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Apparent synonymous mutation *F9* c.87A > G causes secretion failure by in-frame mutation with aberrant splicing

Koya Odaira[a,1], Shogo Tamura[a,*,1], Nobuaki Suzuki[b], Misaki Kakihara[a], Yuna Hattori[a], Mahiru Tokoro[a], Sachiko Suzuki[a], Akira Takagi[a], Akira Katsumi[c], Fumihiko Hayakawa[a], Shuichi Okamoto[d], Atsuo Suzuki[e], Takeshi Kanematsu[e], Tadashi Matsushita[b,e], Tetsuhito Kojima[a,*]

[a] Department of Pathophysiological Laboratory Science, Nagoya University Graduate School of Medicine, Nagoya, Japan
[b] Department of Transfusion Medicine, Nagoya University Hospital, Nagoya, Japan
[c] Department of Transfusion Medicine, National Center for Geriatrics and Gerontology, Obu, Japan
[d] Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, Nagoya, Japan
[e] Department of Clinical Laboratory, Nagoya University Hospital, Nagoya, Japan

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ABSTRACT

Introduction: Hemophilia B is an X-linked recessive bleeding disorder caused by coagulation factor IX (FIX) gene (*F9*) mutations. Several *F9* synonymous mutations have been known to cause hemophilia B; however, the deleterious mechanisms underlying the development of hemophilia B have not been completely understood. To elucidate the molecular pathogenesis causing hemophilia B, we investigated the synonymous *F9* mutation: c.87A > G, p.(Thr29=).

Materials and methods: The influence of *F9* c.87A > G on mRNA splicing was analyzed by exon-trap assay and in silico prediction. We prepared FIX expression vectors using mutant *F9* cDNA and transfected HepG2 cells to investigate intracellular transport and extracellular secretion of FIX. Intracellular kinetics of the expressed FIX was examined by treatment with the proteasome inhibitor MG132.

Results: Exon-trap analysis revealed that *F9* c.87A > G resulted in almost (99.1%) aberrant splicing (r.83_88del). In silico analysis predicted that *F9* c.87A > G influenced the splicing pattern by generating an available aberrant 5′ splice site. The aberrant *F9* mRNA (r.83_88del) was translated to a mutant FIX p.Cys28_Val30delinsPhe with an in-frame mutation at the signal peptide cleavage site. Simultaneously, a small amount (0.9%) of mutant *F9* r.87A > G translating into WT FIX p.Thr29 = was also observed. The mutant FIX was abnormally retained in the endoplasmic reticulum (ER) and was not extracellularly secreted. It appeared to be intracellularly degraded via proteasome-dependent degradation machinery.

Conclusion: *F9* c.87A > G was found to cause abnormal mRNA splicing, r.83_88del, and produce the mutant FIX p.Cys28_Val30delinsPhe. The mutant FIX is an abnormal protein with extracellular secretory defects and is intracellularly eliminated via proteasome-dependent ER-associated degradation.

Abbreviations: A, adenine; Ala, alanine; BSA, bovine serum albumin; bp, base pair; CHX, cycloheximide; Cys, cysteine; DMEM, Dulbecco's modified Eagle's medium; DNA, deoxyribonucleic acid; EGF domain, epidermal growth factor domain; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; ERAD, ER-associated degradation; FBS, fetal bovine serum; FVIIa, activated factor VII; FIX, factor IX; FIX:C, FIX activity; FXIa, activated factor XIa; G, guanine; Gla domain, γcarboxyglutamic domain; Glu, glutamic acid; HB, hemophilia B; HSF, human splicing finder; ICC, immunocytochemistry; IgG, immunoglobulin G; mRNA, messenger ribonucleic acid; MT, mutant; n.d., not detected; Phe, phenylalanine; PBS, phosphate buffered saline; PCR, polymerase chain reaction; qRT-PCR, quantitative RT-PCR; R, purine base; RT, reverse transcription; SDS, sodium dodecyl sulfate; SPase, signal peptidase; SRP, signal recognition particle; T, thymine; Thr, threonine; Val, valine; WT, wild type; 5′ss, 5′ splice site

* Corresponding authors at: Department of Pathophysiological Laboratory Science, Nagoya University Graduate School of Medicine, 1-1-20 Daiko-Minami, Higashi-ku, Nagoya 461-8673, Japan.
E-mail addresses: stamura@met.nagoya-u.ac.jp (S. Tamura), kojima@met.nagoya-u.ac.jp (T. Kojima).
[1] K.O. and S.T. contributed equally to this work, and either has the right to list him first in bibliographic documents.

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1. Introduction

Coagulation factor IX (FIX) is a vitamin K-dependent single chain glycoprotein that mediates blood coagulation as a major component in the intrinsic pathway. FIX is encoded by the *F9* gene spanning 33.5 kb on the long arm of X chromosome (Xq27.1) [1]. FIX is primarily synthesized as a precursor FIX, including a signal- and pro-peptide (28 and 18 residues, respectively) in the hepatocytes, and a signal- and propeptide are proteolytically removed prior to secretion. Circulating FIX, not the activated form, has a Gla domain, two EGF-like domains, an activation peptide, and a serine protease domain. Activation of FIX is achieved by either FVIIa/tissue factor complex or FXIa in the presence of Ca^{2+} [2].

Hemophilia B (HB) is an X-linked recessive bleeding disorder characterized by quantitative or qualitative defect of FIX. The frequency of HB is 1 in approximately 30,000 male births. Based on FIX activity (FIX:C), HB is classified as severe (< 1 IU/dL), moderate (1%–5 IU/dL), or mild (5%–40 IU/dL) [3]. FIX defect of HB is caused by various types of *F9* mutation, including nonsynonymous mutation (e.g., a missense or nonsense mutation) and *F9* structure abnormality. Additionally, some synonymous mutations could also influence FIX production because of an aberrant splicing, mRNA instability, or abnormal translation [4]. In *F9* gene, 16 synonymous mutations have been reported and at least 7 mutations are considered to be the causes of HB [5]. However, the molecular pathogenesis of these synonymous mutations causing HB have not been fully understood in most cases.

In this study, we identified a *F9* mutation c.87A > G in a patient with moderate HB. The mutation was previously suspected as a synonymous mutation, p.(Thr29=), and implied to disrupt *F9* mRNA splicing [6–8]; however, the pathogenic mechanism of *F9* c.87A > G has not been assessed. Here we demonstrated that *F9* c.87A > G affected *F9* mRNA splicing pattern, and the aberrant mRNA was translated as an abnormal FIX with an in-frame-mutation at the signal peptide cleavage site. The in-frame mutant FIX had defects in the maturation process and intracellular kinetics, resulting in a diminished FIX secretion.

2. Patient and methods

2.1. Patient and DNA sample

Patient was a 55-year-old Japanese male who diagnosed with moderate hemophilia B (FIX:C = 2.5 IU/dL). Genomic DNA of the patient was isolated from peripheral blood leukocytes as per a previously described method [9] after obtaining written informed consent. This study was approved by the Ethics Committee of Nagoya University Graduate School of Medicine (Identification number: 2015-0391).

