

ORIGINAL ARTICLE

A mouse model of the protease-activated receptor 4 Pro310Leu variant has reduced platelet reactivity

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Abstract

Background: Protease-activated receptor 4 (PAR4) mediates thrombin signaling on platelets and other cells. Our recent structural studies demonstrated that a single nucleotide polymorphism in extracellular loop 3 and PAR4-P310L (rs2227376) leads to a hyporeactive receptor.

Objectives: The goal of this study was to determine how the hyporeactive PAR4 variant in extracellular loop 3 impacts platelet function *in vivo* using a novel knock-in mouse model (PAR4-322L).

Methods: A point mutation was introduced into the PAR4 gene *F2rl3* via CRISPR/Cas9 to create PAR4-P322L, the mouse homolog to human PAR4-P310L. Platelet response to PAR4 activation peptide (AYPGKF), thrombin, ADP, and convulxin was monitored by α IIb β 3 integrin activation and P-selectin translocation using flow cytometry or platelet aggregation. *In vivo* responses were determined by the tail bleeding assay and the ferric chloride-induced carotid artery injury model.

Results: PAR4-P/L and PAR4-L/L platelets had a reduced response to AYPGKF and thrombin measured by P-selectin translocation or α IIb β 3 activation. The response to ADP and convulxin was unchanged among genotypes. In addition, both PAR4-P/L and PAR4-L/L platelets showed a reduced response to thrombin in aggregation studies. There was an increase in the tail bleeding time for PAR4-L/L mice. The PAR4-P/L and PAR4-L/L mice both showed an extended time to arterial thrombosis.

Conclusion: PAR4-322L significantly reduced platelet responsiveness to AYPGKF and thrombin, which is in agreement with our previous structural and cell signaling studies. In addition, PAR4-322L had prolonged arterial thrombosis time. Our mouse model provides a foundation to further evaluate the role of PAR4 in other pathophysiological contexts.

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Xu Han and Elizabeth A. Knauss are considered as co-first authors and contributed equally to this study.

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KEYWORDS

animal model, blood platelets, protease-activated receptor 4, single nucleotide polymorphisms, thrombin receptor

1 | INTRODUCTION

Platelets play a pivotal role in primary hemostasis, thrombosis, inflammation, and vascular biology. These anuclear discoid cells circulate in the bloodstream to patrol the integrity of the vascular system [1]. Upon injury, platelets quickly activate, change shape, release granule contents, and aggregate to form the hemostatic plug in the presence of fibrinogen. Platelet activation can be triggered by many physiological agonists, including thrombin, the most potent platelet agonist and a key protease in coagulation [2,3]. Thrombin signals through 2 protease-activated receptors (PARs), PAR1 and PAR4, on the surface of human platelets [4]. PARs belong to the GPCR superfamily and have a unique activation mechanism whereby the N-terminus is enzymatically cleaved to unmask the tethered ligand [5]. The new N-terminus interacts with the endogenous ligand-binding site to induce a global structural rearrangement that activates downstream signaling [6,7]. PAR1 and PAR4 both signal through $G\alpha_q$ and $G\alpha_{12/13}$, however with different kinetics [8]. PAR1 leads to rapid signaling that is quickly dissipated, whereas PAR4 leads to prolonged signaling [4,9,10]. This sustained signaling associated with PAR4 activation is essential for thrombosis, highlighting PAR4 as a promising target for antiplatelet therapies [11–13].

Over the past 10 years, platelet thrombin receptors have been appealing targets for antiplatelet therapies, which led to the first-in-class FDA-approved PAR1 inhibitor vorapaxar. However, targeting PAR1 comes with a significant risk of bleeding, which outweighs its clinical benefits in preventing cardiovascular events [14,15]. In recent years, PAR4 has become a rising star as a safer antiplatelet and antithrombotic target for a number of reasons. First, targeting PAR4 signaling without inhibiting PAR1 allows platelets to continue to respond to low levels of thrombin and preserves normal hemostasis [12]. Second, pharmacologic inhibition of PAR4 prevents thrombin-mediated PAR4 activation at high concentrations that are associated with pathologic thrombosis. Third, since the prolonged signaling mediated by PAR4 activation is associated with factor V release from α -granules and microparticle generation [16], selectively inhibiting PAR4 would not only prevent thrombus formation but also reduce platelet procoagulant activity [11]. Collectively, this has culminated in the development of a number of PAR4 antagonists in the form of pepducins, small-molecule compounds, and function-blocking antibodies [17]. Two small-molecule PAR4 inhibitors from Bristol Myers Squibb, BMS-986120 and BMS-986141, were the subject of clinical trials and proved to be efficient in preventing cardiovascular events with a good safety profile [12,13,18]. PAR4's unique properties have made it an attractive therapeutic target to prevent thrombosis

without hindering normal hemostasis. Therefore, it is essential that we fully understand the mechanisms underlying PAR4 signaling.

The tethered ligand mechanism was proposed in 1991 by Vu et al. [5]; however, the molecular mechanism of receptor activation is only recently understood. Recently, we used amide hydrogen/deuterium exchange (H/D exchange) mass spectrometry with purified full-length PAR4 to examine the conformational dynamics of the tethered ligand mechanism following activation by thrombin [19]. This study revealed that PAR4 activation requires a coordinated rearrangement of extracellular loop 3 (ECL3) and threonine at position 153 in the ligand-binding site formed by transmembrane domain 3 (TM3) and TM7. Within ECL3, there is a single nucleotide polymorphism (SNP) in which the proline at 310 is replaced with a leucine (PAR4-310P/L, rs2227376). This natural sequence variant of PAR4 had significantly lower receptor reactivity, as measured by calcium mobilization in HEK293 cells. Natural sequence variants of PAR4 (eg, rs773902 [PAR4-120A/T] and rs2227346 [PAR4-296F/V]) affect the receptor reactivity and subsequent platelet function [20–23]. Therefore, we hypothesize that the hyporeactive PAR4-P310L variant would reduce platelet responsiveness to thrombin stimulation.

To test the impact of the PAR4-310P/L polymorphism *in vivo*, we used CRISPR/Cas9 to introduce a point mutation into PAR4 to generate the mouse homolog of this variant, PAR4-P322L. PAR4-P322L significantly reduced platelet responsiveness to PAR4 activation peptide (PAR4-AP; AYPGKF) and thrombin, while ADP and GPVI signaling were not affected. Further, platelet aggregation was dramatically decreased in the platelets from mice that carried one (PAR4^{P/L}, heterozygous) or 2 (PAR4^{L/L}, homozygous) alleles of PAR4-P322L. Additionally, PAR4^{L/L} mice displayed slightly extended tail bleeding compared with wild types. PAR4-P322L also delayed arterial occlusion in the ferric chloride (FeCl₃)-induced carotid artery thrombosis model.

2 | METHODS

2.1 | Materials

Recombinant Cas9 nuclease and single guide RNA (sgRNA) were purchased from PNA Bio. Targeting oligonucleotide was obtained from IDT. Human α -thrombin (catalog #HCT-0020, specific activity greater than 2989 U/mg) was purchased from Haematological Technologies. PAR4-AP AYPGKF-NH₂ was purchased from Tocris Bioscience. Fluorescein isothiocyanate (FITC)-conjugated P-selectin antibody and phycoerythrin-conjugated JON/A antibody were

purchased from Emfret Analytics. All other reagents were from Thermo Fisher Scientific except where noted.

