and stability of the mRNA [38].

In factor (F)IX (FIX), the importance of the context where the U1 snRNAs binds is highlighted by the analysis of a A to T mutation occurring at position + 13 downstream exon 5 of *F9* gene. This mutation is associated with mild hemophilia B and activates a cryptic donor splice site (Fig. 3). We tested whether an ExSpeU1, designed to bind to the not conserved intronic sequence of the cryptic donor splice site (Fig. 3A), improves selection of the correct one. Expression of this ExSpeU1 + 13 partially rescued the splicing pattern and led to an increase in levels of correctly spliced mRNA from approximately 8% to 40% (Fig. 3B–C). Interestingly, this ExSpeU1 did not stimulate usage of the cryptic donor splice site where it binds, suggesting that it acts on intronic splicing regulatory elements important for donor splice site definition. Thus, fine-tuning of U1 snRNA binding sites might reveal interesting targets for splicing correction.

It should be noticed that an important requirement for developing novel strategies for splicing correction relies also on our knowledge of basic mechanisms of normal and pathological splicing, and very few data are available regarding the architecture of defective splicing in coagulation factor genes. In these genes, the identification of splicing regulatory elements with enhancer and silencers function, and their role in pathological processing of pre mRNA, could reveal novel pathways for therapeutic intervention.

Rescue by spliceosome-mediated RNA trans-splicing (SMaRT)

Normally, splicing occurs in *cis* within the same pre-mRNA molecule. Although to a very low frequency in mammalian cells, the spliceosome machinery can also catalyze splicing between two separate pre-mRNA molecules, leading to a process called RNA trans-splicing [39].

The induced spliceosome-mediated RNA trans-splicing (SMaRT) (extensively reviewed by Yang *et al.* [40]) is a recent

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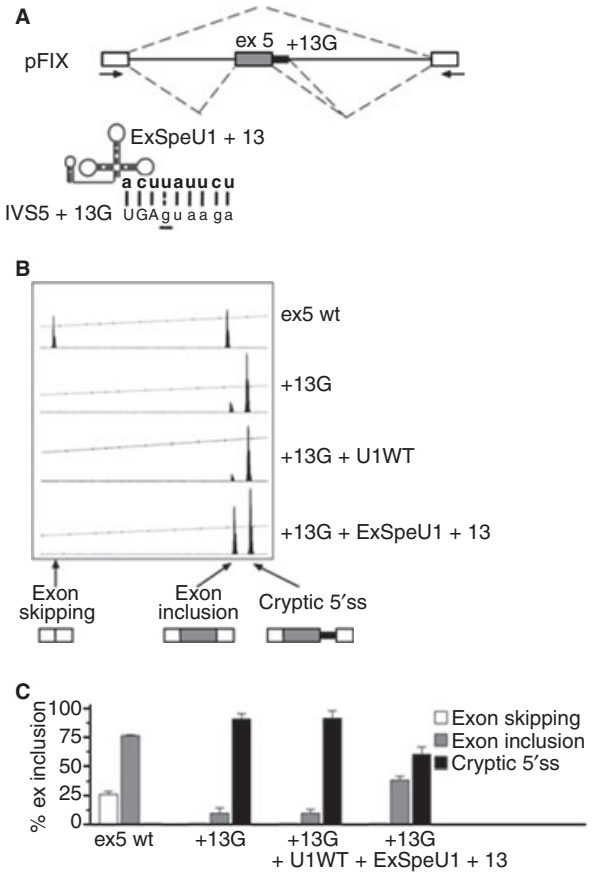