2.2. Polymerase chain reaction (PCR) and direct DNA sequencing

F9 exons and exon/intron boundaries were amplified by polymerase chain reaction (PCR) using KOD FX DNA polymerase (Toyobo, Osaka, Japan) and *F9* specific primers (Supplementary Table 1). PCR products were separated by electrophoresis with 1.5% agarose gel and purified using Fast Gene Gel/PCR Extraction Kit (Nippon Genetics Co. Ltd., Tokyo, Japan). Direct sequencing was performed using a BigDye Terminator v1.1 Cycle Sequencing Kit and ABI Prism 310 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). Mutation was described according to the nomenclature of Human Genome Variation Society (http://www.hgvs.org/mutnomen/).

2.3. In silico analysis of mutant molecules

To predict the influence of c.87A > G on *F9* mRNA splicing, we conducted in silico analyses using Human Splicing Finder, MaxEntScan (http://umd.be/HSF3/), and NetGene2 (http://www.cbs.dtu.dk/services/NetGene2). Alteration of functional signal peptide cleavage site in the mutant FIX owing to *F9* c.87A > G was analyzed using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/).

2.4. Preparation of F9 exon-trap and FIX expression vectors

Cell-based splicing analysis of the mutant *F9* was performed using Exon-trap cloning Vector, pET01, (MoBiTec GmbH, Göttingen, Germany). To prepare mutant *F9* exon-trap vector (pET01-*F9* mutant), *F9* PCR fragment of genomic DNA of patients from the middle of exon 1 to intron 3 was amplified using specific primers (Supplementary Table 1), digested with *Dra*III (Roche diagnostics, Basel, Switzerland) and *Bam*HI (New England Bio Labs, Ipswich, MA, USA.), and ligated into pET01 (Fig. 1C). To obtain WT *F9* exon-trap vector (pET01-*F9* WT), site-directed mutagenesis based on inverse PCR method utilizing highly accurate PCR enzyme KOD-Plus (Toyobo) was performed. Briefly, inverse PCR with KOD-Plus and specific primers (Supplementary Table 1) was performed using pET01-*F9* mutant as a template plasmid. Inverse PCR product was treated with *Dpn*I (Toyobo) to remove methylated template plasmid DNA, phosphorylated by T4 polynucleotide kinase (Toyobo), and self-ligated by Ligation high Ver.2.0 (Toyobo).

To prepare FIX expression vector with high expression efficiency, human *F9* cDNA with a truncated-*F9* intron 1 was inserted into pcDNA3.1 (Thermo Fisher Scientific, Waltham, MA, USA) according to the method described in a report by Enjolras et al. [10] with some modifications (pcDNA3.1-FIXwt). Mutant vector, pcDNA3.1-FIXmt, was prepared by site-directed mutagenesis with a specific primer set (Supplementary Table 1). Each nucleotide sequence of exons, exon/intron boundaries, and coding sequences in vectors were confirmed by direct sequencing as described above (Section 2.2).

2.5. Exon-trap analysis

COS-7 cells were cultured in DMEM containing high glucose (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and penicillin/streptomycin/amphotericin B (Wako) at 37 °C with 5% CO_2. In addition, pET01-*F9* WT and pET01-*F9* mutant were transfected into COS-7 cells by calcium phosphate transfection method as previously described [11] and cultured for 16 h, followed by total RNA extraction using ReliaPrep RNA Cell Miniprep System (Promega, Fitchburg, WI, USA). Extracted RNA sample was subjected to reverse transcription (RT)-PCR and direct sequencing analysis as previously described [12].

2.6. TA cloning

The RT-PCR products derived from pET01-*F9* mutant transfection were inserted into T-vector pMD20 (Takara Bio, Shiga, Japan), which was used to transform DH5α competent cells. Subsequently, the positive clone was selected by colony PCR and the inserted nucleotide alignment was determined by direct sequencing as described above.

2.7. FIX expression

HepG2 cells were cultured in DMEM under the same conditions described for culturing COS-7 cells and transiently transfected with pcDNA3.1-FIXwt or pcDNA3.1-FIXmt using Lipofectamine 2000 (Thermo fisher scientific) according to manufacturer's instructions for 5 h, followed by culture for additional 19 h in DMEM containing 10% FBS. Next, the culture medium was changed to serum-free medium supplemented with 5 μg/mL of vitamin K_1 (Koa Isei Co., ltd, Yamagata, Japan), followed by additional culture under several experimental conditions.