2.2 | Animals

All animal experiments were performed in accordance with the approval from the Case Western Reserve University Animal Ethics Committee. The PAR4-P322L mutation was introduced into the mouse genome using the CRISPR-Cas9 genome-editing system. A guide sequence, 1106/fw (CTATTCAAACCCGAGCCCTG), was validated *in vitro* (sgRNA screening system, Clontech) and obtained from PNAbio. An oligo F2r13 P322L 100-mer: TTTCACACCTAGCAATGTGCTGCTGGTGTGCTGCACTATTCAAACCTGAGCCCTGAAGCCTGGGCAATCTCTATGGAGCCTATGTGCCAGCCTGGCACTC (mutations underlined), designed to mutate P322 and ablate the PAM sequence of 1106/fw with a conservative base change, was obtained from IDT as a polyacrylamide gel electrophoresis-purified ultramer. Mixtures of 100 ng/ μ L Cas9 nuclease, 200 ng/ μ L sgRNA, and 400 ng/ μ L oligo were electroporated into C57BL/6J fertilized oocytes as previously described [24]. Following screening through miSeq by the CWRU Genomics Core, 2 founder mice were then each bred to C57BL/6J mice for further proliferation. The pedigree from each founder was recorded separately. Line 1 was the offspring from the male founder and line 2 was the offspring from the female founder. PAR4 knockout mice (*F2r13*^{-/-}) were purchased from Mutant Mouse Regional Resource Centers.

2.3 | Preparation of murine platelets

Blood was collected from an equal number of male and female mice. For platelet-rich plasma (PRP), blood was collected in sodium citrate, centrifuged at 2300 \times g for 10 seconds, and incubated for 10 minutes at room temperature to obtain PRP. The remaining blood sample was centrifuged again at 2300 \times g for 10 seconds and incubated for 10 minutes at room temperature to obtain more PRP. Platelet concentrations were quantified using a Coulter Counter (Beckman Coulter). Gel-filtered platelets were prepared using Sepharose 2B (Sigma). Columns were packed in H₂O and allowed to stand overnight. Before adding the PRP, 3 volumes of H₂O and 3 volumes of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) Tyrode's buffer (pH 7.4; 10 mM HEPES, 12 mM NaHCO₃, 130 mM NaCl, 5 mM D-glucose, 5 mM KCl, 0.4 mM NaHPO₄, and 1 mM MgCl₂) were passed through the column. The gel-filtered platelet concentrations were quantified using a Coulter Counter.

2.4 | Detecting mouse PAR4 with western blot

Citrated whole blood was collected from PAR4-322 P/P, P/L, L/L, and PAR4-knockout mice. Murine PRP was prepared from the mouse whole blood as described above. After platelets were counted using a

Coulter Counter, equal amounts of platelets (1×10^8) were transferred into a new tube and spun down at 1600 \times g. The pelleted murine platelets were lysed by RIPA buffer (Invitrogen) with protease-inhibitor cocktail (Roche) on ice for 30 minutes. The lysates were further spun down at 21 300 \times g for 20 minutes at 4 °C. The supernatant was transferred to a new tube and mixed with loading dye. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Mouse PAR4 was detected using a goat polyclonal antibody (1 μ g/mL) kindly provided by Dr Steven McKenzie from Thomas Jefferson University. The primary antibody was detected with an anti-goat 800 secondary antibody (0.1 μ g/ml) (LiCor). Total protein loaded was quantified by Revert 700 Total Protein Stain (LiCor) following the commercial protocol [25–27].

2.5 | Flow cytometry

All experiments were analyzed using BD LSRFortessa cell analyzer (BD Biosciences).

Murine PRPs were used to measure the platelet reactivity in response to PAR4-AP (AYPGKF-NH₂), ADP, or convulxin. The activation of platelets was measured by the surface expression of P-selectin using FITC-conjugated P-selectin antibody and the activation of integrin α IIb β 3 was measured using phycoerythrin-conjugated JON/A antibody (Emfret). Specifically, PRP was diluted to 5×10^4 platelets/ μ L with HEPES-Tyrode's buffer (pH = 7.4). Ten microliters of diluted PRP containing 5×10^5 platelets were incubated with 5 μ L FITC-conjugated P-selectin antibody, 5 μ L PE-conjugated JON/A antibody, and 5 μ L agonist for 20 minutes in the dark at room temperature. The negative control was no antibody. The platelets were fixed with 1% formaldehyde prior to analysis.

Gel-filtered murine platelets were used to measure the platelet reactivity in response to thrombin by flow cytometry as described above. Ten microliters of gel-filtered platelets were incubated with 5 μ L FITC-conjugated P-selectin antibody, 5 μ L PE-conjugated JON/A antibody, and 5 μ L agonist for 20 minutes in the dark at room temperature. The cells were then fixed by 1% formaldehyde prior to analysis.

HEK293 Flp-In T-REx cells expressing mouse PAR4 were generated as previously described [19]. Specifically, mouse PAR4-wild type (mPAR4-WT) or mPAR4-P322L, containing an N-terminal V5 epitope, was stably expressed in HEK293 Flp-In T-REx cells following the manufacturer's protocol (Invitrogen). Tetracycline (500 ng/mL) was used to induce expression for 40 hours. The cells were harvested in Versene and washed 3 times with HEPES-Tyrode's buffer (pH 7.4) (10 mM HEPES, 12 mM NaHCO₃, 130 mM NaCl, 5 mM D-glucose, 5 mM KCl, 0.4 mM NaHPO₄, and 1 mM MgCl₂).

To measure the total mouse PAR4 expression, cells were fixed and permeabilized with 4% paraformaldehyde (PFA) for 15 minutes, washed 3 times with HEPES-Tyrode's buffer (pH 7.4), stained with FITC-conjugated anti-V5 antibody for 30 minutes in the dark at room temperature, followed by 3 washes with HEPES-Tyrode's

buffer (pH 7.4) and a 15-minute fixation with 4% PFA. To measure the surface expression of mouse PAR4, cells were directly incubated with FITC-conjugated anti-V5 antibody for 30 minutes, followed by 3 washes with HEPES-Tyrode's buffer (pH 7.4) and a 15-minute fixation with 4% PFA.

2.6 | Platelet aggregation

Gel-filtered murine platelets were used to measure the platelet aggregation in response to thrombin. Platelets were diluted to the final concentration of 5×10^7 platelets/mL in a 300- μ L reaction volume, which contains 295 μ L of gel-filtered platelets and 5 μ L agonist. Platelet aggregation was recorded using the CHRONO-LOG Model 700 Whole Blood/Optical Lumi-Aggregometer paired with AGGRO/LINK 8 program software (Chrono-log Corporation).