Fig. 3. Partial rescue of factor (F)IX) splicing by the ExSpeU1 + 13. (A) Schematic representation of the minigene construct (pFIX) (upper panel). Exonic and intronic sequences are boxes and lines, respectively. The black box represents the sequences inserted by the +13G mutation in the final mRNA and the dotted lines above and below the construct indicate the splicing events in normal and mutated conditions, respectively. The arrows are the primers used in RT-PCR amplification. The lower panel shows the RNA complementarity between the 5′ tails of the ExSpeU1 + 13 and the intronic FIX sequence affected by the +13a/g mutation (underlined). (B) Separation on a denaturing capillary system of fluorescently labeled RT-PCR products obtained from total RNA of HeLa cells expressing the normal (ex5 wt) or the mutated (+13G) minigenes without or upon overexpression of the normal (U1wt) or the modified (ExSpeU1 + 13G) U1 snRNAs. The identity of transcripts deriving from exon 5 skipping, usage of the correct (exon 5 inclusion) or the cryptic (cryptic 5′ss) donor splice sites is depicted at the bottom. (C) Relative percentage of transcripts in the different experimental conditions.

RNA reprogramming strategy in which the final mRNA results from splicing of two independently transcribed RNA molecules. During splicing of the endogenously expressed gene, a new pre-mRNA sequence, driven by an exogenous vector, is trans-spliced into the target to generate a new gene product. Through this approach, depending on the design of the pre-trans-splicing RNA molecules (PTMs), the target mRNA can be modified to include the novel sequences at its 3′ or 5′ region, or within its sequence (Fig. 4).

PTMs consist of three main elements: a binding domain (BD) complementary to a chosen target pre-mRNA, an unpaired splice site and a coding domain containing sequences to be trans-spliced into the target. To prevent direct PTM expression, 3′ exon replacement PTMs should be constructed without translation start codons, 5′ PTMs without polyadenylation signals and double trans-splicing PTMs should lack both these sequence elements.

Hemophilia A was the first human inherited disease in which the therapeutic potential of SMaRT has been successfully demonstrated *in vivo* [41]. Hemophilia A mice created by insertion of the neomycin resistance gene into *F8* exon 16 did not display detectable levels of functional FVIII. It should be noted that replacement gene therapy approaches for this bleeding disorder are made difficult by the large size of the *F8* gene, thus making alternative approaches of great interest.

To induce synthesis of the correct FVIII mRNA, a PTM was designed to trans-splice to exon 15 of the endogenous FVIII pre-mRNA target and to replace the downstream coding sequence. By exploiting adenoviral vector driving the expression of the PTM, the authors demonstrated the presence of the repaired FVIII mRNA in the mouse liver, a significant increase (25% of normal) in circulating functional FVIII and an amelioration of the bleeding phenotype.

Based on these results and on the principle of the SMaRT, virtually all mutations in coagulation factor deficiencies could be approached, with the exception of those significantly affecting gene transcription (i.e. sequence variations in the promoter), which prevent pre-mRNA from being available to trans-splicing.

Although extremely interesting, a number of open issues have to be addressed before translating SMaRT into the clinic. Among them, improvement in SMaRT efficiency for therapeutic purposes will probably require the development of more effective molecules and/or the combination with other approaches that, by specifically targeting the spliceosome, will facilitate the process. For example, an antisense oligonucleotide has been used to selectively block a downstream splice site and facilitate *trans*-splicing [42] in a mouse model of SMA. This approach could be exploited in inherited coagulation disorders, to improve its efficacy in a transcript specific manner.

The potential and limitations of the SMaRT approach have been extensively reviewed elsewhere [40,43] and will not be discussed further in the present review.

As for any gene therapy approach, the induction of trans-splicing or splicing correction by modified U1 snRNA *in vivo* requires a safe and efficient delivery of the expression cassette that should guarantee a long-term correction of the genetic defect. As a result of their limited size, the expression cassettes could fit into adeno-associated virus (AAV) vectors that are extensively used for gene replacement therapy purposes [35]. These studies, and particularly those on gene therapy of hemophilia B in the liver, will hopefully provide the ideal vector and protocol to translate the RNA-based mediated approaches into the clinic.

