The impact of aberrant von Willebrand factor-GPIbα interaction on megakaryopoiesis and platelets in humanized type 2B von Willebrand disease model mouse

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Received: December 22, 2021.
Accepted: February 2, 2022.
Prepublished: February 10, 2022.

https://doi.org/10.3324/haematol.2021.280561

Supplemental Methods

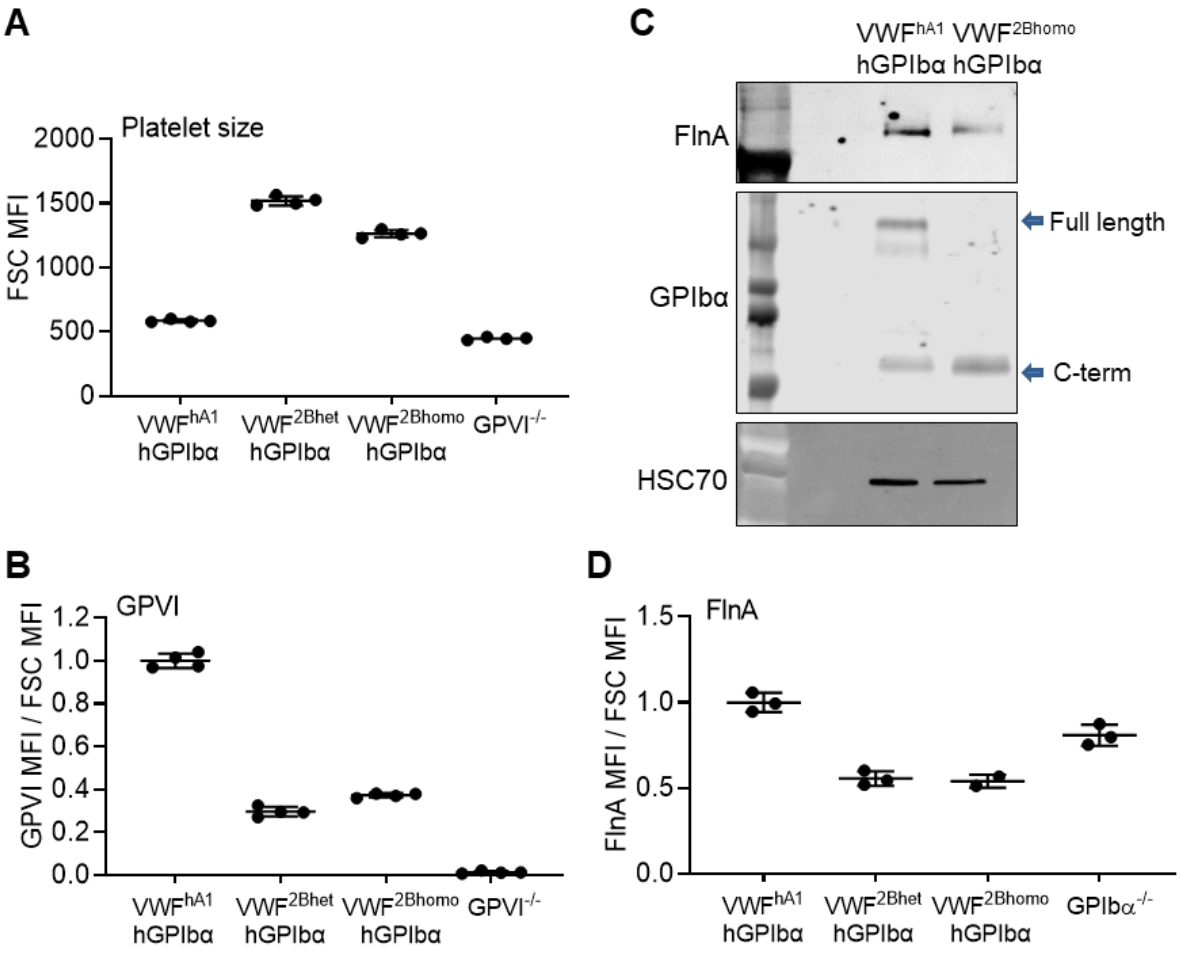
Mouse BM MK analysis. BM cells collected from mouse femurs were suspended in PBS containing 0.5% BSA and 2 mM EDTA and fixed in 1% paraformaldehyde at 4° C for 2 hours.[1] After washing, cells were treated with Fc block at room temperature for 10 minutes, then washed and resuspended in PBS, incubated for 10 minutes at room temperature with Brilliant Violet 421 anti-mouse CD41 antibody (Biolegend, San Diego, CA), propidium iodide (Sigma-Aldrich, St. Louis, MO) in the presence of 50μg/mL RNase A (Qiagen, Germantown, MD) for 30 minutes at room temperature. After staining, cells were analyzed by NovoCyte (ACEA Biosciences, San Diego, CA).

