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THE ROLE OF PLATELET ENDOTHELIAL AGGREGATION RECEPTOR 1 IN MIGRATION, ADHESION, AND PROLIFERATION

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Abstract :- Several investigations have shown that platelet endothelial aggregation receptor 1 (PEAR1) is critical in a number of physiological processes including phagocytosis, megakaryopoiesis, and platelet aggregation. However, few investigations have been conducted to elucidate the role of PEAR1 in endothelial cells, where it is most highly expressed. In this investigation, we utilized shRNA-mediated knockdown of PEAR1 in primary human umbilical vein endothelial cells (HUVECs) to determine the impact of PEAR1 on endothelial cell migration, adhesion, and proliferation. We extended our findings by examining the impact of the well-described PEAR1 variant, rs12041331, on HUVEC function, and evaluated the influence of this polymorphism on circulating endothelial biomarkers in 63 healthy subjects pre- and post-aspirin intervention (325 mg/day for 7 days). We observed that PEAR1 knockdown significantly reduced HUVEC migration, adhesion, and proliferation compared to control cells (P = 0.008, 0.02, and 0.009, respectively). Aspirin exposure largely abrogated the effect of PEAR1 knockdown on migration and adhesion (P = 0.19 and 0.24, respectively); however, proliferation remained significantly reduced compared to control cells (P = 0.01). rs12041331 did not significantly influence PEAR1 expression, and no significant difference in migration adhesion, or proliferation was observed by PEAR1 genotype pre- or post-aspirin administration. Furthermore, no genotypic differences in circulating endoglin, sVCAM-1, sICAM-1, or endothelin-1 levels were observed before or after aspirin treatment. Taken together, these results suggest that PEAR1 significantly impacts endothelial cell migration, adhesion, and proliferation, although further investigation is warranted to determine the influence of genetic variation and aspirin use on these traits.

Keywords: Platelet endothelial aggregation receptor 1, Migration, Adhesion, Proliferation.

Introduction

Platelet Endothelial Aggregation Receptor 1 (PEAR1), first described in 2005 by Nanda and colleagues, is a type 1 transmembrane tyrosine kinase receptor involved in a number of physiological processes including megakaryopoiesis, thrombopoiesis, platelet aggregation, and vasculogenesis[13,15,] While it is most highly expressed on the plasma membrane of endothelial cells and platelets, PEAR1 is found in multiple tissue and cell types, and has been previously implicated in cardiovascular disease risk[8,15]. At this time, an increasing body of evidence has led to a better understanding of the function of PEAR1 in platelets, where it acts to stabilize growing platelet aggregates downstream of platelet activation through secondary activation of glycoprotein IIb/IIIa activation[1,15]. Interestingly, recent investigations also suggest that PEAR1 is critical in endothelial function as a negative regulator of neoangiogenesis and wound healing[23,19].

Multiple genetic studies have consistently observed that a polymorphism in intron 1 of *PEAR1* (rs12041331) significantly impacts platelet- and endothelial-related functions of PEAR1[8,13,]. Indeed, recent work by Izzi and colleagues convincingly showed that genetic variability at rs12041331 results in allele-specific differences in DNA methylation and a subsequent reduction in PEAR1 expression in those who carry the minor allele of this polymorphism[10]; an observation that is consistent with previous reports[14]. Importantly, prior investigations have

also shown that the influence of *PEAR1* rs12041331 on platelet-related phenotypes and cardiovascular disease risk is influenced by antiplatelet therapy withaspirin[8,15].

In this investigation, we sought to better understand the relationship between PEAR1 and endothelial function in order to gain insights regarding the mechanism(s) bywhich this receptor may impact cardiovascular disease risk. Furthermore, we hypothesized that the effect of PEAR1 on endothelial-related phenotypes would be influenced by aspirin administration. To test these hypotheses, we utilized short hairpin RNA (shRNA)-mediated knockdown of *PEAR1* expression to investigate the impact of this gene on endothelial cell migration, adhesion, and proliferation pre- and post-aspirin administration in primary human umbilical vein endothelial cells (HUVECs). We extended our findings by performing experiments of endothelial cell migration, adhesion, and proliferation in the presence and absence of aspirin.in primary HUVECs stratified by *PEAR1* rs12041331 genotype. Finally, we performed a prospective genotype-directed short-term invention of aspirin (325 mg/day for 7 days) in 63 relatively healthy individuals to assess whether rs12041331 genotype was correlated with circulating biomarkers of endothelial function.

