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BRIEF REPORT

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Murine cadherin-6 mediates thrombosis in vivo in a platelet-independent manner

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Abstract

Background: Platelet adhesion is the critical process mediating stable thrombus formation. Previous reports of cadherin-6 on human platelets have demonstrated its role in platelet aggregation and thrombus formation.

Objectives: We aimed to further characterize the importance of cadherin-6 in thrombosis in vivo.

Methods: Cadherin-6 platelet expression was evaluated by western blotting, flow cytometry, and immunoprecipitation. Thrombosis was evaluated using the $FeCl_3$ and Rose Bengal carotid artery models in C57Bl6 mice treated with anti–cadherin-6 or IgG and wild-type or *Cdh6*$^{-/-}$ mice. Platelet function was compared in wild-type and *Cdh6*$^{-/-}$ mice using tail-clip assays, aggregometry, and flow cytometry.

Results: Human platelet expression of cadherin-6 was confirmed at ~3000 copies per platelet. *Cdh6*$^{-/-}$ mice or those treated with anti–cadherin-6 antibody showed an increased time to occlusion in both thrombosis models. Cadherin-6 was not expressed on mouse platelets, and there were no differences in tail bleeding times, platelet aggregation, or platelet activation in wild-type versus *Cdh6*$^{-/-}$ mice.

Conclusions: Cadherin-6 plays an essential role in thrombosis in vivo. However, cadherin-6 is not expressed on murine platelets. These data are in contrast to human platelets, which express a functional cadherin-6/catenin complex. The essential, platelet-independent role for cadherin-6 in hemostasis may allow it to be an effective and safe therapeutic target.

KEYWORDS

arterial thrombosis, cadherin-6, mouse model, platelet adhesion

Essentials

- Cadherin-6 function in thrombus formation was investigated in vivo using two murine models of thrombosis.
- Blocking or deleting cadherin-6 significantly delayed time to occlusion
- Human platelets express cadherin-6, but murine platelets do not.
- Cadherin-6 has an essential role in thrombosis that is independent of platelets.

1 | INTRODUCTION

Arterial thrombosis occurs following rupture of an atherosclerotic plaque. Platelets are activated upon contact with the damaged vessel and potentiate thrombus formation. A primary consequence of platelet activation is the conformational change of platelet integrins to a high-affinity state.[1] The most essential and abundant platelet integrin is $\alpha_{IIb}\beta_3$, which builds platelet aggregates by cross-linking fibrinogen.[2]

Cadherin-6 was proposed as a novel platelet-expressed ligand to $\alpha_{IIb}\beta_3$.[3] Junctional proteins, including cadherins, were first documented on platelets by Elrod et al.[4] E-cadherin has also been identified on platelets.[5] Cadherins are calcium-dependent, single-pass transmembrane adhesion proteins with characteristic extracellular repeats (ECs).[6] Cadherin-6 is a type II classical cadherin, containing five tandem ECs and two tryptophan residues in EC1 that facilitate trans-cadherin binding.[7] Notably, cadherin-6 contains an arginyl-glycyl-aspartic acid (Arg-Gly-Asp; RGD) tripeptide in EC1.[8] RGD domains are well-characterized integrin-binding motifs; $\alpha_{IIb}\beta_3$ binds to RGD sequences on fibrinogen.[9]

Dunne et al[3] first demonstrated a supportive role for cadherin-6 in platelet aggregation and thrombus formation. In their study, platelets adhered to cadherin-6 and this interaction was disrupted by blocking $\alpha_{IIb}\beta_3$. Additionally, ex vivo thrombus formation was reduced with anti–cadherin-6 antibodies. The current understanding of cadherin-6 in thrombosis is solely based on these in vitro and ex vivo studies. The goal of this study was to examine the role of cadherin-6 in vivo. Herein, we demonstrate that cadherin-6 has a significant role in thrombus formation in vivo. However, analysis of murine platelets exposed an absence of cadherin-6, revealing an important species difference and a platelet-independent role for cadherin-6 in thrombosis.