2.7 | Tail bleeding time assay

Both male and female mice at 8 weeks of age were used. The mice were anesthetized by a mix of ketamine (100 mg/kg) and xylazine (10 mg/kg) via intraperitoneal injection. The body weight of the mice was recorded prior to the assay. Animals were placed in a prone position on a heater set to 37 °C. Five millimeters of the tail was removed by a razor blade. The tail was then immediately immersed in a 15-mL conical tube containing 15 mL prewarmed 0.9% saline. The tail was laid vertically to the body with the tip of the tail hanging 4 cm below the heating pad and 2 cm immersed in the saline. Each mouse was monitored for 10 minutes and all rebleeding events were recorded. Initial bleeding time was defined as the first time observing the stop of the bleeding regardless of any rebleeding. Total bleeding time was defined as the sum of bleeding times of all bleeding on/off cycles until a stable cessation occurred (no bleeding for 60 seconds). The experiment was terminated at 10 minutes.

2.8 | Arterial thrombosis

FeCl₃-induced carotid artery injury was performed at Marshall University by Dr Wei Li (approved by IACUC #1033528), as previously described [28,29]. Briefly, 8- to 12-week-old mice were anesthetized by a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) via intraperitoneal injection. The right jugular vein was exposed and injected with 100 μ L of rhodamine 6G solution (Sigma 252433-1G, 0.5 mg/mL in saline, 0.2 μ m filtered) to label platelets. The injection site was ligated with a 6-0 suture to prevent bleeding. The left common carotid artery was exposed and freed from surrounding tissues. One small piece of "U"-shaped black plastic was placed under the vessel to separate the carotid artery from the background fluorescence. Saline was applied to the surgical field to keep the carotid artery moist before transferring

the mouse to the attached Gibraltar Platform of a Leica DM6 FS fluorescent microscope. A 10 \times water lens was positioned above the carotid artery to record baseline flow and vessel wall conditions before injury and after injury. Video imaging was performed using a QImaging Retiga R1: 1.4 Megapixel Color CCD camera system with monochrome mode (Teledyne Photometrics) and StreamPix version 7.1 software (Norpix). Filter paper (1 \times 2 mm) saturated with 7.5% FeCl₃ was placed directly onto the carotid artery at the position with the "U"-shaped plastic underneath. After 1 minute, the filter paper was removed, the carotid artery was rinsed with saline, and then saline was reapplied between the lens and vessel. Blood flow was monitored and recorded until full occlusion or 30 minutes after FeCl₃ injury.

3 | RESULTS

3.1 | Generation of PAR4-P322L mice

Previously, we characterized a naturally occurring variant of human PAR4, PAR4-P310L (rs2227376) [19]. Changing proline to leucine at position 310 in ECL3 significantly reduced PAR4-mediated calcium signaling in response to both PAR4-AP (AYPGKF-NH₂) and thrombin in transfected HEK293 cells. PAR4 is highly conserved between human and mouse. Human and mouse PAR4 share 74.4% sequence identity and 82.2% sequence similarity as analyzed by the Pairwise Sequence Alignment tool EMBOSS Needle. ECL3 spans 15 amino acid residues and has 13/15 (87%) identity, and the 310 position in human PAR4 is homologous to the 322 site in mouse PAR4. Mouse PAR4-P322L also had a reduced PAR4-mediated calcium signaling in transfected HEK293 cells (data not shown).

To determine the impact of the PAR4-P310L polymorphism *in vivo*, we developed a mouse model using CRISPR-Cas9 to introduce the P322L mutation into mouse PAR4. The gRNAs were designed to target exon 2 from 1189 base pairs (bps) to 1287 bps of the *F2r13* locus (Figure 1A). This introduced a C > T substitution at 1232 bps, which changed the proline at 322 to a leucine (Figure 1B, C). These mice are referred to as PAR4^{P/P} (wild type), PAR4^{P/L}, or PAR4^{L/L}. The homozygous PAR4-P322L mice PAR4^{L/L} were obtained by breeding the heterozygous PAR4^{P/L} mice. Litters were born in the expected Mendelian inheritance ratios and equally divided into males and females. The platelet counts, mean platelet volume, red cell count, and leukocyte count for the PAR4^{L/L} were all unchanged compared with their PAR4^{P/P} and PAR4^{P/L} littermates. More importantly, the P322L mutation of PAR4 on the ECL3 did not affect protein expression on mouse platelets, validated by western blot (Figure 1D). We next wanted to determine the surface expression of PAR4-L322. However, there are no reliable antibodies directed to mouse PAR4 that can be used for flow cytometry. To circumvent this, we expressed V5-tagged mPAR4-WT and mPAR4-P322L in HEK293 cells and measured total and surface expression using flow cytometry. There is no difference between mPAR4-WT and mPAR4-P322L (Figure 1E).