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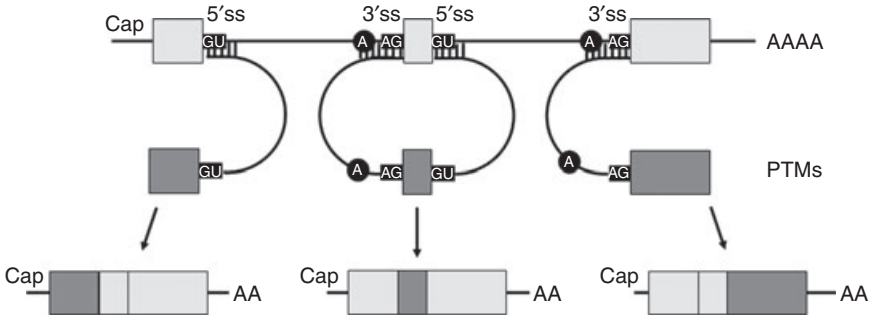

Fig. 4. Spliceosome-mediated RNA *trans*-splicing (SmaRT). The therapeutic trans-splicing is based on the substitution of the 5′ (left), internal (middle) or 3′ (right) exons (light grey rectangles). The three different trans-splicing events are triggered by pre-trans-splicing molecules (PTMs), appropriately designed to reprogram 3′, internal or 5′ exons (dark grey rectangles). Products of SmaRT, in which the new exons are inserted, are shown in the lower panels.

Rescue of coagulation factor expression by translation modulation

Nonsense mutations, translation termination efficiency and ribosome readthrough

Mutations introducing premature stop codons (nonsense mutations) are relatively frequent in coagulation factor deficiencies, particularly in the most severe forms. Nonsense mutations account for approximately 10% of all molecular defects leading to hemophilia A (http://europium.csc.mrc.ac.uk) and B (http://www.kcl.ac.uk/ip/ petergreen/haemBdatabase.html) [44,45].

Depending on its localization in the gene sequence, premature stop codons can trigger the rapid degradation of the mRNA through a process termed nonsense-mediated mRNA decay (NMD) [46]. Moreover, the translation of the mutated mRNA may lead to synthesis of truncated polypeptides that usually are not properly folded and hence degraded. Based on these detrimental mechanisms, nonsense mutations virtually produce null phenotypes.

A multitude of mechanisms and players participate in the exquisitely regulated process of translation termination at nonsense codons (UAA, UAG and UGA) that is essential for the correct expression of proteins. When a stop codon is presented in the A site of the ribosome, it is recognized and bound by release factors that trigger release of the polypeptide chain [47]. However, albeit with a very low rate in normal conditions (10^{-4}), an aminoacyl-tRNA can also enter the ribosomal A site at the stop codon position, thus leading to amino acid misincorporation and keep the the protein synthesis going. This process is termed ribosome readthrough [48]. As the sequence context of the natural stop codon has a role in its functional definition, the premature nonsense triplets could lack the proper definition, which would favour ribosome readthrough [49].

Translation fidelity has a crucial role in the gene expression control and is the target of antibiotic drugs such as aminoglycosides. These drugs are able to specifically bind to the highly conserved 16S ribosomal RNA (rRNA) at the decoding center of the small bacterial ribosomal subunit [50]. The aminoglycosides induce codon misreading and misincorporation of amino acids at a nonsense codon, thus leading to translational readthrough rather than chain termination.

Although with a much lower affinity, aminoglycosides can also bind to the eukaryotic ribosome, thus explaining their toxicity in humans upon long-term administration or at high doses [51].

In the past decades, many studies have focused on turning this side effect into a therapeutic opportunity for human genetic diseases caused by nonsense mutations [52]. The aminoglycoside-mediated restoration of protein biosynthesis in the presence of a nonsense mutation may, even to a lower extent, be functionally significant (Fig. 5).

To date, the restoration of protein and functional levels by aminoglycosides has been demonstrated *in vitro* and *in vivo* for a number of human diseases such as cystic fibrosis (CF) and DMD (reviewed in Zingman *et al.* [53]). Here we will focus on studies conducted in hemophilia and FVII deficiency.