Histology. Cryosections were prepared as previously described,[2-4] fixed, stained, and visualized using a fluorescence microscope (BZ-X700; Keyence) or confocal microscope (LSM 880; Carl Zeiss). In brief, the harvested organs were snap-frozen and cryosectioned using modified Kawamoto's method. After fixation with 4% paraformaldehyde, samples were blocked and permeabilized using 1% Triton X-100 PHEM buffer containing 5% normal goat serum and FcR blocker (INNOVEX bioscience, Richmond, CA), then stained with antibodies followed by fluorescent-labeled secondary antibodies. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI).

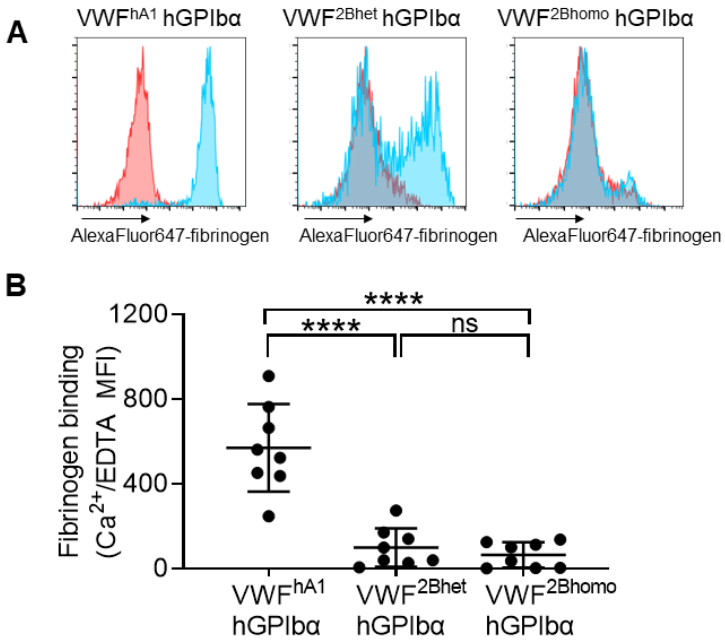
Tail bleeding time assay. For tail bleeding time assay, mice were anesthetized using isoflurane in a precision vaporizer.[5] After clipping 3 mm of the distal tip with a sterile scalpel blade, the tail was immersed into isotonic saline at 37° C and the time to complete cessation of blood flow was recorded as the bleeding time. After 600 seconds, persistent hemorrhage was stopped by cauterizing the tail wound.

Statistical analysis. The statistical analyses were performed using Prism (GraphPad Software, La Jolla, CA). The details of each analysis are specified in the figure legends.