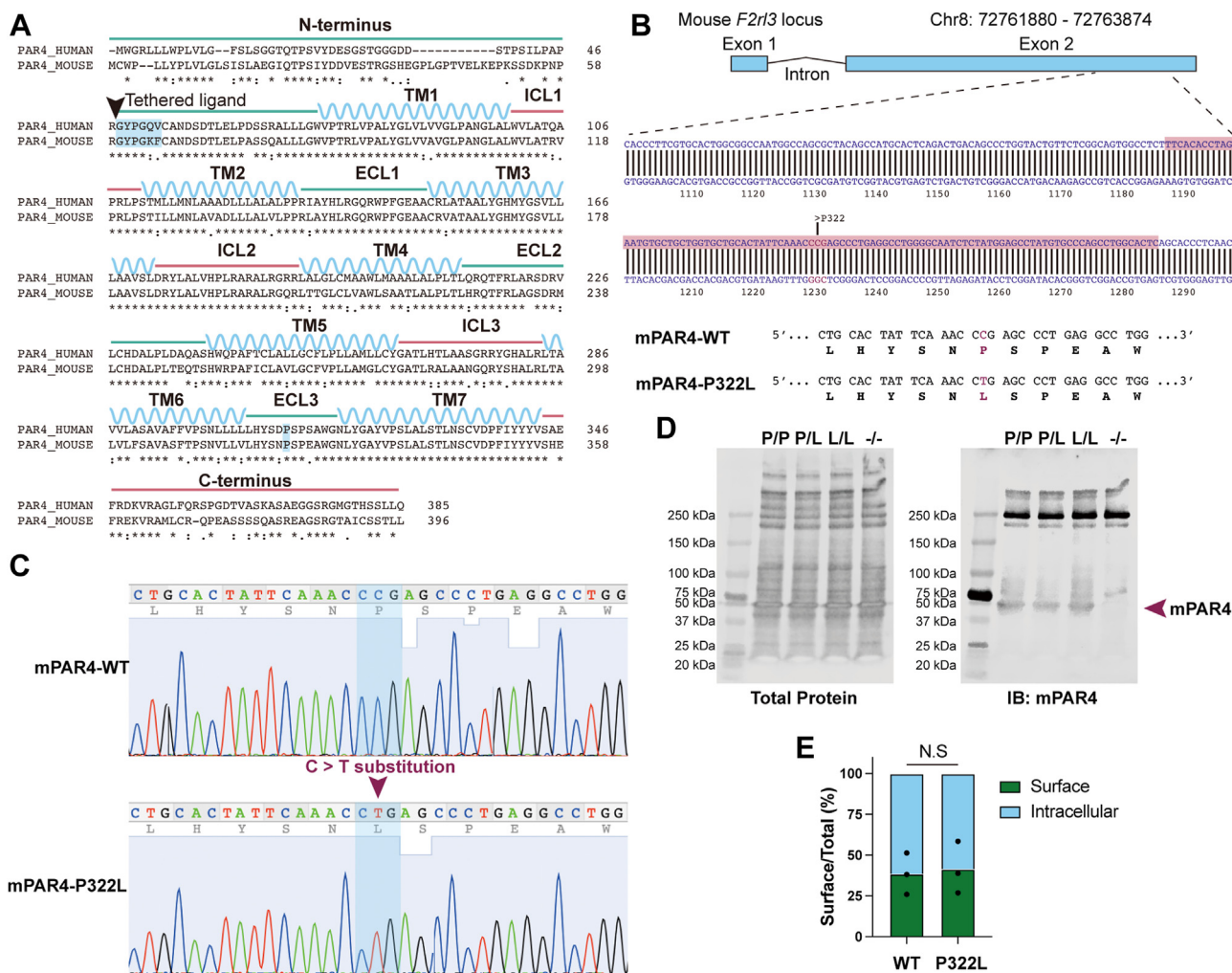


FIGURE 1 Introduction of the C>T substitution in *F2r13* locus. (A) Sequence alignment of human PAR4 and mouse PAR4. P310 at ECL3 (highlighted in blue) in humans is homologous to P322 in mice. (B) gRNAs were targeted against the region of the mouse *F2r13* locus highlighted in red, while the red letters indicate the substitution target site. (C) Sanger sequencing shows the *F2r13* locus around the gRNA target site of a wild-type mouse (top) and a PAR4-P322L homozygous mouse (bottom) in which both alleles contained C>T substitutions. (D) Protein levels of PAR4 were compared across genotypes using an antibody specific for the mouse protein. (E) The total and surface expression levels of mouse PAR4 WT and P322L on HEK293 Flp-In T-REx stable cell lines were compared by flow cytometry using a V5-FITC-conjugated antibody ($n = 3$). FITC, fluorescein isothiocyanate; WT, wild type.

3.2 | PAR4-P322L reduced platelet response to PAR4-AP (AYPGKF)

To characterize the platelet function of the PAR4^{P/L} and PAR4^{L/L} mice in response to PAR4-specific stimulation, murine PRP from PAR4^{P/P}, PAR4^{P/L}, or PAR4^{L/L} littermates was stimulated with 50 to 1600 μM PAR4-AP, AYPGKF-NH₂. Integrin activation and α-granule secretion were measured by flow cytometry using antibodies specific for activated αIIbβ3 or P-selectin (Figure 2A, B). Four hundred micromolar PAR4-AP was sufficient to elicit the maximum activation of the platelets from PAR4^{P/P} mice with wild-type PAR4 on their surface, with a half-maximal effective concentration (EC₅₀) of 191 μM for P-selectin and 144 μM αIIbβ3. These data are summarized in the Table.

PAR4^{P/L} mice required 800 μM PAR4-AP to reach maximum activation, with an EC₅₀ of 372 μM for P-selectin and 312 μM for αIIbβ3. There was no difference in the maximum response to the

PAR4-AP between the platelets from PAR4^{P/L} and PAR4^{P/P} mice. Further, the platelets from the homozygous PAR4^{L/L} mice showed a pronounced reduction in their response to PAR4 stimulation. When the platelets were treated with the highest dose of PAR4-AP, 1600 μM, we saw only 50% of maximal response with P-selectin exposure and 60% with activated αIIbβ3. Taken together, PAR4-P322L decreased receptor reactivity on mouse platelets, which reduced platelet responsiveness to PAR4-AP.

3.3 | PAR4-P322L altered platelet responsiveness to thrombin

Thrombin is the major protease of the coagulation cascade and activates mouse platelets by cleaving PAR4. Gel-filtered platelets from PAR4^{P/P}, PAR4^{P/L}, or PAR4^{L/L} littermates were stimulated with 0.1

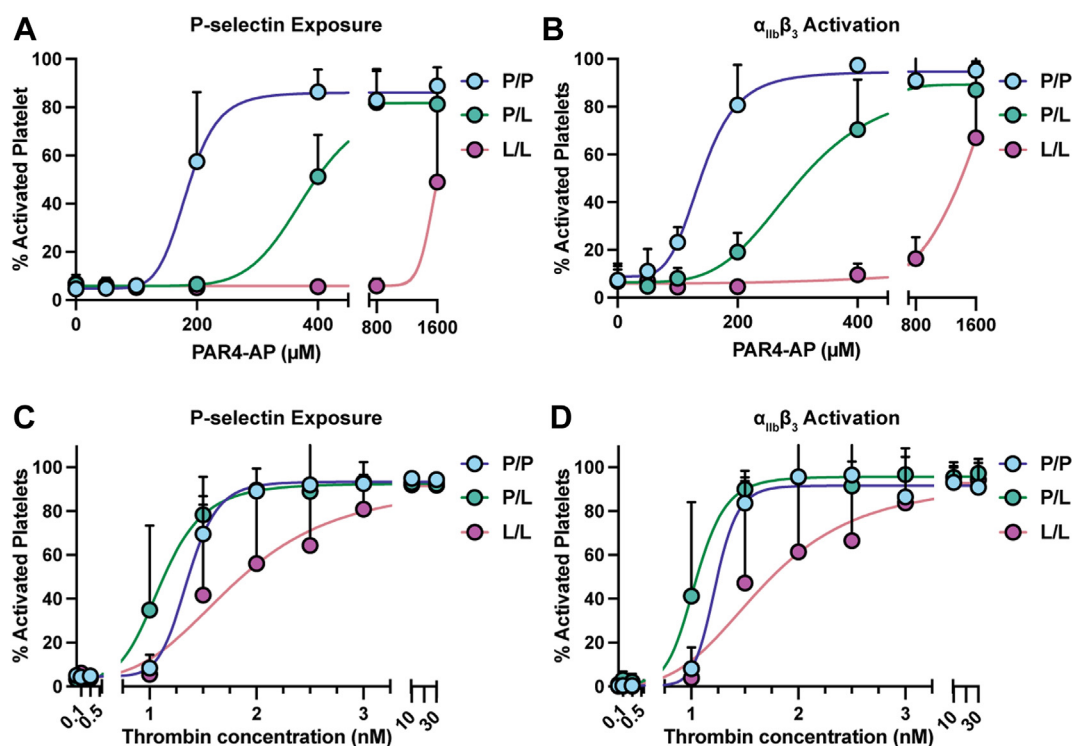


FIGURE 2 Platelets from PAR4-P322L mice were less responsive to PAR4 agonists. PRP from PAR4^{P/P}, PAR4^{P/L}, and PAR4^{L/L} littermates were stimulated with 50 to 1600 μM PAR4-AP, AYPGKF-NH₂. (A, B). Gel-filtered platelets from PAR4^{P/P}, PAR4^{P/L}, and PAR4^{L/L} littermates were stimulated with 0.1 to 30 nM thrombin (C, D). The α -granule secretion (A, C) and integrin $\alpha_{\text{IIb}}\beta_3$ activation (B, D) were measured by flow cytometry using antibodies specific for P-selectin and activated $\alpha_{\text{IIb}}\beta_3$. Data are presented as means \pm SD from 5 independent experiments at each concentration for panels A and B. Data are means from 3 independent experiments at each concentration for panels C and D. PAR4, protease-activated receptor 4; PRP, platelet-rich plasma.