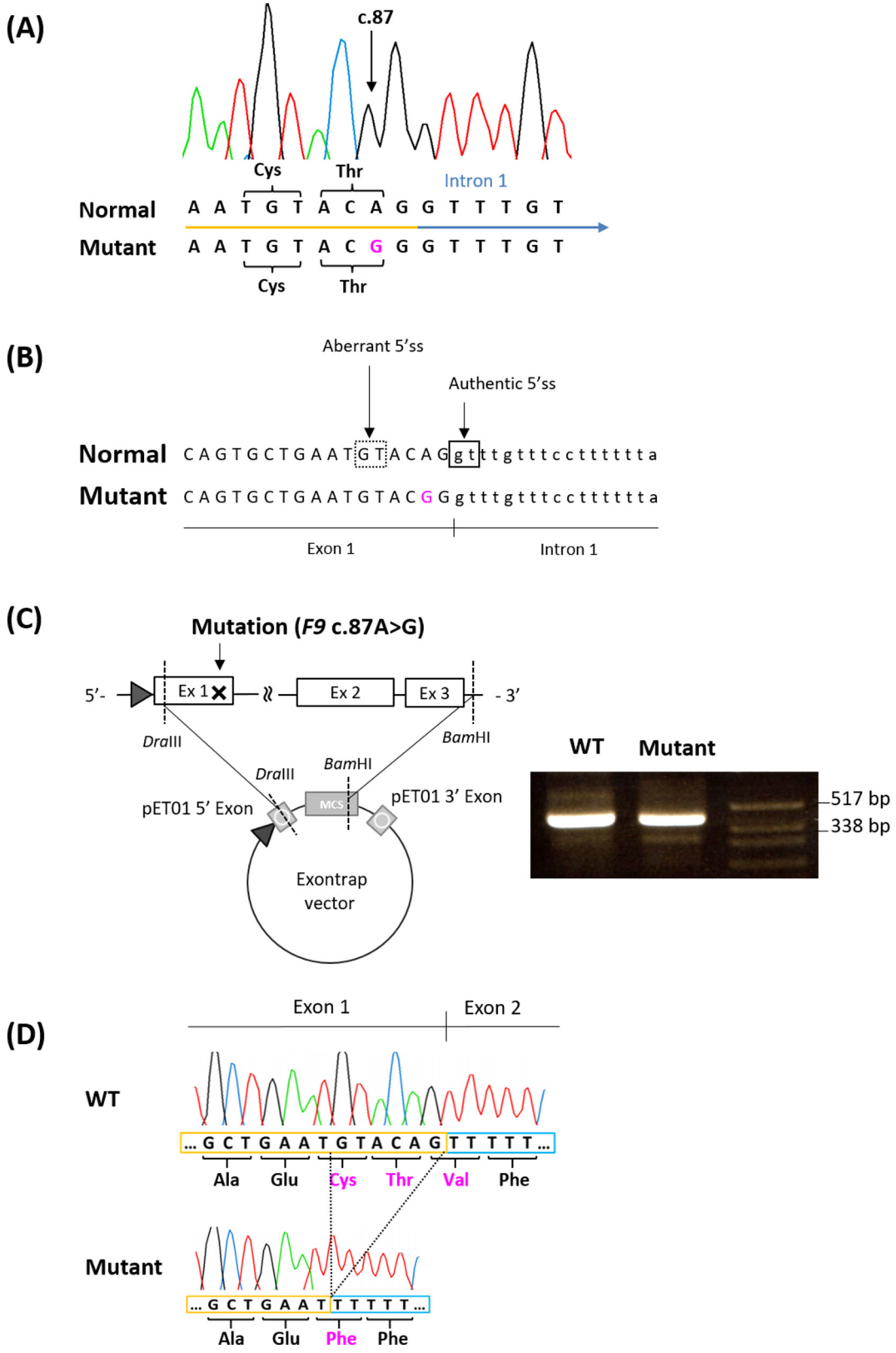

Fig. 1. Cell-based transcript analysis of *F9* c.87A > G.
(A) Direct sequencing data of *F9* c.87A > G mutation identified in the patient with moderate hemophilia B.
(B) Genomic sequences in the flanking region of *F9* exon 1/intron 1 boundary.
Solid and dashed rectangles indicate authentic and aberrant 5′ splice site (5′ss), respectively. DNA sequence is referred to NC_000023.11 in the NCBI database.
(C) Cell-based transcription assay using exon-trap vectors. Left panel: PCR amplicons with or without mutation spanning *F9* exon 1 to intron 3 were inserted into pET01. Right panel: Approximately 439 bp of RT-PCR product was clearly detected in COS-7 transfected with WT and a mutant vector.
(D) DNA sequences of RT-PCR amplicons derived from WT and mutant *F9* exon-trap vectors. Six nucleotides on the 3′ side of exon 1 were deleted in the transcript from the mutant *F9* exon-trap vector (*F9* r.83_88del).

Table 1
In silico splicing simulation of *F9* c.87A > G.

	Authentic 5′ splice site			Aberrant 5′ splice site		
	WT	Mutant	Variation (%)	WT	Mutant	Variation (%)
HSF[a]	86.17	81.32	−5.63	65.28	77.44	18.63
MaxEnt[a]	7.44	3.45	−53.63	−2.22	4.12	285.59
NetGene2[b]	0.55	n.d.	–	n.d.	0.30	–

n.d.: not detected.

[a] Threshold values for HSF and MaxEnt are 65 and 3, respectively. A score, 0 to 100 for HSF and from −20 to +20 for MaxEnt, above the threshold is considered to be a splice site. In the case of mutation occurred, if the WT score is above the threshold and the score variation (between WT and Mutant) is under −10% for HSF or −30% for MaxEnt, the mutation breaks the splice site. In the case that the WT score is under the threshold and the score variation is above +10% for HSF or +30% for MaxEnt, the mutation creates a new splice site.

[b] A score ranges between 0 and 1 for a potential splice site.

2.8. Western blot analysis

After 24 h of culture under serum-free conditions, culture media and cell lysates of the transfected HepG2 cells were collected. Culture media were centrifuged at 200 ×*g* for 10 min and passed through 0.45-μm filter (Merck, Darmstadt, Germany) to remove cellular debris. Proteins in the samples of culture media were concentrated by acetone precipitation method [13]. Precipitated pellet was dissolved with 1× sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% Glycerol). To prepare cell lysate, cells were lysed with 1× sample buffer. Cell lysates or culture medium precipitates were heated at 95 °C for 5 min, and the protein concentration was determined using Bio-Rad *DC*™ Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Cell lysates or culture medium precipitates were subjected to electrophoresis on 10% SDS-polyacrylamide gel with 850 mM 2-mercaptoethanol and 0.04% bromophenol blue, and blotted onto Immobilon®-P Transfer Membrane (Merck). After blocking with 2.5% skim milk, the membranes were incubated with primary antibody against human FIX (B-3, Santa Cruz Biotechnology Inc., Heidelberg, Germany) or beta-actin (Bio Vision Inc., Milpitas, CA, USA) overnight at 4 °C. Subsequently, the membranes were incubated with horseradish peroxidase conjugated secondary antibody (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 90 min. Signals were visualized with an ECL Select™ Western Blotting Detection Reagent (GE Healthcare UK Ltd., Little Chalfont, UK) and Light Capture II (Atto Corporation, Tokyo, Japan), and then quantified using CS Analyzer Ver. 3.0 (Atto Corporation) as previously described [11].

2.9. Enzyme-linked immunesorbent assay (ELISA)

Enzyme-linked immune-sorbent assay (ELISA) for FIX was performed using Factor IX Human SimpleStep ELISA® Kit (Abcam, Cambridge, UK).

2.10. Immunocytochemistry (ICC)

HepG2 cells were seeded onto coverslip in a 12-well plate and transfected with pcDNA3.1-FIXwt or pcDNA3.1-FIXmt. After 24 h of culture in DMEM containing 10% FBS and vitamin K_1 (5 μg/mL), the transfected cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature, and incubated with an endoplasmic reticulum (ER) marker, concanavalin A, Alexa Fluor™ 594 Conjugate (25 μg/mL) (Thermo Fisher Scientific) for 30 min. Subsequently, the cells were immunologically blocked with blocking reagent (2% goat serum and 3% BSA in PBS) and incubated with anti-FIX antibody (B-3, Santa Cruz Biotechnology Inc) overnight at 4 °C, followed by incubation with Alexa 488 conjugated anti-mouse IgG antibody (Thermo Fisher Scientific) for 90 min under conditions devoid of direct light. The coverslips were finally mounted with VECTASHIELD Antifade Mounting Medium containing DAPI (Vector Laboratories, Burlingame, CA, USA) and monitored using AX-80 (Olympus, Tokyo, Japan).

2.11. Inhibition of mRNA translation or proteasome

To inhibit mRNA translation, transfected HepG2 cells were treated with cycloheximide (CHX: 10 μg/mL, Sigma) in the serum-free medium containing vitamin K_1 (5 μg/mL). At each time point (0, 6, 12, and 24 h), cells and culture media were collected and FIX levels were assessed by western blot analysis or ELISA.

To inhibit proteasome, transfected HepG2 cells were treated with MG132 (10 μM, Abcam) in DMEM containing 10% FBS and vitamin K_1 (5 μg/mL) for 12 h. After MG132 treatment, cells were harvested and intracellular FIX levels were evaluated by western blot analysis.

3. Results

3.1. Effects of c.87A > G mutation on F9 mRNA splicing pattern

In the patient with moderate HB, we identified a single-base substitution in *F9* exon 1, c.87A > G (Fig. 1A), which was previously reported as a splicing mutation [6–8]. Based on a genetic code rule, *F9* c.87A > G was implied as a synonymous mutation, p.(Thr29=). On the other hand, this mutation is located in a consensus sequence of 5′ splice site (5′ss), AG/GTRAGT (exon/intron, R indicates a purine base) [15]. We therefore investigated the influence of c.87A > G on *F9* mRNA splicing process in silico (Table 1). Several simulations in silico (HSF, MaxEnt, and NetGene2) suggested that c.87A > G could destroy an authentic 5′ss at exon 1/intron 1 boundary and made available the aberrant 5′ss at the position −6 nucleotides from the boundary (Fig. 1B). These simulations in silico strongly suggested that the aberrant 5′ss was dominantly utilized as a splice site rather than authentic 5′ss in the *F9* transcript with c.87A > G.

