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Inter-chain disulfide bond improved protein *trans*-splicing increases plasma coagulation activity in C57BL/6 mice following portal vein FVIII gene delivery by dual vectors

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Protein *trans*-splicing based dual-vector factor VIII (FVIII) gene delivery is adversely affected by less efficiency of protein splicing. We sought to increase the amount of spliced FVIII protein and plasma coagulation activity in dual-vector FVIII transgene in mice by means of strengthening the interaction of inteins, protein splicing elements, thereby facilitating protein *trans*-splicing. Dual-vector delivery of the FVIII gene in cultured cells showed that replacement of Met226 in the heavy chain and Asp1828 in the light chain with Cys residues could facilitate inter-chain disulfide linking and improve protein *trans*-splicing, increasing the levels of spliced FVIII protein. In this study, C57BL/6 mice were coadministered dual vectors of intein-fused human FVIII heavy chain and light chain with Cys mutations via portal vein injection into the liver. Forty-eight hours post-injection, plasma was collected and analyzed for FVIII antigen concentration and coagulation activity. These mice showed increased circulating FVIII heavy chain polypeptide (442±151 ng mL^{-1} vs. 305±103 ng mL^{-1}) and coagulation activity (1.46±0.37 IU mL^{-1} vs. 0.85±0.23 IU mL^{-1}) compared with control mice co-administered dual vectors expressing the heavy and light chains of wild-type FVIII. Moreover, coagulation activity was similar to that of mice receiving a single vector expressing FVIII (1.79±0.59 IU mL^{-1}). These findings indicate that improving protein *trans*-splicing by inter-chain disulfide bonding is a promising approach for increasing the efficacy of dual-vector based FVIII gene transfer.

coagulation factor VIII, protein *trans*-splicing, dual-vector gene delivery, plasma coagulation activity

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Protein *trans*-splicing based dual-vector cotransfer of FVIII heavy and light chain genes is effective in overcoming the capacity limit of adeno-associate virus (AAV) vectors. This strategy uses split intein, a protein splicing element, linked to the FVIII heavy and light chains, resulting in functionally peptide-joined intact FVIII protein, with intein fragments removed post-translationally [1]. As an inter-peptide *trans*-splicing reaction, its efficiency depends on the strength of the molecular interactions, although splicing information located in the intein molecules and protein splicing occur spontaneously without any auxiliary factors or energy consumption [2]. In our recent study, in which the FVIII gene was transfected into cultured cells using the dual-vector, the spliced FVIII protein, with coagulation activity recovered, was observed, but these cells contained unspliced precursors of intein fused heavy and light chain polypeptides [1]. These nonfunctionally unspliced protein precursors may be immunogenic. The amount of spliced FVIII protein depends on its splicing efficiency; this, in turn, determines the coagulation activity and the efficacy of gene therapy. Another dual-vector gene delivery strategy based on intracellular

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formation of heterodimeric FVIII causes obvious chain imbalance because of inefficient heavy chain secretion, which reduces efficacy of transgene and may also destabilize host cells, with excessive retention of intracellular heavy chain polypeptide even inducing apoptosis [3,4].

Protein splicing reactions are not dependent on any cellular mechanisms, with an ability of protein splicing intracellularlly before secretion and extracellularlly after secretion of heavy and light chains to produce intact FVIII protein. Moreover, peptide bonded light chain to heavy chain shows a *cis* promotive effect on secretion of heavy chain [1]. Improved protein *trans*-splicing between two peptides, in protein splicing based dual-vector FVIII gene delivery, may increase the amount of spliced FVIII protein and procoagulant activity, thus enhancing the results of gene therapy. Using cultured cells, we recently demonstrated that an interchain disulfide bond between B domain-deleted FVIII (BDD-FVIII) heavy and light chains can increase the amount of protein-spliced BDD-FVIII protein and its coagulation activity [5]. We have expanded these findings to a mouse model, by injecting two vectors, one each encoding the BDD-FVIII heavy and light chain, into the portal vein of mice, suggesting a strategy of gene therapy for patients with hemophilia A.