2 | METHODS

2.1 | Animals

C57BL/6J and Cdh6$^{-/-}$ (B6.129S6-Cdh6^{tm1sma}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Platelets from FVB and 129x1/SVJ mice were also used to assess cadherin-6 expression. All animal studies were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University School of Medicine.

2.2 | Preparation of platelet-rich plasma

With approval by the Case Western Reserve University Institutional Review Board, human whole blood was obtained by venipuncture from healthy donors after obtaining informed consent. Murine whole blood was collected retro-orbitally; platelet count was obtained using a Hemavet 950FS (Drew Scientific Inc, Miami Lakes, FL, USA). Whole blood was collected into either acid citrate dextrose (2.5% sodium citrate, 71.4 mmol/L citric acid, 2% D-glucose) or sodium citrate (0.15 mol/L). The blood was centrifuged at 200 *g* for 15 minutes (human) or 2300 *g* for 20 seconds (murine) at room temperature to obtain platelet-rich plasma (PRP). Platelet concentrations were quantified using a Coulter counter.

2.3 | Western blot

The 5×10^7 platelets in PRP were directly suspended into SDS Laemmli buffer. Chinese hamster ovary (CHO) cells were transfected with 5 μg of human cadherin-6 in pIRES-puro or mouse cadherin-6 in pCDNA3.1 via Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 hours, the cells were lysed in RIPA buffer (1% NP-40, 0.5% deoxycholate, 0.1% SDS), and 100 μg of protein was loaded.

For quantitative western blotting, recombinant human cadherin-6 Fc chimera protein (~130 kD) or recombinant mouse cadherin-6 protein (~90 kD) was used to generate a standard curve (R&D Systems, Minneapolis, MN, USA; catalog # 2715-CA or 6826-CA, respectively). For mouse samples, the gel was loaded with 0.0023, 0.0115, 0.023, 0.046, and 0.23 μg of protein per lane. Based on loading 5×10^7 platelets, this is the equivalent of 300, 1500, 3000, 6000, or 30 000 molecules/platelet, respectively. The same strategy was used for human samples. The formula is shown below:

$$\frac{\#\ \text{molecules Cdh6}}{\text{platelet}} \times 5 \times 10^7\ \text{platelets} \times 90\ \text{kDa} = \mu\text{g loaded on gel}$$

Samples were resolved by SDS-PAGE, transferred to nitrocellulose, probed with mouse monoclonal anti–cadherin-6 antibody (1:500, R&D Systems, catalog # MAB2715), then IRDYE 800CW donkey anti-mouse IgG (LI-COR Biosciences, Lincoln, NE, USA) and imaged using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Human platelet lysate was immunoprecipitated using IgG or anti–cadherin-6 antibody (R&D Systems, catalog # MAB2715) and Protein A beads (Invitrogen). The immunocomplexes were washed three times with Tris buffered saline with 0.1% Triton X-100 and resuspended in 2× sample buffer, and resolved on SDS-PAGE. The membrane was probed with anti–cadherin-6, anti–α-catenin (Life Technologies, catalog # MAS-161986), and anti-β-catenin (Cell Signaling Technology, Danvers, MA, USA; catalog #9582) antibodies.

2.4 | Flow cytometry

Human PRP was stimulated with SFLLRN (25 μmol/L), AYPGKF (250 μmol/L), ADP (5 μmol/L), or convulxin (5 nmol/L). Platelets were incubated with allophycocyanin (APC)-conjugated anti–cadherin-6 antibody (R&D Systems; catalog #FAB2715A) and analyzed using a LSRFortessa (BD Biosciences, San Jose, CA, USA). Cadherin-6 expression was quantified using Quantum Simply Cellular beads (Bangs

Laboratories, Inc, Fishers, IN, USA) to generate a standard curve of antibody-binding sites as previously described.[10-13]

PRP from wild-type and *Cdh6*$^{-/-}$ mice was stimulated with AYPGKF (250-1000 μmol/L) or convulxin (0.5-5 nmol/L). JON/A and Wug.E9 (Emfret Analytics, Eibelstadt, Germany) were used to detect platelet expression of activated integrin αIIbβ3 and P-selectin, respectively.