Supplemental Figures and Legends

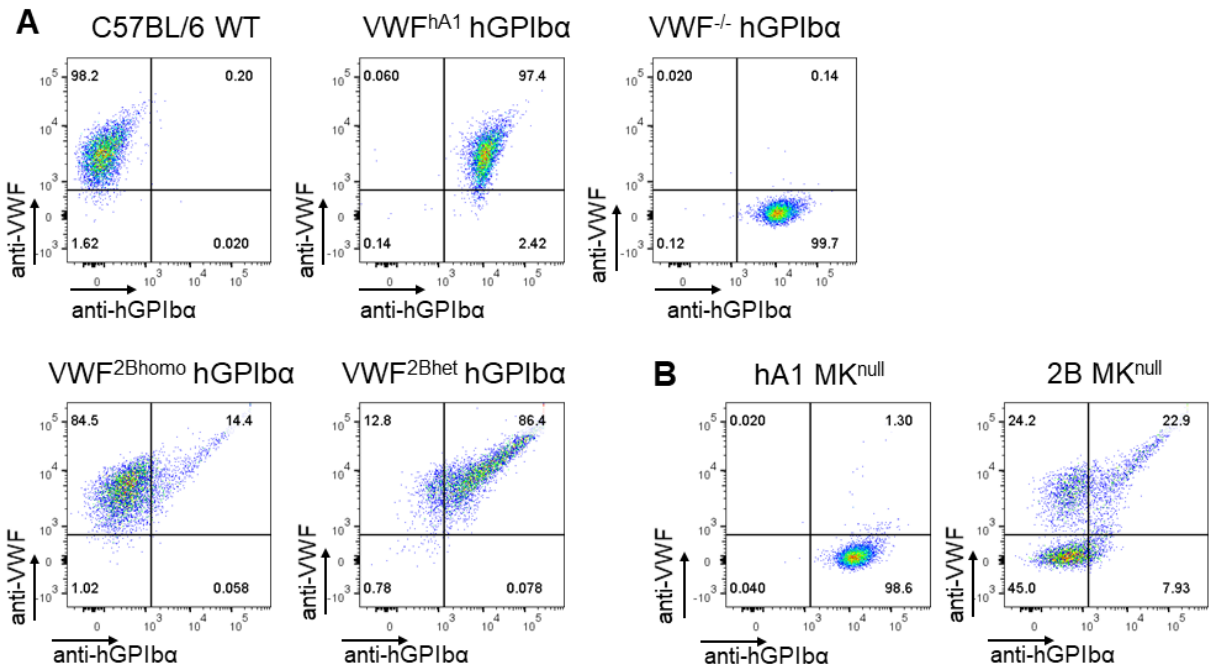


Supplemental Figure 1. Analysis of platelet activation status in type 2B VWD mouse models. (A, B) Whole blood samples collected from each mouse strain were analyzed by flow cytometry. Platelets were gated by staining with Brilliant Violet 421 anti-mouse integrin αIIb antibody (MWReg30) and the expression of GPVI was analyzed by staining with FITC labeled anti-mouse GPVI antibody (JAQ1). Platelet sizes are shown by mean fluorescence intensity (MFI) of forward side scatter (FSC). Due to varied platelet size among mouse strains, MFI of GPVI signal was corrected by that of FSC. (**C**) Platelet lysates were prepared from blood collected from each mouse strain and analyzed by SDS-PAGE, followed by Western Blotting using antibodies against ani-filamin A (FlnA), GPIbα, and HSC70. (**D**) Blood samples collected from each mouse strain were fixed, permeabilized, and stained for intracellular content of FlnA. Data were shown as scatter plots with mean ± SD. n = 4 in (A) and (B), n = 3 in (D).



Supplemental Figure 2. Functional analysis of platelets in response to agonist stimulation.

(A) Blood samples collected from each mouse strain were incubated with were diluted in modified Tyrode's buffer, pH 7.4 containing 2 mM EDTA or 0.2 mM Ca^{2+} and incubated with AlexaFluor 647-labeled fibrinogen in the presence of 100 µM PAR4-AP. After incubation at room temperature for 10 minutes, samples were stained with PE-Cy7 labeled anti-mouse integrin αIIb antibody (MWReg30) and analyzed by flow cytometry. Typical profiles of gated platelet population analyzed for AlexaFluor 647-labeled fibrinogen binding in the presence of 0.2 mM Ca^{2+} (blue) or 2 mM EDTA (red, negative control) are shown. **(B)** Binding was reported as the ratio of the median fluorescence intensity (MFI) in the AlexaFluor 647 fluorescence channel of platelets stimulated in the presence of 0.2 mM Ca^{2+} divided by the value obtained in the presence of 2 mM EDTA (negative control). Data are shown as scatter plots with mean ± SD and were analyzed by one-way ANOVA with Tukey's multiple comparisons test.



Supplemental Figure 3. Flow cytometry analysis of intracellular VWF in platelets. (**A, B**) Blood samples collected from each mouse strain were fixed, permeabilized, and stained with the antibodies against Brilliant Violet 421 anti-mouse integrin αIIb antibody (MWReg30), AlexaFluor 488 labeled anti-hGPIbα (LJ-P3), and AlexaFluor 647 labeled anti-VWF polyclonal antibodies.

References

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