Identification of candidate nonsense mutations of FVIII for ribosomal readthrough therapy

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MATERIALS AND METHODS

Cell lines and culture conditions

HEK293T and HEK293 cells were maintained in Dulbecco Modified Eagle Medium supplemented with 10% FBS at 37 ºC and 5% CO_2 culture conditions. Both cell lines tested negative upon routine mycoplasma testing with the Universal Mycoplasma Detection Kit (ATCC).

Plasmids

Human *F8* cDNA with B domain deletion and the full-length (FL) human *F9* cDNA were fused in-frame with the mature humanized Gaussia luciferase (*Gluc*) cDNA, separated by a short linker sequence (Figure 1A). The resulting fusion genes were cloned into the pMSCV plasmid. Nonsense mutations were introduced into the wild-type plasmids using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). All expression constructs were validated by Sanger DNA sequencing to confirm the presence of the mutation and the absence of unintended changes. To construct FL-FVIII nonsense mutant constructs, mutations were introduced into the pUC19 vectors containing one of three fragments of FVIII (XhoI - KpnI, KpnI - EcoRV, or EcoRV – SalI.

XhoI and SalI are restriction sites used for cloning the FL-FVIII cDNA into pMT2), depending on the location of the mutation. The DNA fragment containing the mutation was sequenced to confirm the presence of the mutation and the absence of unintended mutations, excised from the mutagenized plasmid, and used to replace the same fragment in pMT2-FVIII.[1]

Stable expression cells constructed by retroviral gene transfer

HEK293T cells plated on 60-mm dishes were transfected with a pMSCV-based retroviral expression plasmid (2 μg), together with the pGag-pol (2 μg) and the pVSV-G (0.7 μg) plasmids using FuGENE6 (Promega). Two days after transfection, virus-containing media were harvested and used to infect HEK293 cells in the presence of polybrene (8 μg/mL). The infected cells were selected with puromycin (2 μg/mL) for at least 10 days.

Drug challenge and Gaussia luciferase assay

For cells stably expressing BDD-Gluc and FIX-Gluc fusions, HEK293 cells (2.5 x10^5 cells/mL) were seeded into 24-well plates as triplicates. After 16 hr, spent media were replaced with fresh media containing drugs or DMSO and cultured for additional 48 hr. Sample media (20 μl) were transferred to black-welled 96-well plates and assayed for Gluc activity with 50 μl of 10 μM coelenterazine in 0.1 M Tris (pH 7.4) and 0.3 M sodium ascorbate in a LMAX II384 illuminometer.

Evaluation of FVIII activity and antigen levels

Activities of secreted recombinant FVIII variants (BDD-Gluc or FL-FVIII) after nonsense

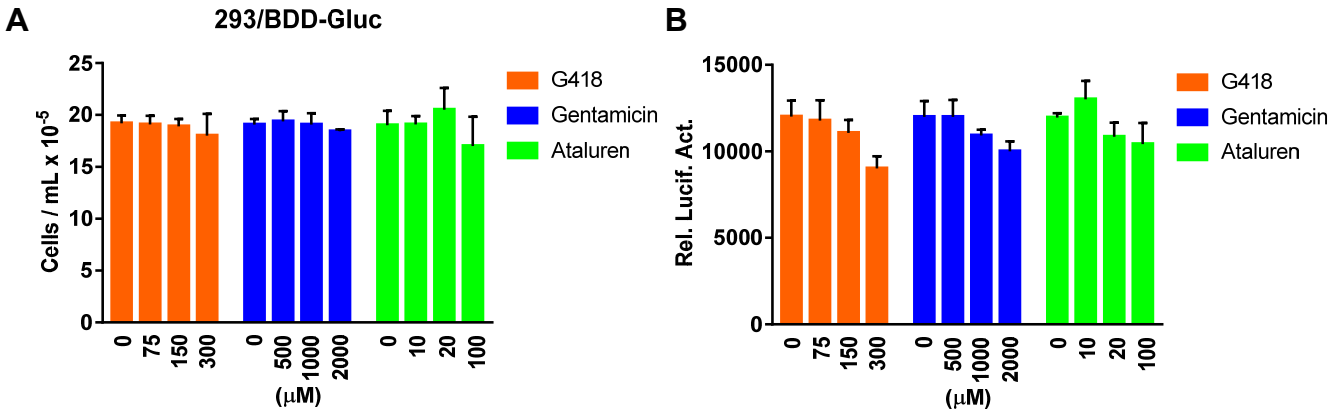
readthrough treatments were measured by the Coatest® SP4 FVIII kit (Diapharma) as described.[2] For cells stably expressing BDD-Gluc fusions, media collected for Gaussia luciferase assay ware also used for FVIII activity assay. For cells transiently expressing FL-FVIII mutants, 18 hour after transfection with FuGENE6, cells were cultured in fresh media containing G418 or DMSO for another 48 hr. Media were collected, stored in -80 ºC for FVIII activity and antigen assays. Antigen levels of the secreted BDD, BDD-Gluc fusion and FL-FVIII proteins were evaluated by the VisuLize FVIII ELISA kit (Affinity Biologicals, Ancaster, Ontario, Canada) as described.[2]