1 Materials and methods

1.1 Materials

The plasmid vector pCMV-F8 expressing human BDD-FVIII, pCMV-HCIntN and pCMV-IntCLC with *Ssp* DnaB intein-fused BDD-FVIII heavy and light chain genes have been described previously [1]. C57BL/6 mice were purchased from Vital River Laboratories (VRL, Beijing, China).

Pfu, a high fidelity DNA polymerase was obtained from Stratagene (Santa Clara, CA, USA), the DNA ligase kit from New England Biolabs (Ipswich, MA, USA), and the HiSpeed Plasmid Midi Kit from Qiagen (Dusseldorf, Germany).

Recombinant human factor VIII protein was obtained from BioChain (Hayward, CA, USA). Monoclonal antibodies against FVIII heavy and light chain, ESH5 and ESH8 were purchased from American Diagnostica (Greenwich, CT, USA). Horseradish peroxidase (HRP) conjugated rabbit anti-human FVIII polyclonal antibody was purchased from Novus (Littleton, CO, USA). Normal pooled human reference plasma and FVIII deficient plasma were obtained from George King Biomedical (Overland Park, KS, USA). A chromogenic FVIII coagulation activity assay kit was purchased from Chromogenix (Milan, Italy).

1.2 Vector construction

Inverse PCR was used to introduce the Met662Cys and Asp1828Cys mutations into the heavy and light chains of BDD-FVIII. DNA fragments were generated using the plasmids pCMV-HCIntN and pCMV-IntCLC as templates and the pairs of primers 5′-TGTGTCTATGAAGACAC-ACTCACC-3′ (forward) and 5′-TTTGTGTTTGAAGGT-ATATCCAG-3′ (reverse) for heavy chain, and 5′-TGTG-AGTTTGACTGCAAAGCCTG-3′ (forward) and 5′-TTTA-GTGGGTGCCATATGATG-3′ (reverse) for light chain, respectively. The resulting DNAs were self-ligated using T4 DNA ligase, leading to the formation of the two circular plasmids, pCMV-SHCIntN and pCMV-IntCSLC.

1.3 Animal procedures

Normal C57BL/6 mice were injected via the portal vein with 200 μg each of the column-purified plasmids, pCMV-SHCIntN and pCMV-IntCSLC, in 2 mL of saline as previously described [6]. As controls, 200 μg of both pCMV-HCIntN and pCMV-IntCLC, the single vector pCMV-F8 for BDD-FVIII, were administered (n=6 per group). After 48 h, blood samples were collected by tail clip using sodium citrate as an anticoagulant at a final concentration of 0.38% (w/v). The blood samples were centrifuged for 10 min at 2000×g at 4°C, and the plasma was aliquoted and used for FVIII assays.

1.4 Plasma FVIII antigen assay

Plasma concentrations of BDD-FVIII HC and LC antigens were measured using chain-specific ELISAs as previously described [7]. Human recombinant FVIII protein was used as the standard and serially diluted in blocking buffer to concentrations of 320 to 10 ng mL^{-1}. Ninety-six well ELISA plates were coated with 2 μg mL^{-1} of monoclonal antibody to human FVIII heavy chain (ESH5; heavy chain-specific ELISA) or light chain (ESH8; light chain-specific ELISA) overnight at 4°C, washed, and incubated with blocking buffer for 2 h at 37°C. After washing, 100 μL diluted standards and plasma samples were added to each well, and the plates were incubated at 37°C for 1 h. The plates were washed, 100 μL of HRP-conjugated rabbit anti-human FVIII polyclonal antibody (2 μg mL^{-1}) were added to each well, and the plates were again incubated for 1 h at 37°C. After a final washing, antigen was detected by incubating with the substrate OPD for 30 min at 37°C. The reactions were stopped by adding 2 mol L^{-1} of H_2SO_4, and the plates were read at a wavelength of 490 nm. Standard curves were plotted from the diluted standards, and the concentrations of human HC and LC in plasma samples produced by the transgenes were deduced from the standard curves.