To verify the in silico predictions, we conducted cell-based exon-trap analysis using pET01-*F9* normal and mutant vectors, which resulted in RT-PCR amplicons of almost similar sizes (Fig. 1C). Amplicon sequencing in the mutant indicated that 6 nucleotides at 3′ side of exon 1 were deleted, which resulted from the aberrant splicing via the aberrant 5′ss (*F9* r.83_88del, Fig. 1D). The frequency of splice variants was evaluated by TA cloning of the RT-PCR amplicons from the transfected cells with pET01-*F9* mutant vector. Among the 111 clones tested, 1 clone (0.9%) possessed a normal transcript pattern spliced via authentic 5′ss (*F9* r.87A > G, p.Thr29=). These findings suggested that *F9* c.87A > G disrupted mRNA splicing balance to predominantly utilize the aberrant 5′ss. The majority of the transcripts originated from the *F9* c.87A > G mutant, r.83_88del, were predicted to be translated into in-frame variants at the signal peptide cleavage site (p.Cys28_Val30delinsPhe, Fig. 1D). In silico simulation using SignalP denoted that signal peptide of wild type (WT) FIX was predicted to be cleaved between 28 Cysteine and 29 Threonine (C- and Y-scores were maximum at position 29, Fig. 2A). Alternatively, in-frame mutant FIX showed an obvious decrease in C- and Y-scores in the in-frame alignment (Fig. 2B). These results suggested that the in-frame mutation disrupted protein processing, particularly signal peptide cleavage.

3.2. Effects of in-frame FIX mutation on extracellular secretion

To investigate abnormality of in-frame mutant FIX, we analyzed the in-frame mutant FIX molecule expressed in HepG2 cells (Fig. 3). The in-frame mutant FIX was detected in cells (Fig. 3A Lysate, and Fig. 3B), and the molecule size was approximately 3 kDa larger than WT FIX. The increase in size by 3 kDa roughly corresponded to the size of signal

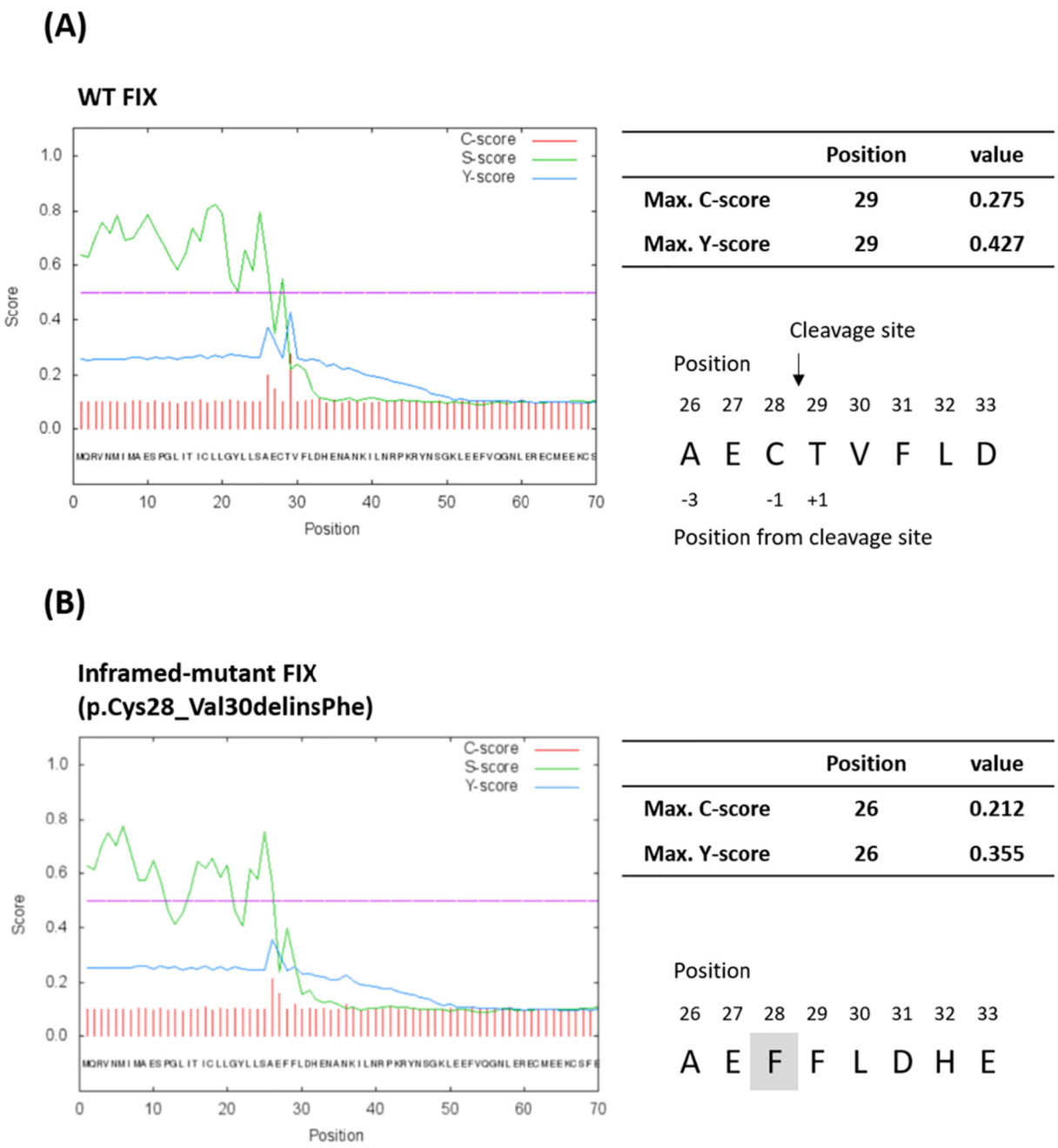

Fig. 2. In silico analysis of the in-frame mutant FIX, p.Cys28_Val30delinsPhe.
Signal peptide cleavage site of WT FIX and in-frame mutant FIX was simulated by an in silico analyzing tool, SignalP4.1. C-score indicates a raw cleavage site score, which is high at the position immediately after the cleavage site. S-score denotes a signal peptide score, which can distinguish the position of signal peptide from the position of the mature part of the protein. Y-score is a combined cleavage score, which is calculated from the C-score and the slope of S-score, and is considered to mark a better cleavage site prediction than the C-score. (A) Simulation results of WT FIX. C- and Y-scores were maximum at position 29 (threonine), indicating that signal peptide could be cleaved between cysteine at position 28 and threonine at position 29. (B) Simulation results of the in-frame mutant FIX p.Cys28_Val30delinsPhe. The in-frame phenylalanine is indicated by shading.

peptide, indicating that the in-frame mutant FIX carried the signal peptide. In culture medium WT FIX was clearly detected to be of the size of mature FIX (60 kDa); however, in-frame mutant FIX did not qualitatively reach detectable levels (Fig. 3A Medium). Quantitative analysis by ELISA also showed that FIX secretion was significantly reduced in the culture medium from transfectants with in-frame mutant FIX expressing vector (Fig. 3C).