SUPPLEMENTAL REFERENCES

1. Pipe SW, Eickhorst AN, McKinley SH, Saenko EL, Kaufman RJ. Mild hemophilia A caused by increased rate of factor VIII A2 subunit dissociation: evidence for nonproteolytic inactivation of factor VIIIa in vivo. *Blood*. 1999;93(1):176-183.

2. Wei W, Misra S, Cannon MV, et al. Molecular mechanisms of missense mutations that generate ectopic N-glycosylation sites in coagulation factor VIII. *Biochem J*. 2018;475(5):873-886.

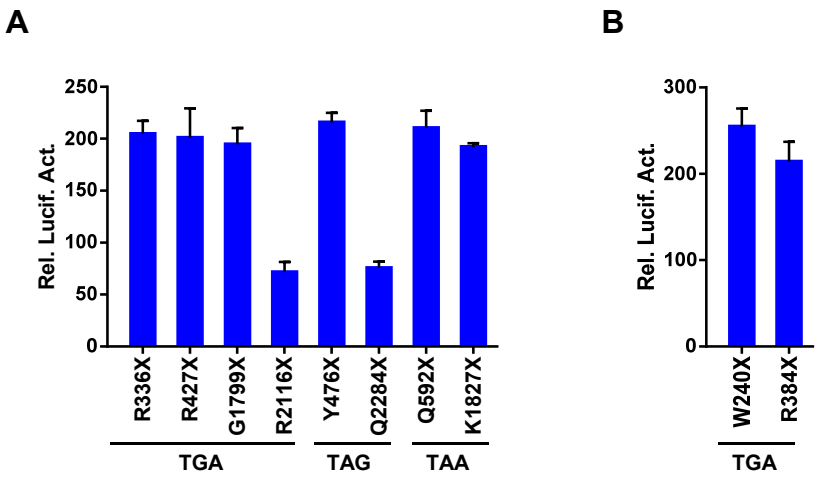
Supplementary Figure S1



Supplemental Figure S1. Gaussia luciferase fusion did not significantly increase or impair the activity or secretion of BDD-FVIII.

HEK293 cells stably expressing the WT BDD-Gluc fusion protein were treated with the indicated concentrations of drugs for 48 hr. Viable cell numbers were determined by trypan blue staining (A). Fusion protein levels in the media were determined by a Gaussia luciferase activity assay (B).