to 30 nM thrombin to determine the response to the endogenous activator. Integrin $\alpha_{\text{IIb}}\beta_3$ activation and α -granule secretion were measured by flow cytometry using antibodies specific for activated $\alpha_{\text{IIb}}\beta_3$ or P-selectin, respectively (Figure 2C, D). Platelets from PAR4^{P/P} and PAR4^{P/L} mice reached maximum activation at 2 nM thrombin. The EC₅₀ for PAR4^{P/P} was 1.4 nM for P-selectin and 1.2 nM for $\alpha_{\text{IIb}}\beta_3$. The EC₅₀ for PAR4^{P/L} was 1.1 nM for P-selectin and 1.1 nM for $\alpha_{\text{IIb}}\beta_3$. Platelets from PAR4^{L/L} mice required more thrombin to reach maximum activation, with an EC₅₀ of 2.1 nM as measured by P-selectin and 1.9 nM for $\alpha_{\text{IIb}}\beta_3$ (see Table). These

findings indicated that PAR4-P322L significantly reduced platelet response to thrombin.

3.4 | PAR4-P322L did not affect platelet responsiveness to ADP and convulxin

We then determined whether PAR4-P322L altered other signaling pathways by testing platelet response to non-PAR4 agonists. Diluted murine PRPs from PAR4^{P/P}, PAR4^{P/L}, or PAR4^{L/L} littermates were

TABLE Platelet activation determined by P-selectin-positive or integrin $\alpha_{\text{IIb}}\beta_3$ activation using flow cytometry.

Genotype	PAR4-AP (AYPGKF) (μM)		Thrombin (nM)	
	$\alpha_{\text{IIb}}\beta_3$ activation	P-selectin	$\alpha_{\text{IIb}}\beta_3$ activation	P-selectin
PAR4-P/P	144 (115-173)	191 (157-225)	1.2 (1.1-1.4)	1.4 (1.1-1.8)
PAR4-P/L	312 (260-366)	372 (348-396)	1.0 (0.7-1.2)	1.1 (0.8-1.3)
PAR4-L/L	~8900	~1544	1.9 (1.3-2.5)	2.1 (1.5-2.7)

Data are presented as EC₅₀ with a \pm 95% CI. The maximum response of PAR4-L/L to AYPGKF is greater than 1600 μM ; EC₅₀ values are estimated. AP, activation peptide; PAR4, protease-activated receptor 4.

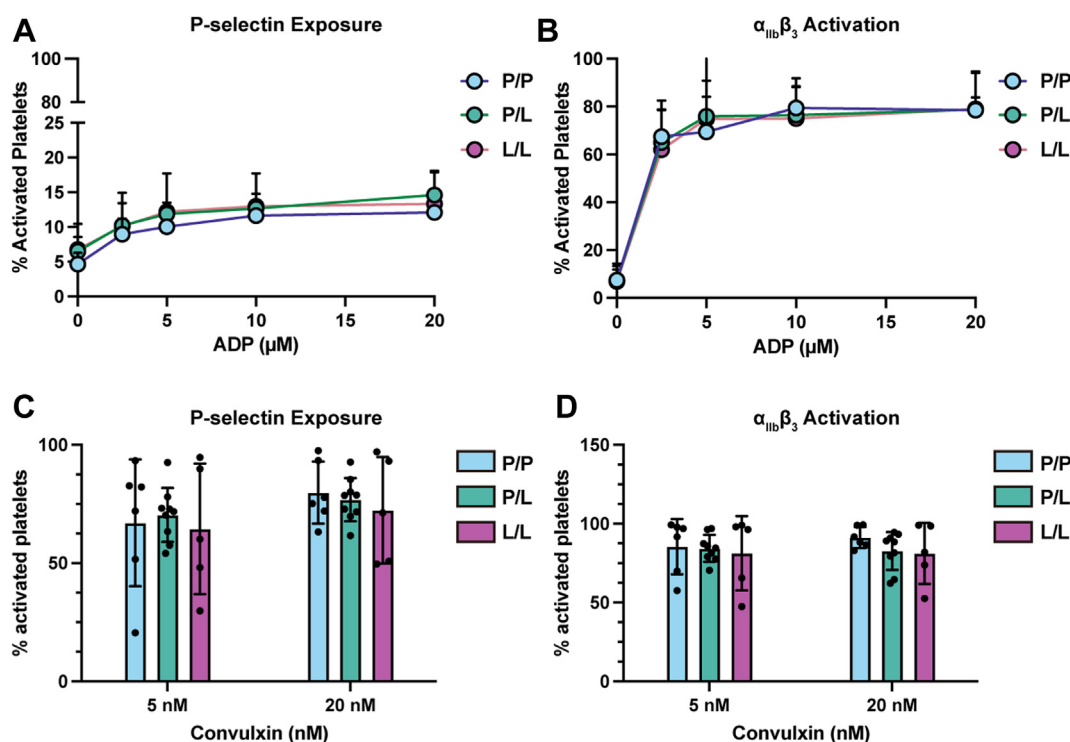


FIGURE 3 Reactivity to non-PAR4 agonists was unaffected in platelets from PAR4-P322L mice. PRP from PAR4^{P/P}, PAR4^{P/L}, and PAR4^{L/L} littermates was stimulated with 2.5 to 20 μM ADP (A, B) or 5 nM or 20 nM convulxin (C, D). The α -granule secretion (A, C) and integrin $\alpha_{\text{IIb}}\beta_3$ activation (B, D) were measured by flow cytometry using P-selectin and activated $\alpha_{\text{IIb}}\beta_3$ specific antibodies. Data are presented as means \pm SD from 5 independent experiments at each concentration. Dots represent individual mice. PAR4, protease-activated receptor 4; PRP, platelet-rich plasma.

stimulated with ADP (2.5-20 μM), the agonist for P2Y1 and P2Y12 receptors (Figure 3A, B), or convulxin (5 nM and 20 nM), a ligand for the platelet collagen receptor glycoprotein VI (Figure 3C, D). Platelet activation was characterized by integrin $\alpha_{\text{IIb}}\beta_3$ activation and α -granule secretion measured by flow cytometry. The platelets from the PAR4^{P/L} or PAR4^{L/L} mice had the same response to ADP and convulxin as wild-type mice (PAR4^{P/P}). Therefore, PAR4-P322L did not change platelet responsiveness to ADP or convulxin, indicating that PAR4-P322L impaired PAR4-mediated platelet reactivity without impacting other signaling pathways.

3.5 | PAR4-P322L diminished thrombin-mediated platelet aggregation

Since PAR4-P322L reduced platelet integrin activation and granule release in response to thrombin, we next evaluated how this mutation impacted platelet aggregation in response to thrombin (0.5-10 nM). Representative tracings are shown (Figure 4A-C). Wild-type (PAR4^{P/P}) platelets reached a maximum aggregation of 74% (\pm 6%) response with 1 nM of thrombin while PAR4^{P/L} platelets required 3 nM and PAR4^{L/L} platelets required 10 nM to reach the same degree of aggregation (Figure 4D). Overall platelet aggregation as measured by area under the curve plateaued at 1 nM for PAR4^{P/P}, 3 nM for

PAR4^{P/L}, and 10 nM for PAR4^{L/L} (Figure 4E). The rate of aggregation was also delayed in PAR4^{P/L} and PAR4^{L/L} (Figure 4F). Altogether, this showed that the PAR4-P322L mutation significantly impaired platelet aggregation.