To further investigate the in-frame mutant FIX, intracellular FIX localization was observed (Fig. 3D). In transfectant with WT FIX, intracellular FIX was mainly stained as a diffuse pattern. On the other hand, the in-frame mutant FIX was stained not only as a diffuse pattern but also as a punctate pattern. Moreover, the FIX punctate signal was detected in a co-localized manner with the ER marked by concanavalin A. These observations suggested that the in-frame mutant FIX remained in the ER, causing defects in extracellular secretion.

3.3. Intracellular elimination via proteasome-mediated degradation of in-frame mutant FIX

Considering that the in-frame mutant FIX abnormally accumulated

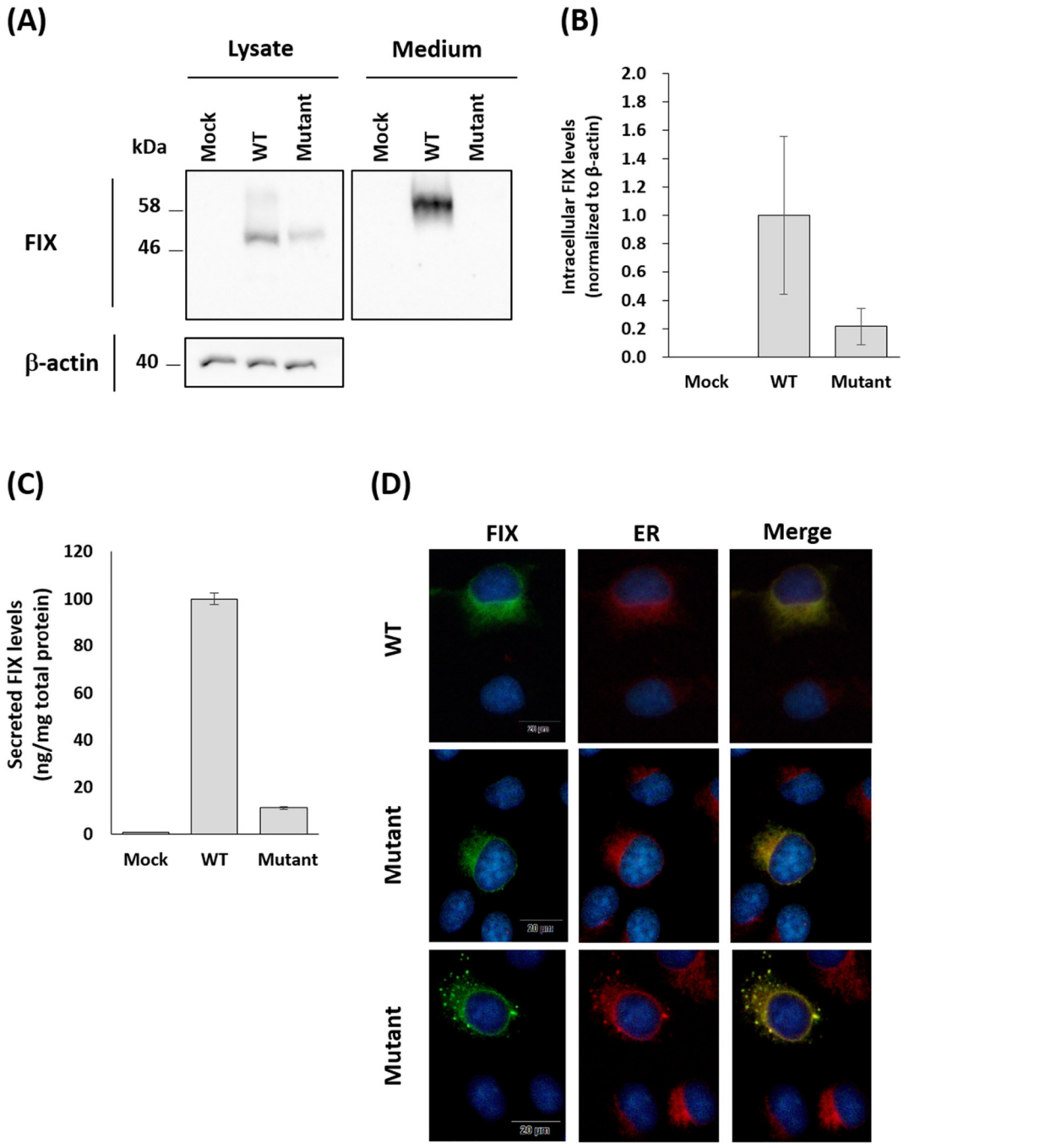

Fig. 3. Expression analysis of in-frame mutant FIX in HepG2 cells.
(A) Western blot analysis of intracellular and extracellular FIXs. Herein, 3 μg of protein was loaded in each lane. Cell lysates and culture media from HepG2 transfectants with pcDNA3.1 (Mock), pcDNA3.1-FIXwt (WT), and pcDNA3.1-FIXmt (Mutant) were prepared. Beta-actin was considered as the loading control. (B) Quantified data of intracellular FIX levels obtained by western blot analysis were normalized to beta-actin. Data were obtained from three independent experiments. Error bar reflects S.E.M. (C) Secreted FIX levels quantified by ELISA were corrected by total amount of protein in each culture medium. Data were achieved from three independent experiments. Error bar reflects S.E.M. (D) Representative immunocytochemical images of HepG2 transfectants expressing WT or in-frame mutant FIX (green). Endoplasmic reticulum (ER) was marked by staining with concanavalin A (red) and nuclei was stained with DAPI (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in the ER, we examined the intracellular dynamics of this mutant FIX. We first performed a protein-chasing experiment by treatment with mRNA translation inhibitor, CHX. At several time points after treatment with CHX, the amount of intracellular FIX gradually decreased in WT FIX, whereas that of extracellular FIX proportionally increased in a time-dependent manner (Fig. 4A, B, and C, WT). These findings indicated that the synthesized WT FIX was readily secreted. Conversely, the amount of intracellular FIX decreased in the in-frame mutant FIX in a manner similar to that in WT FIX; however, its extracellular FIX level was not augmented (Fig. 4A, B, and C, mutant FIX). These observations indicated that the in-frame mutant FIX was disposed through intracellular protein degradation systems.