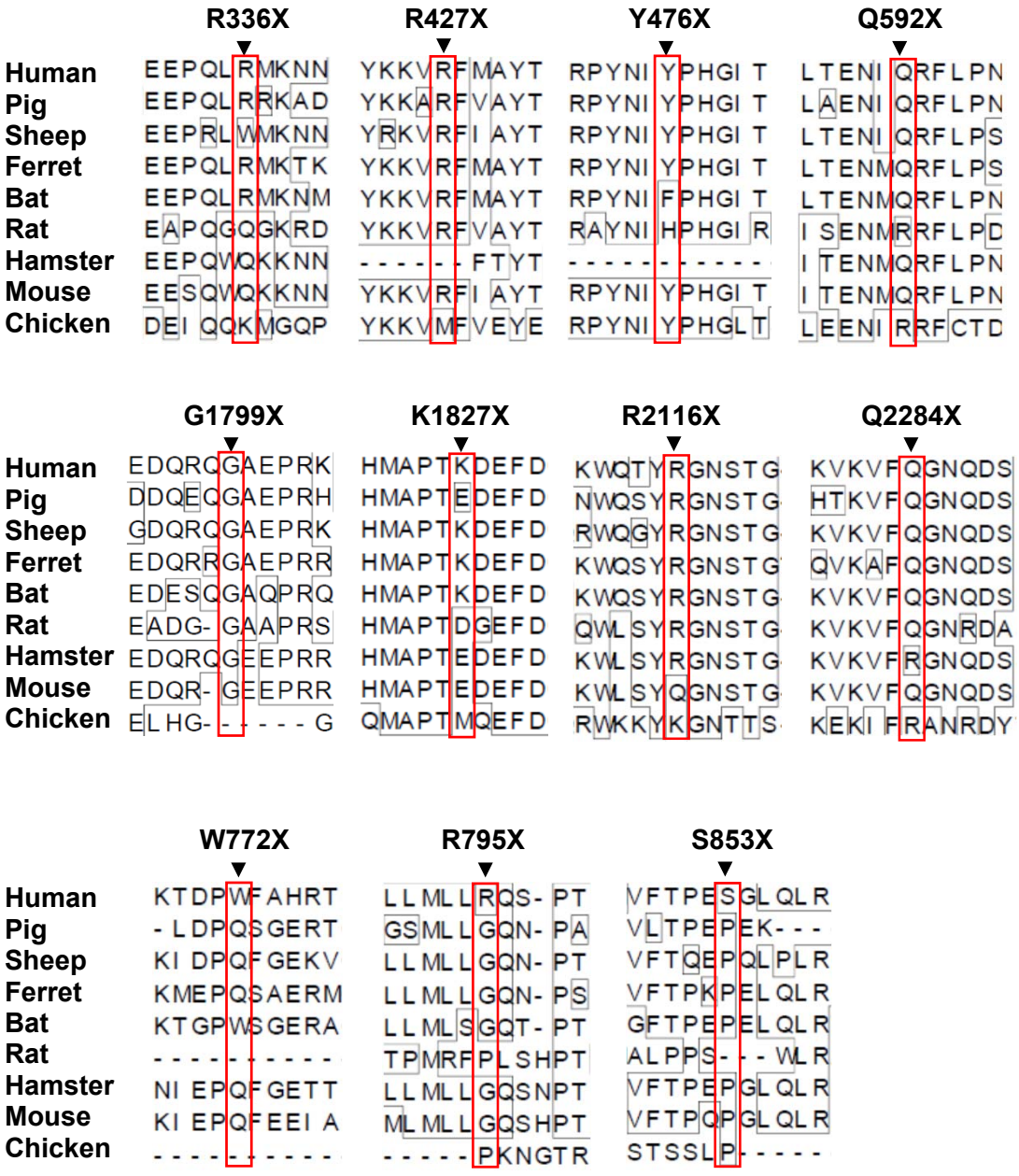
Supplementary Figure S2



Supplemental Figure 2. Background readthrough levels of FVIII nonsense mutants tested.

Relative luciferase activities in conditioned media of HEK293 cells stably expressing the FVIII BDD-Gluc (A) and the FIX-Gluc (B) fusion proteins with the indicated nonsense mutations without G418 treatment.

Supplementary Figure S3



Supplemental Figure S3. Alignment of amino acid sequences flanking *F8* nonsense mutations.

Alignment of FVIII protein sequences of the indicated species was performed with the Clustal V method using the DNASTAR Lasergene MegaAlign program. Amino acid residues that are mutated to PTCs in human are marked with arrowheads. Residues that are aligned with human PTCs are boxed in red rectangles.