2.5 | Carotid artery thrombosis models

Mice (8-12 weeks) were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and the carotid artery was exposed. In some experiments, mice received sheep polyclonal anti-human cadherin-6 (R&D Systems; catalog # AF2715) or sheep IgG (0.8 mg/kg) intravenously 15 minutes before initiation of thrombosis. Based on >98% sequence homology between mouse and human cadherin-6, the anti-human antibody was expected to cross-react; this was verified via western blot. Rhodamine 6G (100 μL) was injected via the right jugular vein to label platelets. Thrombosis was induced via the $FeCl_3$ model[14,15] with a minute-long topical application of filter paper saturated with 7.5% $FeCl_3$, and vessel occlusion was monitored for 30 minutes via intravital microscopy. Images were captured with a QImaging Retigo Exi 12-bit mono digital camera and Streampix version 3.17.2 (NorPix, Montreal, QC, Canada).

In the Rose Bengal model[16] 4,5,6,7-tetrachloro-3, 6-dihydroxy-2,4,5,7-tetraio-dospiro (isobenzofuran-1(3H),9[9H] xanthan)-3:1 dipotassium salt (50 mg/kg in 0.9% saline, Fisher Scientific, Hampton, NH, USA) was injected retro-orbitally before catalyzing vessel injury with a 540-nm laser. A Doppler flow probe was used to monitor vessel occlusion for 90 minutes.

Because some animals did not reach full occlusion (right-censored data), the data were plotted on Kaplan-Meier survival curves, and the log-rank (Mantel-Cox) test was used to compare curves.[17]

2.6 | Tail-clip assay

Three millimeters was clipped from the tails of anesthetized wild-type and *Cdh6*$^{-/-}$ mice, and the tail was placed in 37° saline. Time to cessation of bleeding was observed for 10 minutes.

2.7 | Platelet aggregometry

PRP was stimulated with AYPGKF (50 μmol/L) or convulxin (0.5 nmol/L). Platelet aggregation was measured under constant stirring (1200 rpm) with a Chrono-log Model 700 aggregometer (Chrono-Log Corp., Havertown, PA, USA) using Aggrolink8 version 1.3.98.