To investigate this hypothesis, we subsequently performed inhibitory experiments on proteasome-dependent protein degradation by adding a potent non-specific proteasome inhibitor, namely MG132. Treatment with MG132 intensified intracellular FIX levels in both WT-

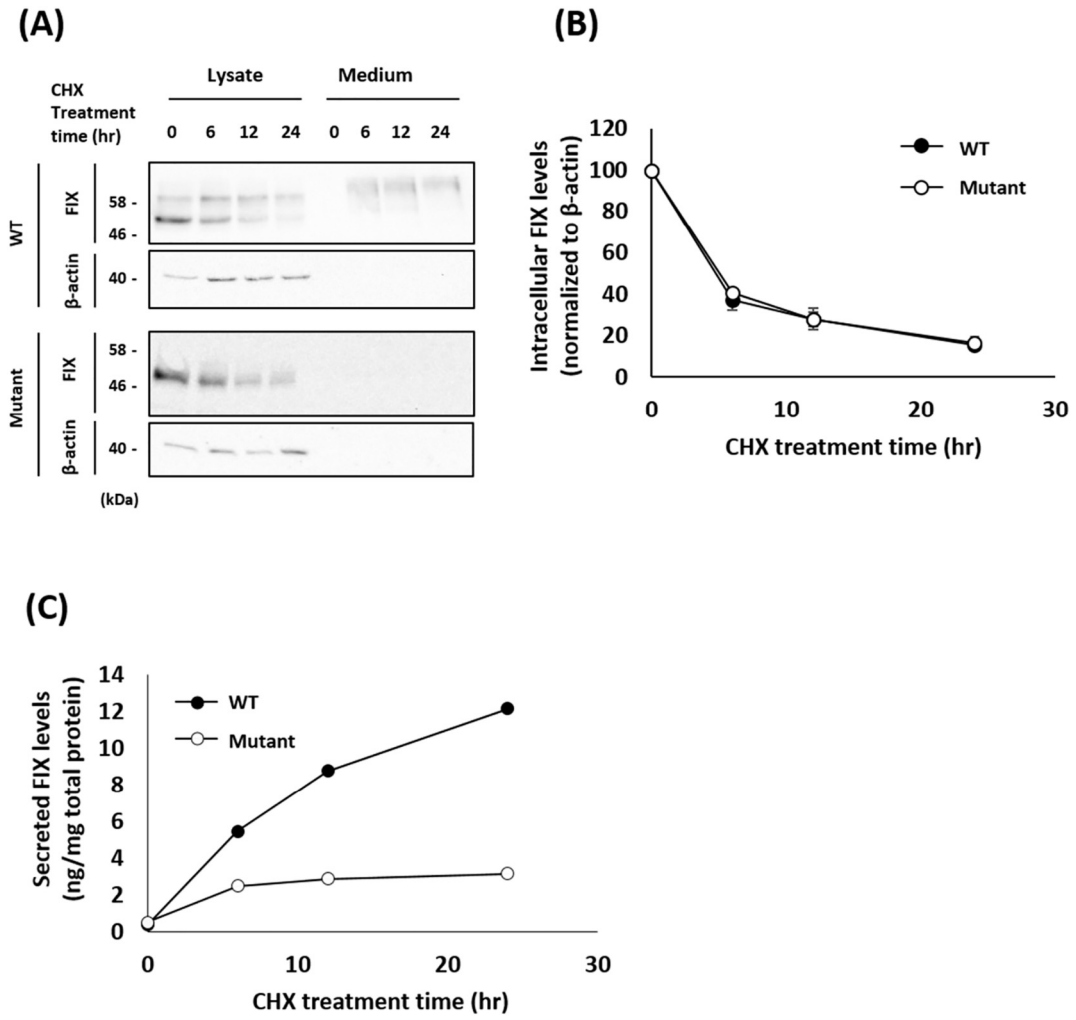

Fig. 4. Effects of the translation inhibitor cycloheximide (CHX) on FIX expression in HepG2 cells.
(A) Western blot analysis of the intracellular and secreted FIXs subjected to CHX treatment. Upper and lower panels show representative western blotting images of WT and in-frame mutant FIX expressed by each HepG2 transfectant, respectively. Beta-actin was detected as a loading control. (B) Intracellular WT and in-frame mutant FIX levels under CHX treatment were quantified by western blot analysis. Band intensity was normalized to beta-actin. Data were obtained from three independent experiments. Error bar reflects S.E.M. (C) Secreted WT and in-frame mutant FIX levels were quantified by ELISA. Data were corrected by the total amount of protein in each culture medium.

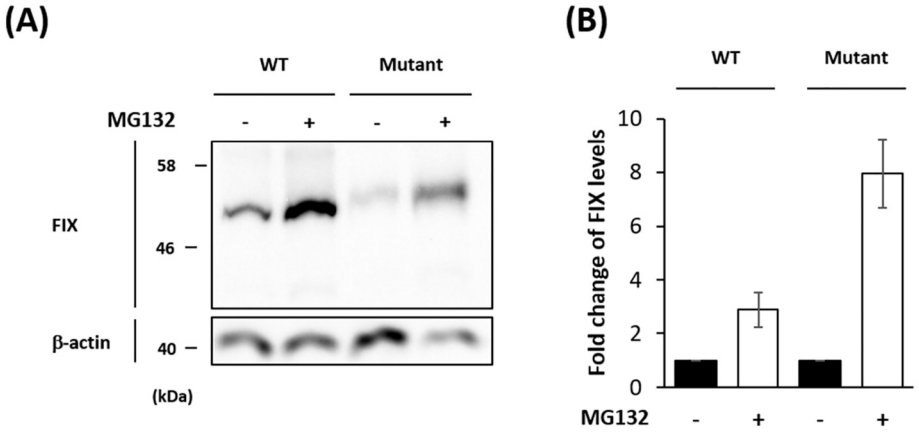

Fig. 5. Effects of proteasome inhibitor, MG132, on FIX expression in HepG2 cells.
(A) Western blot analysis of the intracellular FIXs under MG132 treatment. Beta-actin was considered as the loading control. (B) Intracellular WT and in-frame mutant FIX levels in cells under MG132 treatment were quantified by western blot analysis. Band intensity was normalized to beta-actin. Data were achieved from three independent experiments. Error bar reflects S.E.M.

and mutant-FIX transfectants (Fig. 5A), and increased the intracellular FIX of WT FIX by 2.89 ± 0.66 fold and that of mutant FIX by 7.95 ± 1.29 fold, respectively (Fig. 5B). These results showed that in-frame mutant FIX along with WT FIX was intracellularly degraded via proteasome-dependent degradation machinery.

4. Discussion

In the present study, we demonstrated the molecular pathogenesis of *F9* c.87A > G mutation. The *F9* c.87A > G affected the splicing of *F9* mRNA by disrupting the 5′ss consensus sequence at exon 1/intron 1