Aminoglycoside-mediated rescue

James *et al.* [54] tested the effect of the aminoglycoside gentamicin (7 mg kg^{-1} once a day) in severe hemophilia A and B patients with nonsense mutations (Fig. 5). In one patient with hemophilia A (S1395X mutation) and one with hemophilia B (R333X mutation) the treatment produced a transient shortening in the activated partial thromboplastin time (approximately 20 s) and an increase in FVIII/FIX activity (approximately 2%). However, such an effect on functional parameters could not be detected in the other three patients.

Notably, in the hemophilia A patient displaying a 1.6% increase in FVIII activity the plasma FVIII antigen levels were remarkably higher (7%), thus suggesting that ribosome readthrough induced the synthesis of dysfunctional molecules. This hypothesis was corroborated by the observation of a persistent increase in FIX antigen levels (approximately 2%) in one hemophilia B patient (R252X mutation) in whom FIX activity levels did not appreciably change upon treatment.

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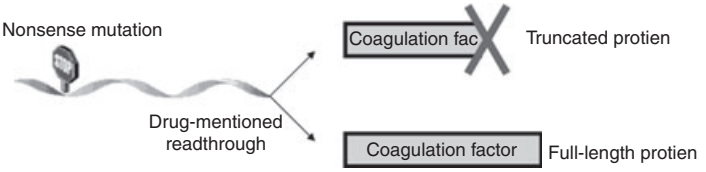

B

	Mutation	Cellular model		Mouse model		Patients	
		Drug	Response	Drug	Response	Drug	Response
FVII deficiency	K316X	G418	Yes (>3%)[57]				
	K316X	Gentamicin	Yes (>2%)[57]			Gentamicin	Sub-therapeutic[58]
	VV364X	G418	Yes (>3%)[57]				
	VV364X	Gentamicin	Yes (>2%)[57]			Gentamicin	Sub-therapeutic[58]
FIX deficiency	R29X			G418	No[56]		
	R29X			Gentamicin	No[56]		
	R252X					Gentamicin	No[54]
	R333X					Gentamicin	Yes (2%)[54]/NO[55]
	R338X			G418	Yes, (>8%)[56]		
	R338X			Gentamicin	Yes, (>3%)[56]		
FVIII deficiency	R427X					Gentamicin	No[54]
	S1395X					Gentamicin	Yes (1.6%)[54]
	R2116X					Gentamicin	No[54]

Fig. 5. Aminoglycoside-mediated ribosome readthrough in coagulation factor deficiencies. (A) Schematic representation of the effect of premature nonsense codons and of ribosome readthrough on protein products. (B) Summary of results obtained with the aminoglycosides neomycin (G418) and gentamicin in factor (F)VII, FIX or FVIII deficiencies caused by nonsense mutations. The percent within parentheses indicate the activity levels measured upon treatment in the experimental models or patients.

A negligible effect on FIX activity levels was also previously reported by Srivastava *et al.* [55] in four hemophilic B patients treated with gentamicin.

A remarkable variation of FIX levels in response to aminoglycosides geneticin and gentamicin has been documented in hemophilic B mice expressing the human FIX mutations R338X and R29X [56]. Geneticin treatment resulted in a significant increase (approximately 5%) in antigen and activity in R338X mice but not in R29X mice. Noticeably, residual antigen could be detected at 3 weeks, and activity levels could be detected at 6 days.

The aminoglycosides-mediated rescue of FVII expression impaired by the K316X and W364X nonsense mutations was explored by us both in cellular models [57] and in patients [58].

In a fluorescent model with the FVII-green fluorescent protein (GFP) chimera, geneticin treatment of cells expressing the nonsense variants resulted in an appreciable fluorescence expression, to indicate partial restoration of full-length protein synthesis. Consistently, expression studies with the native FVII revealed that geneticin (G418) and gentamicin induced a dose-dependent increase of secreted FVII molecules with activity levels reaching 3% to 4% of wild-type FVII (Fig. 5). However, the increase in secreted protein levels was more pronounced, thus indicating the synthesis of dysfunctional proteins [57].