1.5 Plasma FVIII coagulation activity assay

Coatest chromogenic assays were used to measure transgene produced human FVIII coagulation activity in mouse

plasma as described with some modifications [6]. Total plasma FVIII activity consists of both mouse and transgene generated human FVIII activities. Normal pooled human plasma was used as a standard and defined as 1 mL of this plasma containing one unit FVIII activity (IU mL^{-1}) and 200 ng FVIII protein (ng mL^{-1}). This standard was serially diluted with FVIII-deficient human plasma, such that FVIII activity ranged from 1 (undiluted) to 0.0313 IU mL^{-1}. 10 μL of each standard or mouse plasma diluted into FVIII-deficient plasma were incubated at 37°C for 15 min in the presence or absence of 2 μL of the anti-human FVIII light-chain antibody ESH8 (1 mg mL^{-1}) and immediately placed on ice to deplete human FVIII activity. After serial reactions according to kit instructions, plates were read at 405 nm and standard curves were plotted based on the difference in absorbance of standard samples incubated in the presence or absence of ESH8 versus corresponding FVIII activity. The absorbance of plasma samples was also measured at 405 nm and FVIII activity was obtained from standard curves. The difference in FVIII activity between ESH8-untreated and ESH8-treated samples represents human FVIII activity.

1.6 Statistical analysis

All results are presented as mean±SD and compared using two-tailed Student's *t*-tests, with $P<0.05$ considered statistically significant.

2 Results

2.1 Structures of BDD-FVIII and its intein fused heavy and light chains

The full length human FVIII molecule consists of 2351 amino acid (aa) residues with a 19-aa N-terminal signal peptide sequence and a domain arrangement from the N- to the C-terminus of A1-A2-B-A3-C1-C2. A functional, smaller version of FVIII, B-domain-deleted FVIII (BDD-FVIII), results from the removal of most of the centrally located B domain from Ile761 to Asn1639. Cleavage of BDD-FVIII before Ser1657 generates separate heavy (HC) and light (LC) chains. The N-part of *Ssp* DnaB intein (IntN) was added to the C-terminus of HC and the C-part of *Ssp* DnaB intein (IntC), preceded by the signal sequence (SS) of FVIII, was added to the N-terminus of LC. These steps yielded HCIntN and IntCLC, respectively. The residues in HCIntN and IntCLC corresponding to the Met662 and Asp1828 residues of the FVIII were each mutated to Cys, generating SHCIntN and IntCSLC (Figure 1).

2.2 Transgenes produced plasma concentrations of BDD-FVIII light and heavy chains

We found that injection of the BDD-FVIII light chain vector alone had no effect on the plasma concentration of BDD-FVIII light chain (Figure 2). When heavy and light chain genes were coinjected into mice, the plasma light chain concentrations in the absence and presence of the Cys mutation were 827±197 and 897±208 ng mL^{-1}, respectively ($P>0.05$). Moreover, light chain concentrations were similar in mice administered the light chain gene alone. The light chain detected in plasma has three sources: light chain in intracellularlly protein *trans*-splicing produced and secreted BDD-FVIII, light chain in post-secretion spliced BDD-FVIII, and directly secreted non-spliced light chain precursor. Our findings therefore suggest that secretion of light chain is independent of inter-chain disulfide linking. The single vector BDD-FVIII transgene had a mean plasma light chain concentration of 521±112 ng mL^{-1}, all bound quantitatively to heavy chain as secreted heterodimeric BDD-FVIII. The reduced amount of secreted light chain in single- than in dual-vector BDD-FVIII transgene may be due to the binding of heavy chain to the ER chaperone protein BiP,