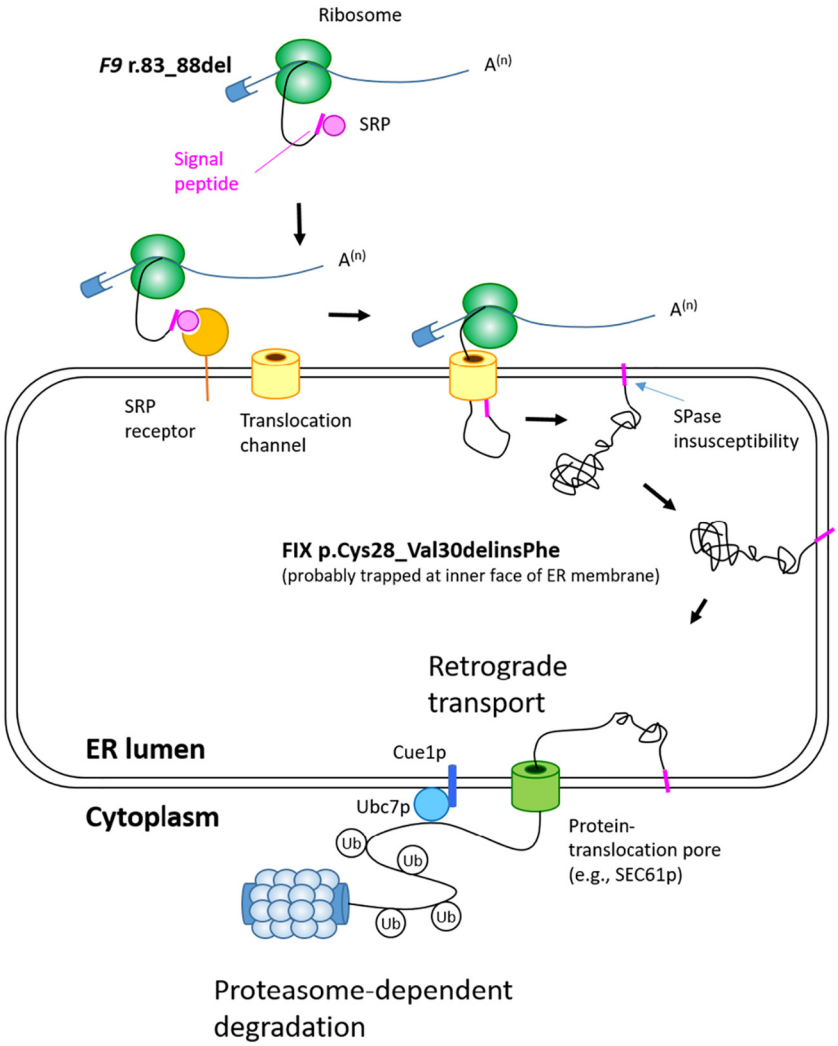

Fig. 6. Illustration of the hypothetical mechanism by which the in-frame mutant FIX is intracellularly eliminated via the ER-associated degradation (ERAD) system.
In general, signal peptide is recognized by signal recognition particle (SRP)/SRP receptor, which anchors protein to ER membrane. Anchored protein is transferred into ER lumen through a translocation channel. After protein biosynthesis, mature part of the protein is released by signal peptide cleavage mediated by SPase. If protein is not cleaved by SPase because of abnormal structure or mutation at the signal peptide, the abnormal protein is trapped by the ER inner membrane, followed by elimination from the ER lumen via a retrograde transport system. In the case of the in-frame mutant FIX, as the mutant FIX possesses insusceptibility to SPase, synthesized mutant FIX could be trapped onto the inner face of the ER membrane and translocated back to the cytoplasm. After retrograde transport, the mutant FIX could be ubiquitinated by an ubiquitin-conjugating enzyme located on the cytoplasmic surface of the ER membrane (e.g., Ubc7p/Cue1p complex) and subsequently degraded via proteasome.

boundary, and induced aberrant mRNA splicing via an aberrant 5′ss. The aberrant transcript *F9* r.83_88del encoded the in-frame mutant FIX p.Cys28_Val30delinsPhe, a mutant at the signal peptide cleavage site. The in-frame mutant FIX was intracellularly retained in the ER, resulting in the failure of extracellular secretion. The abnormally accumulated mutant FIX was excluded by a proteasome-dependent protein degradation system.

F9 c.87A > G is a single nucleotide substitution at the 5′ss consensus sequence at the exon 1/intron 1 boundary (Fig. 1A). The consensus sequence (AG/GTRAGT: exon/intron, R = purine base) universally conserves GT dinucleotides and the other 6 nucleotides affect the activity of donor site [14]. The WT *F9* exon 1/intron 1 boundary matches six nucleotides to the consensus sequence (AG/GTttGT, mismatched nucleotides with the consensus sequence are indicated by lower case letters). *F9* c.87A > G is a mutation of the first nucleotide in the sequence (gG/GTttGT, mutated nucleotides are indicated by letters in square), creating an effective aberrant 5′ss (At/GTAcGg). Moreover, the mutation-based aberrant 5′ss could be more accessible with spliceosomes (5′ss is recognized by U1 small nuclear RNA in spliceosomes [15]) (Tables 1).

The aberrant transcript *F9* r.83_88del is translated into a deleterious FIX, p.Cys28_Val30delinsPhe, which is an in-frame mutation at the signal peptide cleavage site. Signal peptide generally has tripartite regions: a central hydrophobic core region (h-region), a positively-charged N-terminal flanking region (n-region), and a polar C-terminal flanking region (c-region) [16]. Small uncharged amino acids located at position −1 and −3 of the cleavage site in the c-region are important for signal peptide cleavage by signal peptidase (SPase). Moreover, large, aromatic, and charged or polar amino acids are commonly not observed at the immediate flanking region of cleavage site. In the present study, the in-frame mutant FIX lacked 3 residues including a cysteine at position −1, and inserted a newly phenylalanine, which resulted in insusceptibility to SPase.

Impaired signal peptide cleavage causes abnormal intracellular protein trafficking, which mostly retains the mutant protein in the ER. For instance, FX Santo Domingo (FX p.Gly21Arg), a deleterious mutation in the signal peptide at position −3 from the cleavage site, has been shown to exhibit ER retention and extracellular secretion defect [17]. ER retention mutant proteins are eliminated via a proteolytic system called ER-associated degradation (ERAD) or simply ER degradation [18]. In soluble proteins, mutant proteins either trapped on the inner surface of ER membrane or residing in the ER lumen are recognized by an ER quality control system and transported back to the cytoplasm, which is referred to as the retrograde transport. Retrograde transport proteins are ubiquitinated and degraded via a proteasome-dependent degradation machinery. In the present case, the in-frame mutant FIX p.Cys28_Val30delinsPhe was retained in the ER and intracellularly degraded in a proteasome-dependent manner (Fig. 3D and 5). We considered that the in-frame mutant FIX was eliminated through an ERAD system due to abnormal ER retention associated with uncleaved signal peptide (Fig. 6).

The patient carrying *F9* c.87A > G was diagnosed as having

moderate HB (FIX:C = 2.5 IU/dL). The moderate phenotype could be explained by the frequency of splice variant of *F9* c.87A > G. Although abundant transcripts were spliced to an abnormal pattern associated with the aberrant 5′ss, approximately 1% of transcripts were processed into normal patterns using authentic 5′ss. The *F9* mRNA with a normal splicing pattern should be able to undergo normal protein processing and extracellular secretion, resulting in the formation of properly circulating FIX.

5. Conclusion

F9 c.87A > G is a single-base substitution mutation, causing an aberrant splicing pattern by making an aberrant 5′ss available instead of the original 5′ss. The nomenclature at RNA and protein levels of *F9* c.87A > G should be described as *F9* r.[87A > G, 83_88del] and p.[Thr29=, Cys28_Val30delinsPhe], respectively. Additionally, FIX p.Cys28_Val30delinsPhe is an in-frame mutant at the signal peptide cleavage site, which is abnormally retained in the ER and is intracellularly disposed via a proteasome-dependent protein degradation system. However, the silent mutant FIX p.Thr29 = which is translated from *F9* r.87A > G can contribute to blood coagulation as a proper FIX and explain the moderate phenotype of the patient.