Methods

Reagents and Antibodies

Endothelial Basal Growth Medium 2 (EBM2) and all added supplements were purchased from Lonza (Walkersville, Maryland). Acetylsalicylic acid (ASA), gelatin solution (0.5%), as well as endoglin, sVCAM-1, and sICAM-1 ELISA kits were purchased from Sigma Aldrich (St. Louis, Missouri). Trypsin and Dulbecco's phosphate buffered saline (dPBS) were purchased from Mediatech, Inc. (Manassas, Virginia).

GentraPuregene Cell Kit was purchased from Qiagen (Valencia, California). RIPA lysis buffer and all added components were purchased from Teknova (Hollister, California). Gateway LR Clonase II Enzyme Mix was purchased from Invitrogen (Carlsbad, California). pENTR1A no ccDB (w48-1) and pCD/NL-BH*DDD (plasmid 17531) were purchased from Addgene (Cambridge, Massachusetts). Polybrene Transfection Reagent was purchased from EMD Millipore (Billerica, Massachusetts). cOmplete™ Protease Inhibitor Cocktail Tablets and the Rapid DNA Ligation Kit were purchased from Roche Diagnostics (Indianapolis, Indiana). TaqMan® SNP genotyping reagents, NuPAGE™ 4-12% Bis-Tris Mini Gels, XCellSureLock™ Mini-Cell Electrophoresis System, NuPAGE® MES SDS Running Buffer (20X), NuPAGE® Transfer Buffer (20X), Pierce™ BCA Protein Assay kit, and Pierce™ECLWesternBlottingSubstratewerepurchasedfrom ThermoFisherScientific (Waltham, Massachusetts). Laemmli Sample Buffer (2X), 2-Mercaptoethanol, and nitrocellulose membranes were purchased from Bio-Rad Laboratories(Hercules,California). Human PEAR1 antibody (antigen affinity-purified polyclonal goat-IgG) and donkey anti-goat IgG horseradish peroxidase-conjugated antibody were purchased from R&D Biosystems (Minneapolis, Minnesota). GAPDH (14C10) rabbit monoclonal antibody was purchased from Cell Signaling Technology, Inc. (Beverly, Massachusetts). Donkey anti-rabbit IgG horseradish peroxidase – conjugated antibody was purchased from Santa Cruz Biotechnology (Dallas, Texas). Luminata™Classico and Luminata™Cresendo Western horseradish peroxidase substrate were purchased from EMD Millipore (Billerica, Massachusetts). HEK293T cells and XTT Cell Proliferation Assay Kits were purchased from ATCC (Manassas, Virginia). IbidiibiTreat culture dishes with a µDish culture insert were purchased from Ibidi USA Inc. (Madison, Wisconsin). CytoSelect™ Cell Adhesion Assay Kits, polybrene transfection reagent, pVSVG envelope vector, and pSMPUWlentiviral vector were purchased from Cell Biolabs, Inc. (San Diego, California). Endothelin-1 ELISA kits were purchased from Enzo Life Sciences (Farmingdale, NewYork).

Cell culture

The umbilical vein was injected with 0.2% collagenase solution and incubated for 8 minutes at 37 ºC, after which the cord wasgently squeezed to dislodge and collect detached cells. Collected cells were centrifuged at 400 x g

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for 6 minutes and re-suspended in media for culture. Isolated HUVECs were cultured in complete EBM2 containing 2% FBS, 0.04% hydrocortisone, 0.4% hFGF, 0.1% VEGF, 0.1% R3-IGF, 0.1% ascorbic acid, 0.1% hEGF, 0.1% gentamicin- amphotericin-B [GA-1000], and 0.1% heparin and incubated under normal growth conditions (37 ºC, 5% CO_2). DNA from each cell line was extracted using a GentraPuregene Cell Kit according to the manufacturer's instructions. *PEAR1* rs12041331 genotype of each cell line was obtained using a TaqMan® SNP genotyping assay performed on an Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems 7900HT Sequence Detection System) according to the manufacturer's protocol. Six primary HUVEC lines that were wild-type for *PEAR1* rs12041331 (i.e. G/G homozygotes) were randomly selected to assess the effect of *PEAR1* knockdown on endothelial migration, extracellular matrix adhesion, and proliferation. Nine additional primary HUVEC cell lines (4 G/G, 3 G/A, and 2 A/A for rs12041331) were randomly selected to assess the functional impact of *PEAR1* rs12041331 genotype on endothelial migration, extracellular matrix adhesion, and proliferation.