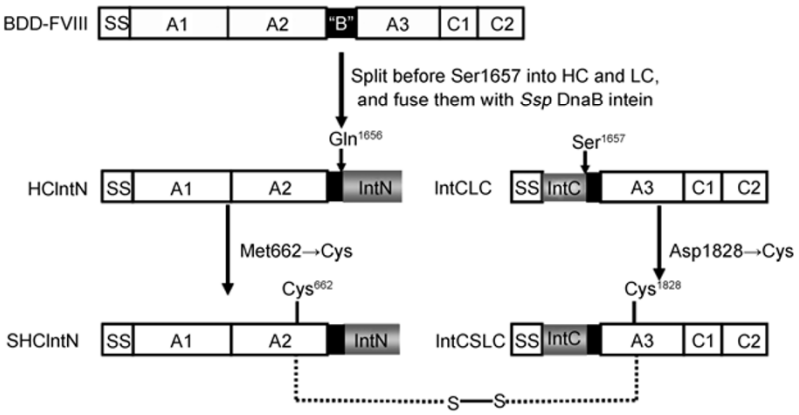

Figure 1 Schematic representation of BDD-FVIII and the intein-fused heavy and light chains. BDD-FVIII was generated from FVIII by deleting most of its B-domain. BDD-FVIII was split into heavy chain (HC) and light chain (LC), and these were fused with the N- and C-parts of *Ssp* DnaB intein (IntN, IntC), respectively, generating HCIntN and IntCLC. Site-directed mutagenesis was used to insert Cys mutations into Met^{662} of HC in HCIntN and Asp^{1828} of LC in IntCLC, resulting in SHCIntN and IntCSLC. SS, signal sequence of FVIII.

which hindered the secretion of light chain resulting in a lower plasma concentration [8].

Chain-specific ELISA was used to determine the plasma concentration of heavy chain generated by transgene (Figure 3). Plasma heavy chain concentration was very low in mice injected with intein-fused heavy chain gene alone, whether wild-type or Cys mutated, but was significantly higher in mice injected with dual-vector encoding Cys mutated than wild-type heavy and light chains (442±151 ng mL^{-1} vs. 305±103 ng mL^{-1}, $P<0.05$). The secreted heavy chain includes intracellularly spliced and secreted and post-secretion spliced BDD-FVIII, as well as unspliced intein-fused heavy chain precursor. These findings demonstrate that linking via an inter-chain disulfide bond improves protein *trans*-splicing, resulting in more spliced BDD-FVIII and more heavy chain secreted with light chain under *cis*-promotion of light chain. The increase in secretion of light and heavy chains resulted in an increase in heterodimeric BDD-FVIII, as shown by the increase in coagulation activity. Moreover, increased secretion of heavy chain reduced its cellular retention and resultant chain imbalance and enhanced cell stability [4]. The plasma concentration of heavy chain secreted by single vector BDD-FVIII transgene was 478±125 ng mL^{-1}, similar to the concentration of light chain.

2.3 Dual-vector transgene generated plasma coagulation activity

The coagulation activity of plasma resulting from injection of transgene was determined by chromogenic analysis (Figure 4). The FVIII coagulation activity in plasma of mice co-injected with intein-fused and Cys mutated BDD-FVIII heavy and light chain genes was significantly higher than that of intein-fused, wild-type BDD-FVIII heavy and light chain genes (1.46±0.37 IU mL^{-1} vs. 0.85±0.23 IU mL^{-1}, $P<0.05$), indicating that the inter-chain disulfide linkage between the two mutated Cys residues enhanced the interaction of the two chains. Thus, inter-molecular *trans*-splicing was similar to intra-molecular *cis*-splicing in producing more spliced BDD-FVIII protein. A *cis*-prompting

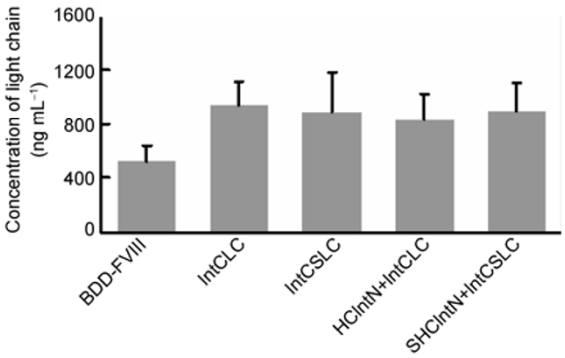

Figure 2 Concentration of light chain in plasma.