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Authors' contribution

K. O. and S. T. designed and performed the research, analyzed data, and drafted the manuscript. N. S. designed the project and collected and analyzed the clinical data. M. K., Y. H., M. T., S. S., performed the research and analyzed data. A.T. developed and supervised the project. A.K., F. H., S. O., A. S., T. Kanematsu., and T. M. developed the project and collected and analyzed the clinical data. T. Kojima designed the project, analyzed data, and drafted the manuscript.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

References

[1] S. Yoshitake, B.G. Schach, D.C. Foster, E.W. Davie, K. Kurachi, Nucleotide sequence of the gene for human factor IX (antihemophilic factor B), Biochemistry 24 (1985) 3736–3750.

[2] F. Giannelli, P.M. Green, S.S. Sommer, M.C. Poon, M. Ludwig, A. Schwaab, P.H. Reitsma, M. Goossens, A. Yoshioka, M.S. Figueiredo, G.G. Brownlee, Haemophilia B: database of point mutations and short additions and deletions - eighth edition, Nucleic Acids Res. 26 (1998) 265–268, https://doi.org/10.1093/nar/26.1.265.

[3] F. Peyvandi, I. Garagiola, G. Young, The past and future of haemophilia: diagnosis, treatments, and its complications, Lancet 388 (2016) 187–197, https://doi.org/10.1016/s0140-6736(15)01123-x.

[4] Z.E. Sauna, C. Kimchi-Sarfaty, Understanding the contribution of synonymous mutations to human disease, Nat. Rev. Genet. 12 (2011) 683–691, https://doi.org/10.1038/nrg3051.

[5] A.C. Goodeve, HemophiliaB: molecular pathogenesis and mutation analysis, J. Thromb. Haemost. 13 (2015) 1184–1195, https://doi.org/10.1111/jth.12958.

[6] S. Saad, G. Rowley, L. Tagliavacca, P.M. Green, F. Giannelli, C.R. Rizza, P. Glangrande, P.B.A. Kernoff, C. Lee, E. Goldman, A. McGraw, B.T. Colvin, G. Lucas, M. Layton, R.S. Mibashan, F.E. Preston, M. Makris, V.E. Mitchell, G.D.O. Lowe, G.F. Savidge, B.J. Hunt, T. Baglin, A. Green, D.N. Whitmore, J.M. Dudley, D. Bevan, G.L. Scott, G.R. Standen, E.E. Mayne, P. Winter, F.G.H. Hill, J.T. Wilde, D.R. Prangnell, P. Jones, G. Dolan, R.F. Stevens, M. Super, A. Aronstam, M. Wassef, A.L. Bloom, D.J. Bowen, D.A. Montgomery, P. Boltonmaggs, J. Hayes, C.A. Ludlam, B. Gibson, I.M. Hann, D.M. Swirsky, C.N. Simpson, N.J. Dodd, G. Taylor, D.S. Thompson, A. Copplestone, M. Chisholm, A. Worsley, C. Costello, R. Lee, J. Tolmie, M.W. McEvoy, M. Rowlands, M. Winter, J.S. Lilleyman, N. Oconnor, J. Behrens, A. Oakhill, H.M. Daly, D.J. Perry, First report on UK database of haemophilia B mutations and pedigrees, Thromb. Haemost. 71 (1994) 563–570.

[7] D. Belvini, R. Salviato, P. Radossi, F. Pierobon, P. Mori, G. Castaldo, G. Tagariello, grp AHs, Molecular genotyping of the Italian cohort of patients with hemophilia B, Haematologica 90 (2005) 635–642.

[8] C.H. Miller, J. Benson, D. Ellingsen, J. Driggers, A. Payne, F.M. Kelly, J.M. Soucie, W.C. Hooper, Hemophilia Inhibitor Res Study I. F8 and F9 mutations in US haemophilia patients: correlation with history of inhibitor and race/ethnicity, Haemophilia 18 (2012) 375–382, https://doi.org/10.1111/j.1365-2516.2011.02700.x.

[9] T. Kojima, M. Tanimoto, T. Kamiya, Y. Obata, T. Takahashi, R. Ohno, K. Kurachi, H. Saito, Possible absence of common polymorphisms in coagulation factor IX gene in Japanese subjects, Blood 69 (1987) 349–352.

[10] N. Enjolras, M.H. Rodriguez, J.L. Plantier, O. Attali, C. Negrier, The three in-frame ATG, clustered in the translation initiation sequence of human factor IX gene, are required for an optimal protein production, Thromb. Haemost. 82 (1999) 1264–1269.

[11] A. Suzuki, N. Sanda, Y. Miyawaki, Y. Fujimori, T. Yamada, A. Takagi, T. Murate, H. Saito, T. Kojima, Down-regulation of PROS1 gene expression by 17 beta-estradiol via estrogen receptor alpha (ER alpha)-Sp1 interaction recruiting receptor-interacting protein 140 and the corepressor-HDAC3 complex, J. Biol. Chem. 285 (2010) 13444–13453, https://doi.org/10.1074/jbc.M109.062430.

[12] Y. Nakamura, M. Murata, Y. Takagi, T. Kozuka, Y. Nakata, R. Hasebe, A. Takagi, J. Kitazawa, M. Shima, T. Kojima, SVA retrotransposition in exon 6 of the coagulation factor IX gene causing severe hemophilia B, Int. J. Hematol. 102 (2015) 134–139, https://doi.org/10.1007/s12185-015-1765-5.

[13] Acetone precipitation of proteins, Tech Tip #49, https://tools.thermofisher.com/content/sfs/brochures/TR0049-Acetone-precipitation.pdf?CID=Social_LAB.

[14] R.P. Ketterling, J.B. Drost, W.A. Scaringe, D.Z. Liao, J.Z. Liu, C.K. Kasper, S.S. Sommer, Reported in vivo splice-site mutations in the factor IX gene: severity of splicing defects and a hypothesis for predicting deleterious splice donor mutations, Hum. Mutat. 13 (1999) 221–231, https://doi.org/10.1002/(sici)1098-1004(1999)13:3<221::aid-humu6>3.3.co;2-l.

[15] Y. Lee, D.C. Rio, Mechanisms and regulation of alternative pre-mRNA splicing, in: R.D. Kornberg (Ed.), Annual Review of Biochemistry, vol 84, Annual Reviews, Palo Alto, 2015, pp. 291–323.

[16] B. Martoglio, B. Dobberstein, Signal sequences: more than just greasy peptides, Trends Cell Biol. 8 (1998) 410–415, https://doi.org/10.1016/s0962-8924(98)01360-9.

[17] M. Racchi, H.H. Watzke, K.A. High, M.O. Lively, Human coagulation factor X deficiency caused by a mutant signal peptide that blocks cleavage by signal peptidase but not targeting and translocation to the endoplasmic reticulum, J. Biol. Chem. 268 (1993) 5735–5740.

[18] R.K. Plemper, D.H. Wolf, Retrograde protein translocation: ERADication of secretory proteins in health and disease, Trends Biochem. Sci. 24 (1999) 266–270, https://doi.org/10.1016/s0968-0004(99)01420-6.