3.6 | PAR4-P322L extended tail bleeding time

The hemostatic plug is formed in response to vascular injury with the goal of preventing blood loss. The tail bleeding assay was used to characterize the hemostatic function of the homozygous PAR4^{L/L} and heterozygous PAR4^{P/L} mice. Both male and female mice at the age of 8 weeks were used. The body weight ranged from 23 to 26 g in the male mice and from 16 to 20 g in the female mice. We measured the time to initial cessation of bleeding within 10 minutes of tail snip (Figure 5A). The time to initial cessation of bleeding was unchanged in PAR4^{P/L} mice, which averaged 225 ± 96 seconds, when compared with that in wild-type PAR4^{P/P} littermates, at 189 ± 117 seconds. PAR4^{L/L} mice did take significantly longer to initially stop bleeding (326 ± 152 seconds). To account for rebleeding due to unstable clots, we also measured the total bleeding time over 10 minutes (Figure 5B). The total bleeding time was also unchanged between PAR4^{P/P} and PAR4^{P/L} mice, 262 ± 152 vs 235 ± 84 seconds, respectively.

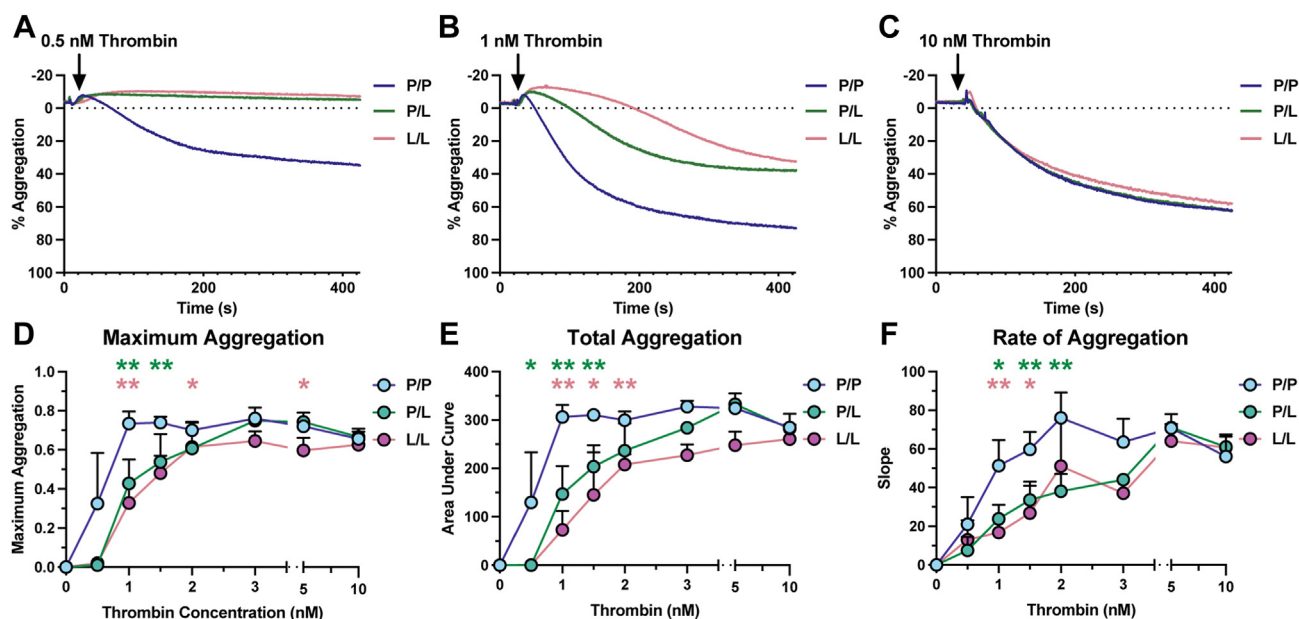


FIGURE 4 Aggregation of platelets from P322L mice had a reduced response to thrombin. Representative tracing of thrombin-mediated platelet aggregation (A-C). Gel-filtered platelets from PAR4^{P/P}, PAR4^{P/L}, and PAR4^{L/L} littermates were stimulated with 0.5 nM (A), 1 nM (B), and 10 nM (C) thrombin. (D) Maximum aggregation of gel-filtered platelets from PAR4^{P/P}, PAR4^{P/L}, and PAR4^{L/L} was compared in response to 0.5 to 10 nM thrombin stimulation. (E) Area under curve of PAR4^{P/P}, PAR4^{P/L}, and PAR4^{L/L} platelet aggregation was compared in response to 0.5 to 10 nM thrombin stimulation. (F) The aggregation rate of PAR4^{P/P}, PAR4^{P/L}, and PAR4^{L/L} platelet aggregation was compared in response to 0.5 to 10 nM thrombin stimulation. Data are representative of 3 independent experiments. Dots represent individual mice. PAR4, protease-activated receptor 4.

3.7 | PAR4-P322L mice had an increased time to arterial occlusion

Global PAR4 knockouts are protected against arterial thrombosis in several mouse models [30,31]. Our results using FeCl₃ carotid artery

injury are consistent with these reports. All PAR4^{P/P} mice fully occluded by 17 minutes (12 ± 3.4 minutes), while PAR4^{-/-} mice were unable to develop thrombi by 30 minutes (Figure 6A). PAR4-P322L mice showed an increased time to occlusion compared with wild types, with 55% of PAR4^{P/L} and 18% of PAR4^{L/L} mice unable to develop

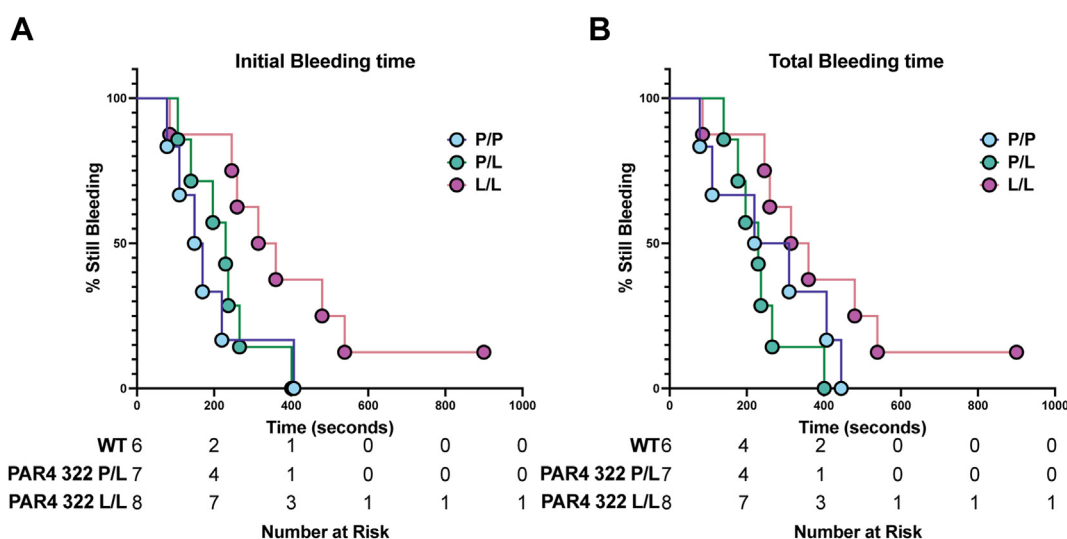


FIGURE 5 PAR4-P322L mice had extended tail bleeding time. Tail bleeding assay was used to evaluate the impact of the PAR4-P322L on hemostatic function. (A) Initial bleeding time was defined as the first time observing the stop of the bleeding regardless of any rebleeding. (B) Total bleeding time was defined as the sum of bleeding times of all bleeding on/off cycles until a stable cessation occurred (no bleeding for 60 seconds). The experiment was terminated at 10 minutes. The data were presented as the percentage of mice that were still bleeding at a specified time point. PAR4, protease-activated receptor 4.