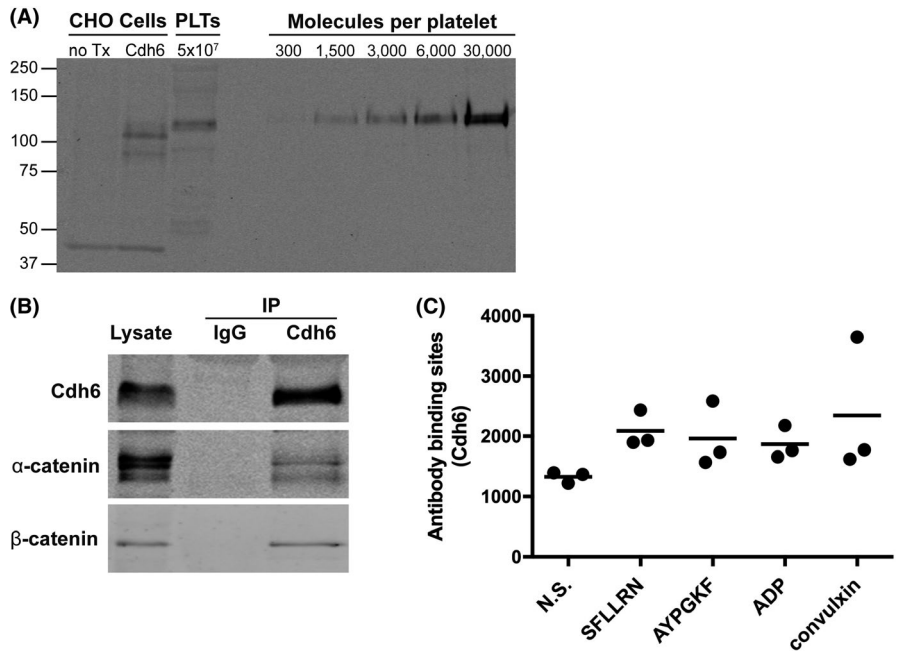

FIGURE 1 Human platelets express cadherin-6. (A) Human platelet lysate was probed with mouse monoclonal anti–cadherin-6; CHO cells transfected with human cadherin-6 (Cdh6) was used as a control. A standard curve was generated using recombinant human cadherin-6 Fc chimera protein. The blot shown is representative of n = 2. (B) Human platelet lysate was immunoprecipitated with anti-cadherin-6 antibody. Cadherin 6, α-catenin, and β-catenin were detected by western blot. (C) Quantitative flow cytometry was performed using an APC-conjugated anti-cadherin-6 antibody on nonstimulated platelets or those activated with PAR1 (SFLLRN, 25 μmol/L) or PAR4 (AYPGKF, 250 μmol/L) stimulating peptides, ADP (5 μmol/L), or convulxin (5 nmol/L). CHO, Chinese hamster ovary; PAR, protease-activated receptor; PLTs, platelets