Aspirin dosing

The impact of *PEAR1* knockdown and rs12041331 genotype on endothelial cell migration, adhesion, and proliferation was assessed in the presence and absence of ASA, the active ingredient in aspirin. For all *in vitro* experiments, ASA was dissolved in complete EBM2 at a concentration of 1 mM, consistent with physiological plasma concentrations of salicylate after therapeutic high-dose aspirin treatment[12] andpreviously published *in vitro* studies evaluating the impact of aspirin on endothelial function[163-165]. For the extracellular adhesion assay, each cell line was treated with ASA for 24 hours. After incubation under normal growth conditions, all cells were collected, counted, and re-suspended at a density of 5 x 10^5 cells/ml in serum-free EBM2. For migration and proliferation assays, untreated cells were re-suspended at a density of 5 x 10^5 cells/ml in complete EMB2 with and without freshly prepared ASA and incubated for 24 hours under normal growth conditions prior to testing.

Measurement of endothelial cell proliferation

An XTT Cell Proliferation Assay Kit was utilized to evaluate HUVEC proliferation in each treatment group. Briefly, aspirin-treated and non-treated cells (100µl at 5.0 x 10^{-5} cells/ml) were added to 96-well microtiter plates and proliferation was determined colorimetrically at 24 hours using a Perkin-Elmer Victor X3 plate reader (PerkinElmer, Inc., Waltham, Massachusetts). All measurements were made in triplicate.

Biomarker testing

Circulating levels of endoglin, endothelin-1, sVCAM-1, and sICAM-1were measured pre- and post-aspirin administration through the use of commercially available enzyme-linked immunosorbent assays according to manufacturer's instructions. Each sample was measured in duplicate using a Perkin-Elmer Victor X3 plate reader (PerkinElmer, Inc., Waltham, Massachusetts) and concentrations of each sample were calculated using a 4-paramater logistic curve. Duplicate samples with a coefficient of variation ≤ 15% were re-tested.

Statistical Analysis

PEAR1 expression after shRNA knockdown was statistically evaluated using a paired t-test implemented in R. For experiments evaluating the influence of *PEAR1* knockdown on endothelial cell migration, adhesion, and proliferation, a repeated measures ANOVA implemented in R was used to determine statistical significance.

Comparison groups for each assay of *PEAR1* knockdown were control vs knockdown and ASA-treated control vs ASA-treated knockdown. *PEAR1* genotype-specific *in vitro* tests of endothelial function were conducted using a one-way ANOVA implemented in R. The effect of genotype was assessed assuming an additive model (G/G = 0, G/A = 1, and A/A

= 2). The impact of aspirin on endothelial cell migration, adhesion, and proliferation was assessed by repeated measures ANOVA in R.

The effect of aspirin on circulating biomarkers of endothelial function was assessed by repeated measures

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ANOVA in R. Given the cryptic relatedness between members of the Amish community, mixed models analyses were performed under an additive genetic effect (G/G = 0, G/A = 1 and A/A = 2) using Mixed Model Analysis for Pedigrees (MMAP; http://edn.som.umaryland.edu/mmap/) to assess the association between *PEAR1* rs12041331 and circulating biomarkers of endothelial function. MMAP conditions the genotype-phenotype correlations on the phenotypic correlations among relatives to account for relatedness between study participants. To model the polygenic component, a relationship matrix was developed from the complete Amish pedigree available through published genealogical records[119]. MMAP has been extensively used for genomic analysis in the Amish [7,8,53,91,117,118,128]. All analyses in MMAP were conducted with adjustment for age, sex, and participant relatedness. All statistical analyses were 2-sided and $P < 0.05$ was considered statistically significant.

Results

PEAR1 knockdown impacts endothelial cell migration, adhesion, and proliferation

To determine the role of *PEAR1* in endothelial cell migration, adhesion, and proliferation, primary HUVECs were transfected with lentiviral constructs expressing either a shRNA targeting *PEAR1* or a scrambled control shRNA. Western blot analysis confirmed reduction of PEAR1 protein expression by approximately 49% in HUVECs infected with *PEAR1*-targeting shRNA compared to cells infected with a scrambled control shRNA (P=0.001, Figure 1). Transfection efficiency was assessed through infection of cells with a lentiviral construct expressing GFP.