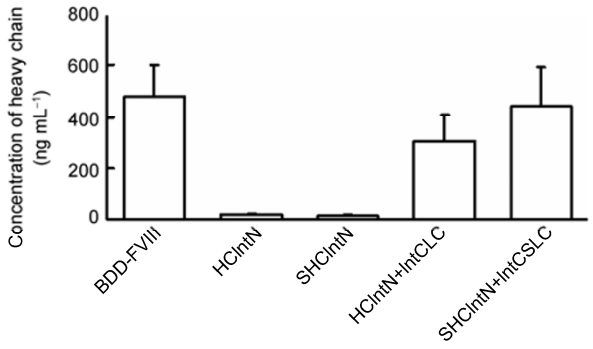

Figure 3 Concentration of heavy chain in plasma.

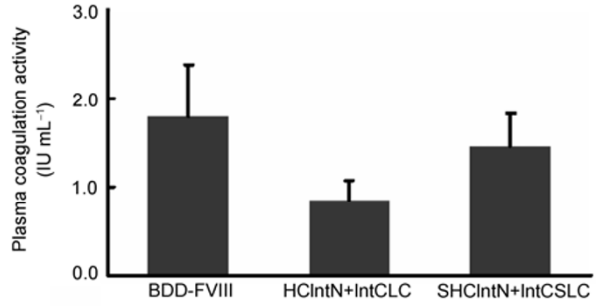

Figure 4 Coagulation activity of plasma.

effect of light chain on heavy chain secretion increased secretion of heterodimeric BDD-FVIII into plasma, resulting in elevated coagulation activity. Injection of single vector BDD-FVIII gene generated a plasma coagulation activity of 1.79±0.59 IU mL^{-1}, similar to that of dual-vector Cys mutant BDD-FVIII transgene, indicating that dual-vector delivery of BDD-FVIII was as effective as a single vector delivery of the BDD-FVIII transgene.

Although all heavy and light chains in BDD-FVIII protein expressed from single vector transgene contributed to coagulation activity (100%), this was not necessarily true for the dual vector strategy. We therefore calculated the ratios of heavy and light chain involved in plasma coagulation activity in the plasma of dual-vector BDD-FVIII gene injected mice (Table 1). We found that injection of intein-fused and Cys mutated heavy and light chain genes increased the contribution of both chains to plasma coagulation activity relative to the co-delivery of wild-type BDD-FVIII. Increased secretion of heavy chain as spliced FVIII effectively decreased the amount of unmatched free light chain.

3 Discussion

Gene therapy is considered promising for the treatment of patients with hemophilia A and may replace transfusion therapy with plasma derived or recombinant FVIII protein. These therapies are costly, generate inhibitory antibodies

Table 1 Contribution of HC and LC produced by transgenes to plasma coagulation activity

Group	Percent functionally active HC	Percent functionally active LC
BDD-FVIII	100	100
SHCIntN+IntCSLC	88.2	47.4
HCIntN+IntCLC	74.4	29.9

and promote the spread of infectious diseases. Effective gene therapy depends on the duration of therapeutic levels of plasma coagulation activity. AAV vectors have been considered ideal gene carriers for hemophilia A gene therapy because of their ability to maintain sustained and stable expression of coagulation factors in targeted liver and skeletal muscle tissues [9−11]. However, the 4.7 kb packaging capacity of AAV vectors has limited their use in the transport of the large FVIII gene. Moreover, although the functional miniature BDD-FVIII cDNA is only 4.5 kb long, it exceeds the maximum capacity of AAV vectors after the addition of sequences of gene expression regulatory elements. Newly synthesized, intracellular FVIII protein is a single-chain polypeptide, 200 kD in size. Post-translational processing results in a heterodimer composed of a heavy and a light chain, and the secreted protein binds to von Willebrand factor (vWF) [12]. An alternative strategy, which overcomes the limited capacity of AAV vectors and was based on the understanding of intracellular FVIII processing, uses two AAV vectors to co-transfer FVIII heavy and light chain genes [13]. However, the primary disadvantage of this approach was the inefficient heavy chain secretion, which was ~1–2 logs less than that of light chain [3,6]. Secretion of excess light chain as a non-matched monomer may induce an immune response in the host [3,6]. Because heterodimers of heavy and light chains only form intracellularly, it is necessary to transfect two vectors encoding both chains into the same target cells [14]. However, transfection of an increased dose of vector encoding heavy chain had little effect on its secretion [7]. Mutations in BDD-FVIII, such as the Phe309Ser mutation in the heavy chain, and modifications, such the addition of up to six glycosylation sites to the B domain, were found to increase BDD-FVIII secretion by almost 20-fold [15,16]. However, transfection of modified heavy chain genes alone did not enhance its secretion of heavy chain, indicating that heavy chain secretion is complex [7]. In the absence of light chain, the heavy chain may fold improperly in the ER or Golgi apparatus. Increasing the efficiency of cell co-transfection is also difficult, with co-transfection of AAV vectors into cultured cells resulting in successful infection of only about 50% [14].