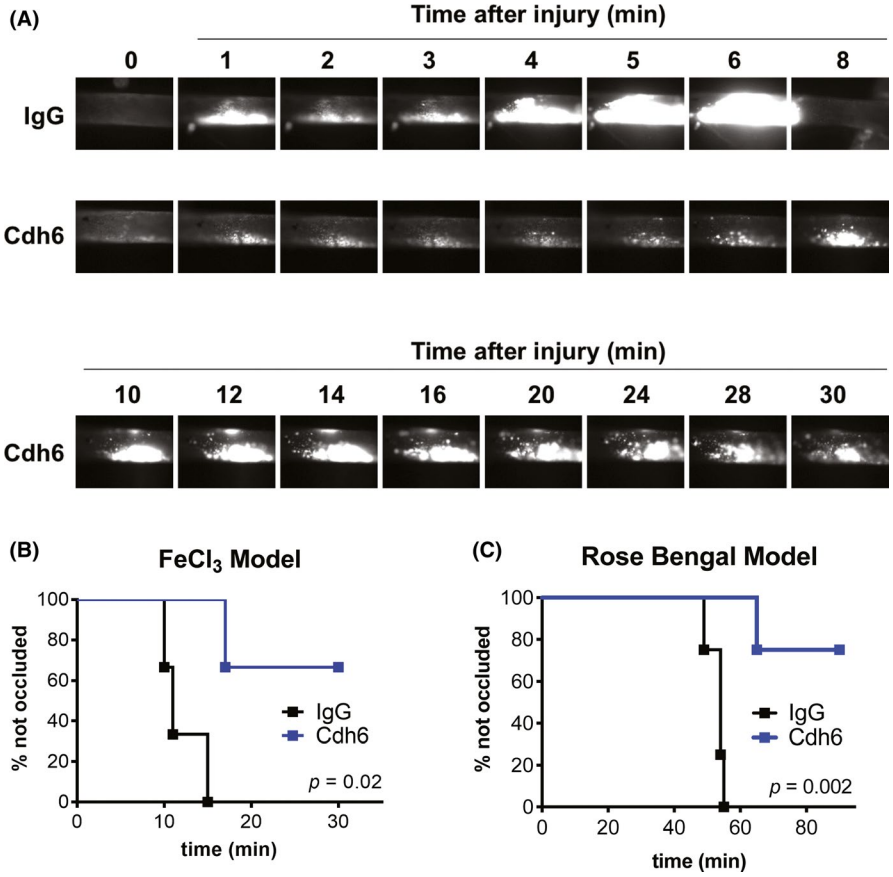

FIGURE 2 Blocking cadherin-6 disrupts thrombosis. C57BL/6J mice were treated with sheep polyclonal anti–cadherin-6 (Cdh6, 0.8 mg/kg) 15 minutes before initiating thrombosis with $FeCl_3$ or Rose Bengal. (A) Intravital microscope images obtained following $FeCl_3$ treatment. In IgG-treated mice at 8 minutes, the proximal end of the thrombus was monitored to accurately detect full occlusion. Time to stable vessel occlusion was observed and represented as Kaplan-Meier curves for (B) $FeCl_3$ (n = 3 per group) and (C) Rose Bengal models (n = 5 for no treatment, n = 4 for IgG or Cdh6). The curves were compared using the Mantel-Cox log-rank test

3 | RESULTS AND DISCUSSION

3.1 | Cadherin-6 is present on human platelets

Previous reports have observed cadherin-6 expression on human platelets.[3] To examine cadherin-6 on human platelets, we designed a western blot to quantify the density of cadherin-6 expression. Purified recombinant human cadherin-6 was used to generate a standard curve to determine the threshold of detection. Cadherin-6 was detected in CHO cells transiently transfected with human cadherin-6 and human platelets isolated from healthy donors (Figure 1A). Based on the standard curve, cadherin-6 expression is ~4500 copies per platelet. Immunoprecipitation of cadherin-6 confirmed associations with α-catenin and β-catenin (Figure 1B). Cadherin-catenin adhesion complexes can link external stimuli to actin cytoskeleton dynamics.[18] Proteomic results from Maguire et al[19] confirm the activation-dependent association of cadherin-6 and β-catenin in human platelets. Platelet surface expression of cadherin-6 was analyzed by quantitative flow cytometry using an APC-conjugated antibody and confirmed the surface density of cadherin-6 to be ~1300 copies per resting platelet (Figure 1C). Cadherin-6 surface expression increased to ~2000 copies when platelets were stimulated with protease-activated receptor (PAR) agonist peptides, ADP, or convulxin. The difference in copy number on the western blot and surface expression by flow cytometry suggests that a portion of intracellular cadherin-6 is translocated to the membrane following stimulation. Together, these results support functional expression of cadherin-6 on human platelets.

3.2 | Cadherin-6 has an essential role during thrombosis in vivo

Previously, fibrinogen and von Willebrand factor double-knockout mice were treated with sheep polyclonal anti–cadherin-6 and evaluated ex vivo for thrombus size. Compared to the IgG control treatment, blocking cadherin-6 resulted in smaller thrombi.[3] To define the effect of blocking cadherin-6 in vivo, we treated wild-type mice with sheep polyclonal anti–cadherin-6 antibody or sheep IgG. Thrombosis was induced in the carotid artery using the $FeCl_3$ or Rose Bengal model. Whereas $FeCl_3$ is applied externally, traverses the vessel, and causes severe endothelial denudation, Rose Bengal causes a milder endothelial injury that more closely mimics the intravascular etiology of arterial thrombosis. In the $FeCl_3$ model, mice treated with control IgG antibody formed full occlusions by 8 minutes, confirmed by monitoring flow at the proximal end of the thrombus (Figure 2A). In contrast, mice treated with anti–cadherin-6 showed impaired accumulation of platelets at the injured vessel, and 66% failed to form a stable occlusion after 30 minutes (Figure 2B). Similarly, 75% of anti–cadherin-6–treated mice never formed a full occlusion in the Rose Bengal model (Figure 2C).