The pilot clinical study with gentamicin (3 mg kg^{-1} once a day) was conducted in two patients bearing the K316X and W364X mutations [58]. At some time-points prothrombin time (PT) values were slightly shortened (approximately 10 s) and, using activated FX generation assays, the FVII activity (0.1%–0.25% of pooled normal plasma [PNP]) was significantly above the baseline (Fig. 5). However, the FVII antigen and coagulant activity levels were undetectable.

Altogether *in vitro* and *in vivo* data, both in humans and animal models, revealed a low and extremely variable response to aminoglycosides, thus making it difficult to define their therapeutic potential. Several elements might explain the variable response:

1 the levels of the mutated mRNA, differentially affected by nonsense mediated decay;
2 the presence of nonsense mutations that may undergo readthrough with differential efficiency, depending on the nonsense triplet and the sequence context [59–62];
3 the introduction of amino acids other than the natural one at the nonsense positions, which could impair protein biosynthesis, stability and/or function. This would explain discrepancies between antigen and activity levels detected upon treatment in some hemophilic patients [54] and in cellular models of FVII deficiency [57]. Therefore, the beneficial effects could be low or negligible for nonsense mutations occurring at crucial functional positions of the protein; and
4 the different duration and method of application of the aminoglycosides between studies, and variability in drug metabolism among individuals.

The toxicity issue should be also considered. In fact, the clinical benefit of gentamicin, and of aminoglycosides in general,

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is limited because high concentrations and/or long-term treatments can cause severe side effects such as kidney damage and hearing loss [63]. Novel molecules, able to discriminate between premature and normal termination codons, are needed to improve selectivity and efficacy, and thus safety.

A high-throughput screening revealed a new small molecule, PTC124 (Ataluren, PTC therapeutics, South Plainfield, NJ, USA), which can readthrough premature termination codons [64,65] and thus potentially treat a variety of genetic diseases. The molecule demonstrated its correction efficacy in a number of human disease models [66] and, in phase I safety studies, was found to be well tolerated [65].

Phase II clinical trials of PTC124 have been initiated in patients with different human genetic diseases caused by nonsense mutations, including CF, DMD or Becker Muscular Dystrophy (BMD) and hemophilia.

In a study involving 23 CF individuals, more than half of the patients showed convincing changes in the nasal chloride-channel defect in the first cycle of treatment, but this improvement was observed in only one-third of them in the second cycle [67]. In a second trial, PTC124 treatment significantly increased the proportion of nasal epithelial cells expressing apical full-length cystic fibrosis transmembrane conductance receptor (CFTR) and induced a nasal chloride transport response or hyperpolarization in 50% and 47% of patients [68]. However, in a phase II clinical trial in DMD/BMD patients, a 48-week treatment of PTC124 did not result in a significant change in 6-min walking distance [69]. The ongoing clinical trial in hemophilia A and B patients will provide insights into this promising 'mutation specific' therapeutic approach for coagulation factor deficiencies.

General conclusion

The intervention at the post-transcriptional (modulation of cis-splicing or induction of trans-splicing) and translational (induction of ribosome readthrough) levels represents an innovative therapeutic approach for coagulation factor diseases. Although the modulation of cis-splicing and of translation termination is mutation specific, it must be underlined that splicing and nonsense mutations are relatively frequent in the severe coagulation factor deficiencies.

One might argue that the extent of rescue of expression levels so far obtained by strategies at the RNA level is moderate and, for *in vivo* application of modified U1 snRNA and SMaRT, depends on a safe and efficient delivery of expression cassettes. However, even a very low increase in plasma levels of coagulation factors would result in a significant amelioration of a clinical phenotype of patients, thus encouraging further studies aimed at demonstrating their clinical translatability.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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