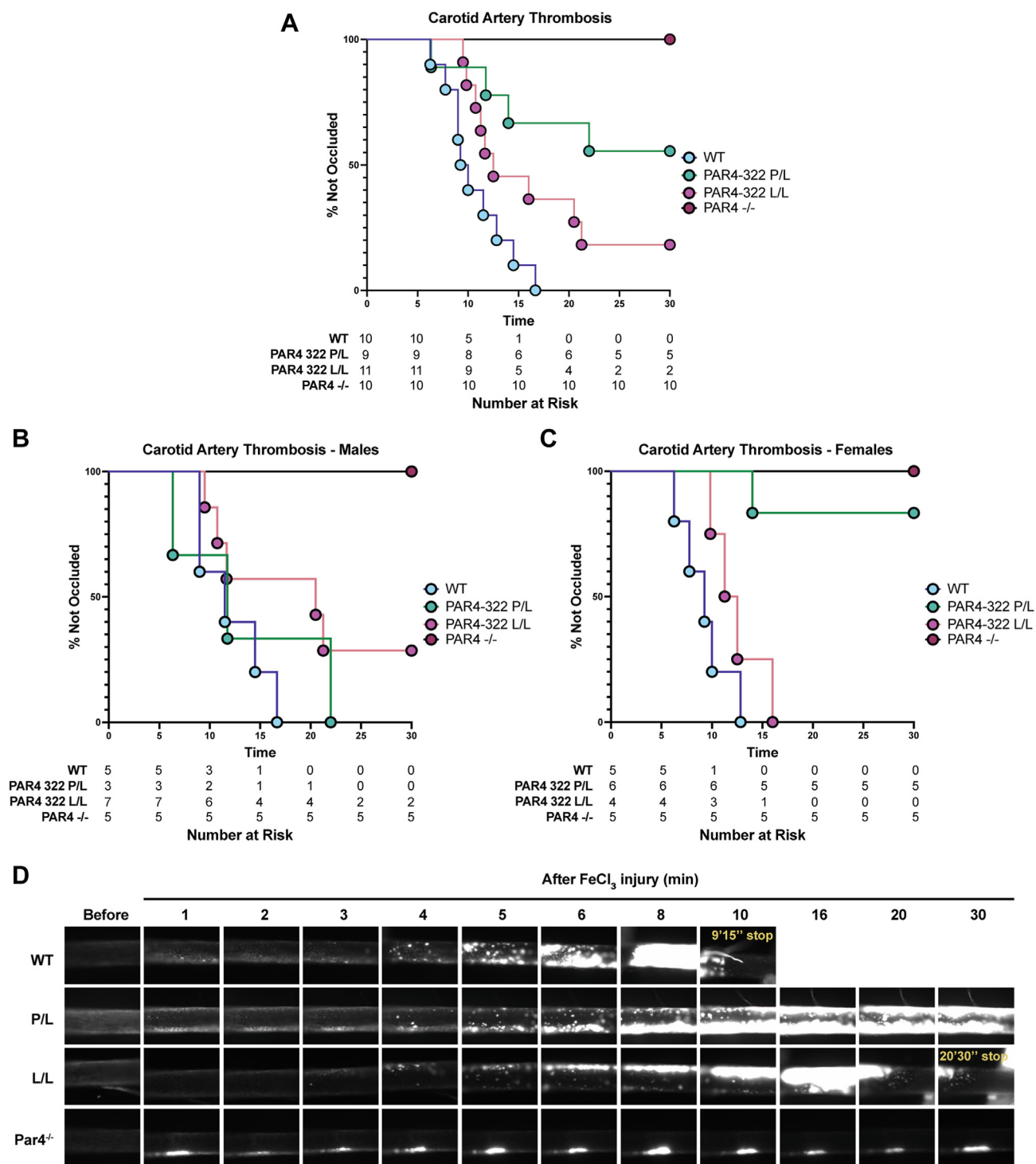


FIGURE 6 PAR4-P322L mice have longer arterial occlusion times. Arterial thrombosis in our mice was assessed with the ferric chloride-induced carotid artery injury model. (A) The time to occlusion was visually determined as the moment blood flow stopped. The time to complete occlusion was determined in males (B) and females (C). Rhodamine 6G was used to label white blood cells and platelets in real-time over 30 minutes. Representative images are shown (D). PAR4, protease-activated receptor 4.

stable thrombi. Interestingly, there was a difference in the trends between male and female mice. Male PAR4^{P/L} mice showed similar time to occlusion when compared with wild types, while 29% of male PAR4^{L/L} mice were unable to fully occlude (Figure 6B). In females,

PAR4^{L/L} mice were similar to wild types; however, 83% of female PAR4^{P/L} mice were unable to develop stable thrombi (Figure 6C). Representative images over 30 minutes for each genotype are shown (Figure 6D).

4 | DISCUSSION

PAR4 is one of the key thrombin receptors on the surface of human platelets. This G-protein-coupled receptor (GPCR) is activated via a unique tethered ligand mechanism and relays extracellular stimulation across the cell membrane [32]. Our previous structural studies demonstrated that a PAR4 SNP in the ECL3, PAR4-P310L (rs2227376), has a reduced receptor function in exogenously expressing HEK293 cells [19].

Here, we designed a mouse model to investigate how the hyporeactive leucine allele at PAR4-P310 impacts PAR4-mediated platelet function. A point mutation was introduced into the mouse PAR4 gene F2r13 via CRISPR/Cas9 to create PAR4-P322L, the mouse homolog to human PAR4-P310L. Platelets from the heterozygous (PAR4^{P/L}) and homozygous (PAR4^{L/L}) mice showed a significantly reduced response to both PAR4-AP and thrombin stimulation compared with wild-type mouse platelets. The PAR4-P322L mutation had no impact on other signaling pathways, such as P2Y12 or GPVI, but did extend tail bleeding time in homozygous PAR4^{L/L} mice. The mutation also extended the time to occlusion in the FeCl₃ model of arterial thrombosis.