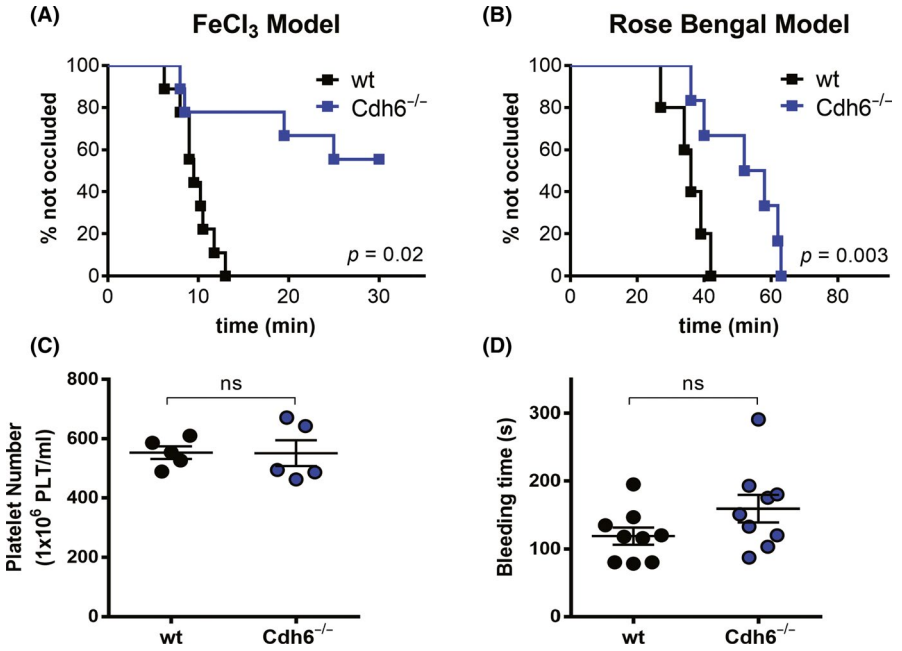

FIGURE 3 Thrombosis is delayed in CDH6 knockout mice. Thrombosis was initiated in *Cdh6*$^{-/-}$ and wild-type mice using the (A) $FeCl_3$ (n = 9 per group) or (B) Rose Bengal (n = 5 for wild-type and n = 6 for *Cdh6*$^{-/-}$) models and time to vessel occlusion was observed. The vessels were monitored until a full occlusion was formed or the experiment ended and represented as Kaplan-Meier curves. The curves were compared using the Mantel-Cox log-rank test. (C) Platelet count was measured in whole blood from wild-type and *Cdh6*$^{-/-}$ mice and compared using Student's *t* test. (D) *Cdh6*$^{-/-}$ and wild-type mice were anesthetized before clipping 3 mm of tail to measure time to bleeding cessation; significance was evaluated with Student's *t* test, and bars represent mean ± SEM