We recently developed an alternative dual-vector strategy to transfer the FVIII gene. Using protein *trans*-splicing, we found that the target cells expressed FVIII heavy and light chain, which were ligated to form intact FVIII protein by fused intein, a protein splicing element, yielding a product similar to that of the FVIII transgene. The light chain in spliced FVIII protein has a *cis*-prompting effect on heavy chain secretion. The secreted heavy and light chains can also be spliced, suggesting that protein splicing is independent of any cellular mechanism(s), and suggesting that it is not necessary for both chains to enter the same target cell [1,7]. The ability of protein splicing extracellularly is a unique advantage over non-protein splicing-based dual vector co-transgenic technology. However, protein *trans*-splicing occurs between the FVIII heavy and light chain polypeptides, so the efficiency of splicing determines the amounts of spliced FVIII protein and coagulation activity. Protein *trans*-splicing may be improved by enhancing the interactions of FVIII heavy and light chains. Mutation of Met662 in the A2 domain of the heavy chain and Asp1828 in the A3 domain of light chain to Cys resulted in the formation of a disulfide bond between these two Cys residues. This linkage between heavy and light chains did not affect FVIII activity while prolonging its half-life following thrombin activation. Coinjection of two vectors encoding the FVIII heavy and light chain with Cys mutations into mice through the portal vein increased plasma coagulation activity, suggesting that the secretion of disulfide cross-linked heterodimeric FVIII had increased [18]. Fusing intein sequences to the FVIII heavy and light chains, followed by dual-vector transfection into cultured cells, increased the secretion of spliced FVIII protein and activity indicating that the inter-chain disulfide bond improved protein *trans*-splicing [5]. In this study, we co-injected vectors encoding intein-tagged and Cys-mutated FVIII heavy and light chains into the livers of mice via the portal vein. We found that the procoagulation activity of mouse plasma was markedly increased, indicating an increased secretion of spliced FVIII protein. The disulfide bond helps to join the heavy and light chains, making inter-peptide *trans*-splicing similar to intra-peptide *cis*-splicing resulting in increased FVIII protein and plasma coagulation activity following secretion. The increased secretion into plasma of heavy chain peptide decreases its intracellular deposition, reducing cell pressure and reducing the amount of nonfunctional, unmatched plasma light chain polypeptide. The increased amounts of heavy and light chain peptides in plasma contribute to coagulation activity, indicating that these two chains are present as heterodimeric FVIII derived from spliced FVIII. As a result, chain imbalance is also alleviated. Because the spliced protein is similar to that expressed by the single vector FVIII transgene, ligation by protein splicing may increase the secretion of glycosylation modified heavy chain, thus enhancing coagulation activity [19].

These findings indicate that protein *trans*-splicing-based

dual-vector approach produces a spliced FVIII identical in structure to physiologically synthesized cellular FVIII and with similar plasma coagulation activity. Thus, transgene efficacy may be increased by enhancing protein *trans*-splicing by the introduction of inter-chain disulfide bonds. In addition, although the A and C domains of FVIII and coagulation factor V (FV) have 40% sequence homology, the secretion of FVIII is less efficient than that of FV, indicating that FVIII is present in a particular configuration and that its cellular trafficking and processing are complex [16,20]. Our results indicate the need for methods to improve the secretion of spliced FVIII and coagulation activity, based on methods that include point mutations and glycosylation.

As a novel biotechnology, protein *trans*-splicing has been used widely in protein research [21]. Its applications in gene therapy, via the dual-vector transfer of disease-causing large genes, require further exploration.

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