Further study of the PAR4-P310L variant will reveal more about how mutations in GPCRs impact structure and function. PARs belong to the class A GPCRs, which all share an architecture of 7 transmembrane domains connected by extracellular and intracellular loops. There is compelling evidence that the extracellular loops are more than just linkers connecting the transmembrane domain. Instead, they are key mediators for critical aspects of GPCR function [3]. ECL2 plays a key role in ligand recognition and interaction for PAR1, PAR2, and PAR4 [7]. Our structural approach using amide hydrogen deuterium exchange coupled with mass spectroscopy revealed an unrecognized role of ECL3 in PAR4 activation [19]. We proposed that a coordinated movement of ECL3 is required for PAR4 activation. The 2 conserved prolines (Pro310 and Pro312) likely confer rigidity of the loop that is essential for the rearrangement of ECL3 to expose the ligand-binding site. Mutating Pro310 to leucine decreased PAR4 activation in response to both thrombin and PAR4-AP, but did not impact expression [19]. Here, we show that the expression and trafficking of mouse PAR4-P322L were the same as those of wild type (Figure 1E). We hypothesize that mutating Pro310 (human) or Pro322 (mouse) leads to a flexible ECL3 that impairs activation, creating a hyporeactive receptor.

We observed a more dramatic impact of the P322L mutation on activation by PAR4-AP vs thrombin. The activation of PARs by the protease-generated tethered ligand is fundamentally different from that of a soluble activation peptide. Specifically for PAR4, the tethered ligand induces a global structural rearrangement of the receptor, whereas the PAR4-AP does not [33]. In addition, PAR4-AP is a soluble ligand that can diffuse from the receptor. This exacerbates any reduced affinity of PAR4-AP for the mutant receptor. In comparison, the tethered ligand generated by thrombin is attached to the receptor, which eliminates diffusion and minimizes the impact of the decreased affinity. We have observed similar differences between thrombin and

PAR4-AP activation with mutants of human PAR4 (eg, T153A, P310L, and P312L) [19]. The mouse platelet data are consistent with our previous findings and other reports that the PAR4-AP does not have signaling properties identical to the endogenous ligand.

PAR4 serves as a major thrombin signal initiator on platelets, and polymorphisms other than P310L have been described and implicated in diseases like stroke and thrombosis. The rs773902 SNP (PAR4-120Ala/Thr) results in an amino acid switch at position 120, which is located within transmembrane domain 2 near the ligand-binding site as determined by H/D exchange [19]. The Thr120 variant is more sensitive to agonists than Ala120 [20]. The PAR4-Thr120 variant renders the receptor less sensitive to PAR4 antagonists and worsens stroke outcomes in a humanized mouse model [21,23]. Further, individuals who express PAR4-Thr120 are at a higher risk of ischemic stroke [23]. A less common PAR4 variant is the PAR4-296Phe/Val resulting from the SNP at rs2227346. Phe296 is located within a conserved switch region in transmembrane domain 6 that is important for GPCR activation [20]. Val296 leads to a dysfunctional PAR4. There have been no studies directly examining the impact of Leu310 on bleeding or thrombosis. However, it is associated with a 15% reduction in relative risk for VTE in the INVENT consortium database [19,34]. The minor allele frequency for rs2227376 (Leu) was 0.015. With our growing knowledge of platelets and their role in the early stages of VTE, it is likely that PAR4-P310L is impacting platelet function in these individuals that results in a degree of protection from thrombosis.

The conserved nature of PAR4 across species, and specifically ECL3, reinforces the idea that this loop is essential for function and permits introducing a point mutation in mice to study the impact on hemostasis and thrombosis in a physiological context. While PAR4 is highly conserved between species, there are key differences in signaling and function that should be considered when interpreting our findings. PAR4 is present on platelets in both mice and humans. Since it is not an efficient thrombin substrate, it requires a cofactor for low concentrations of thrombin [35–37]. Human platelets express PAR1, while mouse platelets express PAR3 [38]. Our data in mouse platelets suggests that the P322L mutation predominantly impacts thrombin-mediated PAR4 activation at low thrombin concentrations. Since the mutation is in ECL3, it is unlikely that it impacts PAR4's ability to dimerize with itself, other PARs, or P2Y12 [39]. Recent studies with mice expressing human PAR4 also suggest that human and mouse PAR4 are more different than previously understood [40]. Renna et al. [40] showed that human PAR4 is more sensitive to thrombin and activates platelets to a greater degree than the mouse counterpart. It is likely that the P322L mutation in a more sensitive, humanized PAR4 would have a greater impact on signaling at low concentrations of thrombin. Based on our data, it would be beneficial to study this mutation in a humanized setting.

Our *ex vivo* data from platelets expressing PAR4-P322L have a dramatic reduction in their response to PAR4 stimulation. Specifically, our platelet data suggest a gene-dosage response where the decrease in reactivity is more pronounced in those homozygous for the leucine allele (PAR4^{L/L}) compared with that in heterozygous mice (PAR4^{P/L}). In

the FeCl₃ model of carotid artery thrombosis, however, we see a flip in this trend. Heterozygous (PAR4^{P/L}) mice had a more dramatic phenotype with 55% of mice unable to develop stable thrombi in comparison with 18% of PAR4^{L/L} mice. Additionally, we saw a distinct difference in clot development between the male and female PAR4^{P/L} mice that was not observed in the PAR4^{L/L} or wild-type PAR4^{P/P} mice. Eighty-three percent of female PAR4^{P/L} mice formed unstable thrombi that were unable to fully occlude the artery. This was not seen in PAR4^{P/L} males. It is unclear why the female PAR4^{P/L} mice occluded later than PAR4^{L/L} mice. This is in contrast to *ex vivo* experiments where PAR4^{L/L} platelets are less reactive than PAR4^{P/L} mice (Figures 2 and 4). Notably, there was no difference between male and female mice in the *ex vivo* studies. PAR4 has also been extensively characterized in platelet function and hemostasis, and there is no recorded difference in PAR4 function between males and females. If sex has an impact on PAR4 function, it is a subtle phenotype seen only when PAR4 function is reduced, as in our P322L mice. The PAR4-P322L mutation in our mouse model is global and present in all cells expressing PAR4. Cells other than platelets express PAR4, including endothelial cells, and their response to the PAR4-P322L mutation might be different than platelets. While endothelial PAR4 could be influencing our phenotype, it is likely that platelets are predominant mediators of thrombin signaling in thrombosis. Lee et al. [41] recently developed a PAR4-floxed mouse with deletion specifically on platelets. These mice also had an increased time to occlusion in the FeCl₃ carotid artery injury model. There was also reduced venous thrombosis in these mice. We know that PAR4-P310L is associated with a reduced risk of VTE, and growing evidence shows that platelets help drive clot development [42–44]. In fact, platelet dysfunction and platelet–neutrophil aggregation have been found to be possible risk factors for VTE [45,46]. Since PAR4 activation also promotes platelet–neutrophil interactions and thrombin generation, it is likely that thrombin-mediated PAR4 activation is driving thrombosis via platelets [47,48]. Going forward, we can use our PAR4-P322L model to further study the underlying mechanism of PAR4 contributions to VTE alone and in combination with other pathologies to highlight the benefit of targeting PAR4 therapeutically.

AUTHOR CONTRIBUTIONS

Study Design: X.H., E.A.K., W.L., M.N. Data Collection: X.H., E.A.K., M.F., W.L., W.J. Data Analysis: X.H., E.A.K., M.F., W.L., M.N. Drafting Manuscript: X.H., E.A.K., M.N. Critical Revisions: S.R., S.M., R.C., D.L.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose

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