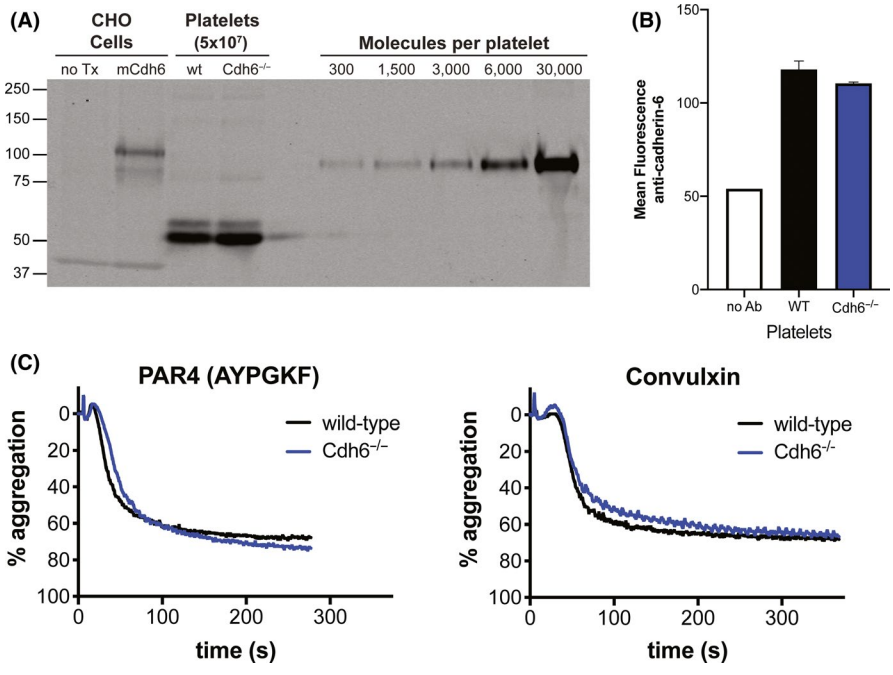

FIGURE 4 Cadherin 6 is absent on murine platelets. (A) Platelet lysates from Cdh6−/− and wild-type mice were probed with mouse monoclonal anti–cadherin-6. CHO cells transfected with mouse cadherin-6 (Cdh6) was used as a control. A standard curve was generated using recombinant mouse cadherin-6 protein. (B) Surface expression of cadherin-6 was measured on platelets from wild-type or Cdh6−/− mice via flow cytometry with an APC-conjugated cadherin-6 antibody. (C) Representative (n = 3) aggregometry tracings of Cdh6−/− and wild-type platelets stimulated with AYPGKF (150 µmol/L) and convulxin (0.5 nmol/L). CHO, Chinese hamster ovary; PAR, protease-activated receptor

To further explore the significance of cadherin-6 in vivo, we evaluated thrombosis in global cadherin-6 knockout (*Cdh6*$^{-/-}$) mice. In the $FeCl_3$ model, 55% of *Cdh6*$^{-/-}$ mice failed to form a stable occlusion after 30 minutes, whereas wild types reached full occlusion at 10 ± 2 minutes (Figure 3A). In the Rose Bengal model, loss of cadherin-6 resulted in significantly delayed occlusion; wild types formed full occlusions in 35 ± 2.5 minutes whereas *Cdh6*$^{-/-}$ mice formed full occlusions in 51.8 ± 4.7 minutes ($P = .03$) (Figure 3B). We confirmed equivalent platelet counts in wild-type and knockout mice (Figure 3C). Interestingly, antibody treatment caused a greater defect in thrombosis than genetic deletion of cadherin-6, which may be explained by off-target effects of the antibody. Nonetheless, thrombosis was impaired in both models when cadherin-6 was knocked out globally, indicating a significant role of cadherin-6 in vivo during arterial thrombosis.

To evaluate the role of cadherin-6 in hemostasis, wild-type and cadherin-6 mice were subjected to the tail-clip assay. In this model, 3 mm of tail is clipped and time to cessation is measured. The tail bleeding time is sensitive to defects in both platelet function and coagulation.[20] There was not a significant difference in the bleeding time in wild-type and *Cdh6*$^{-/-}$ mice (Figure 3D). These results imply cadherin-6 is not required for normal hemostasis.

3.3 | Cadherin-6 is not present on murine platelets

Given our observations that cadherin-6 is expressed on human platelets and contributes to thrombosis in mice, we examined cadherin-6 expression and function on murine platelets. A western blot was performed using CHO cells transfected with murine cadherin-6, platelets isolated from wild-type and *Cdh6*$^{-/-}$ mice, and purified recombinant murine cadherin-6. Surprisingly, there was no expression of cadherin-6 in platelets from wild-type C57BL/6J mice (Figure 4A). The standard curve generated by the recombinant protein demonstrated that sensitivity of the western blot is at least 300 of copies per platelet. Western blotting additionally confirmed the absence of cadherin-6 expression on platelets from FVB and 129x1/SVJ mice (data not shown). Wild-type platelets incubated with APC-conjugated anti–cadherin-6 antibody were analyzed by flow cytometry and had the same signal as *Cdh6*$^{-/-}$ platelets, confirming an absence of platelet cadherin-6 (Figure 4B). Further, Rowley et al[21] performed RNA sequencing with murine and human platelets and found no CDH6 RNA in murine platelets; human platelets contained small amounts of CDH6 RNA. Together, these results confirm that cadherin-6 is not expressed on mouse platelets.

The absence of cadherin-6 on platelets implies that wild-type and *Cdh6*$^{-/-}$ platelets should be indistinguishable in platelet function assays. Platelet aggregation induced with either PAR4-activating peptide (150 μmol/L) or convulxin (0.5 nmol/L) was identical in wild-type and *Cdh6*$^{-/-}$ mice (Figure 4C). Flow cytometry was performed on *Cdh6*$^{-/-}$ and wild-type platelets stimulated with PAR4-activation peptide or convulxin to evaluate molecular markers of platelet activation. Activated *Cdh6*$^{-/-}$ platelets did not display significantly different integrin $\alpha_{IIb}\beta_3$ activation or P-selectin exposure (data not shown). The trend toward greater integrin activation and P-selectin exposure in *Cdh6*$^{-/-}$ platelets is curious but does not culminate in altered platelet aggregation and contradicts the defective thrombosis observed in vivo.

4 | DISCUSSION

Although cadherin-6 expression differs on human and murine platelets, platelets from both species adhered to immobilized cadherin-6.[3] Our results support a role for cadherin-6 in platelet adhesion. Together, significantly delayed occlusion in *Cdh6*$^{-/-}$ mice and absence of cadherin-6 on murine platelets indicate cadherin-6 expression on a cell type that supports platelet adhesion to the injured vessel. Cadherin-6 has been identified in vascular smooth muscle cells,[22] and Notch3 signaling was shown to control cadherin-6 vessel expression.[23] Identifying the cell type responsible for cadherin-6-dependent platelet adhesion in murine thrombosis will lend further insight into mechanisms of cadherin-6 in human thrombosis.

Cadherin-6 is likely participating in the robust network of adhesion interactions that stabilize a developing thrombus. Since cadherin-6 contains an RGD integrin-binding motif in EC1, it likely interacts heterotypically with platelet $\alpha_{IIb}\beta_3$. Dunne et al[3] used Arg-Gly-Asp-Ser peptide to inhibit platelet adhesion to immobilized cadherin-6, supporting this hypothesis. In humans, homotypic interactions between platelet- and vessel-expressed cadherin-6 may reinforce and stabilize platelet aggregates at the vessel wall. Further defining these interactions will help to evaluate the potential therapeutic utility of cadherin-6.

Platelet-cadherin-6 interactions may be important in settings beyond arterial thrombosis. Tumor cells can activate platelets to promote tumor growth, invasion, and immune evasion.[24] Cadherin-6 overexpression has been identified in ovarian and renal cancers,[25,26] inciting the possibility that tumor-expressed cadherin-6 mediates tumor-platelet cross-talk. Treatment of colorectal cancer or melanoma cells with an antibody targeting the cadherin-17 RGD motif reduced their metastatic potential in vivo.[27] Further, human platelets were shown to interact with colorectal cancer cells in a cadherin-6–dependent manner.[28] Inhibiting tumor-platelet cross-talk is a promising strategy to limit tumor progression and metastasis; RGD cadherins may be promising molecular targets.

Herein, we have demonstrated an essential role for cadherin-6 for thrombosis in vivo. Cadherin-6 remains a poorly understood member of the cadherin superfamily, although it carries significant therapeutic potential in multiple clinical settings. Understanding the expression, regulation, and participation of cadherin-6 across different pathologies is necessary for harnessing its potential.

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RELATIONSHIP DISCLOSURE

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

EGB, WL, and MTN designed the experiments. EGB, MF, WL, ERZ, MMM, and MTN conducted experiments and analyzed the data. EGB and MTN wrote the manuscript. MF, WL, ERZ, and MMM provided critical feedback on the manuscript.

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