The impact of PEAR1 on endothelial cell migration was functionally evaluated *in vitro* using a modified version of the well-described scratch assay[167]. Differences in HUVEC migration between cells transfected with either *PEAR1* or control shRNA- expressing lentiviral vectors were assessed 6 hours post-insert removal. We observed that shRNA-mediated knockdown of *PEAR1* significantly reduced HUVEC migration comparedtocellsinfectedwithcontrolshRNA(P=0.008,95.4 ±24.7μmand118.5±

26.4 μm, respectively, Figure 2).

Endothelial cell adhesion to extracellular matrix proteins is critical for angiogenesis, vasculogenesis, and maintenance of vascular endothelium[31]. The adhesive properties of HUVECs infected with either *PEAR1* or control shRNA-expressing lentiviral vectors to fibronectin, collagen I, collagen IV, laminin I, fibrinogen, and bovine serum albumin (BSA) were evaluated in 6 independent cell lines. We observed that *PEAR1* knockdown resulted in a strong reduction in cellular adhesion to collagen I compared to cells that were infected with viral particles expressing control shRNA (P =0.008, Figure 3). Further, adhesion to fibronectin and fibrinogen was modestly reduced in cells infected with *PEAR1* shRNA-expressing lentiviral vectors compared to control shRNA-expressing particles (P = 0.04 and 0.04, respectively). No differences in cellular adhesion between *PEAR1* knockdown and control groups were observed when collagen IV, laminin I, or BSA was used to stimulate adhesion (P = 0.12, 0.09, and 0.06, respectively, Figure 3).

Endothelial cell proliferation in HUVECs infected with either *PEAR1* or control shRNA-expressing lentiviral vectors was assessed at 24 hours. Using 6 primary HUVEC lines, we observed that cellular proliferation was significantly reduced in HUVECs infected with *PEAR1*-targeting shRNA compared to cells infected with a scrambled control shRNA (P = 0.003, Figure 4).

Aspirin treatment influences PEAR1-mediated effects on endothelial function

Given that prior investigations have shown that the impact of genetic variation in *PEAR1* is influenced by aspirin treatment[8,104,156,158] , we also evaluated the role of *PEAR1* knockdown on endothelial cell migration, adhesion and proliferation after ASA exposure (1 mM for 24 hours). While we did observe significant differences in endothelial cell migration in untreated *PEAR1* knockdown vs. control HUVECs (see above), no difference in migration between cells infected with *PEAR1*-targeting shRNA compared to cells infected with a scrambled control shRNA was

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observed after ASA exposure (P = 0.13, 113.5 ± 19.4 µm and 103.7 ± 26.4 µm, respectively, Figure 2). Similarly, while differences in cellular adhesion to extracellular matrix proteins (i.e. collagen I) were observed between *PEAR1* knockdown and control groups prior to ASA treatment, nodifference in adhesion to fibronectin, collagen I, collagen IV, laminin I, fibrinogen, and BSA was observed between these groups after exposure to ASA (P = 0.29, 0.18, 0.65, 0.37, 0.16, and 0.97, respectively, Figure 3). Endothelial cell proliferation was significantly different between HUVECs infected with *PEAR1*-targeting shRNA compared to control shRNA after aspirin treatment (P = 0.003), similar to the results observed in untreated cells (Figure 4). The influence of aspirin exposure on endothelial cell migration, adhesion, and proliferation in control HUVECs and *PEAR1* knockdown HUVECs is shown in Table 1 and 2, respectively.

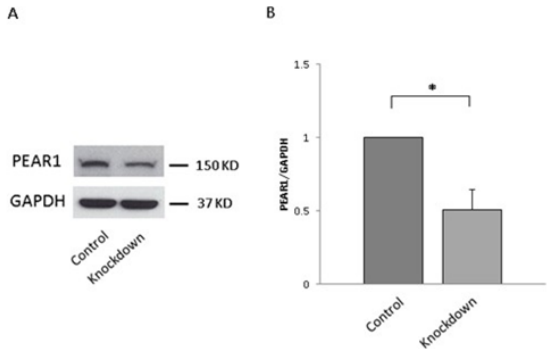

Figure 1. shRNA-mediated *PEAR1* knockdown. A)

Western blot analysis revealed that PEAR1-targeting shRNA (shPEAR1-2058) decreased PEAR1 protein expression relative to control shRNA. A 0.1% SDS-PAGE gel was used for protein separation. **B)** PEAR1 protein levels, relative to GAPDH, was analyzed using ImageJ in 5 independent human umbilical vein endothelial cell lines. Abbreviations: GAPDH, glyceraldehyde 3- phosphate dehydrogenase; PEAR1, platelet endothelial aggregation receptor 1. * P < 0.05.

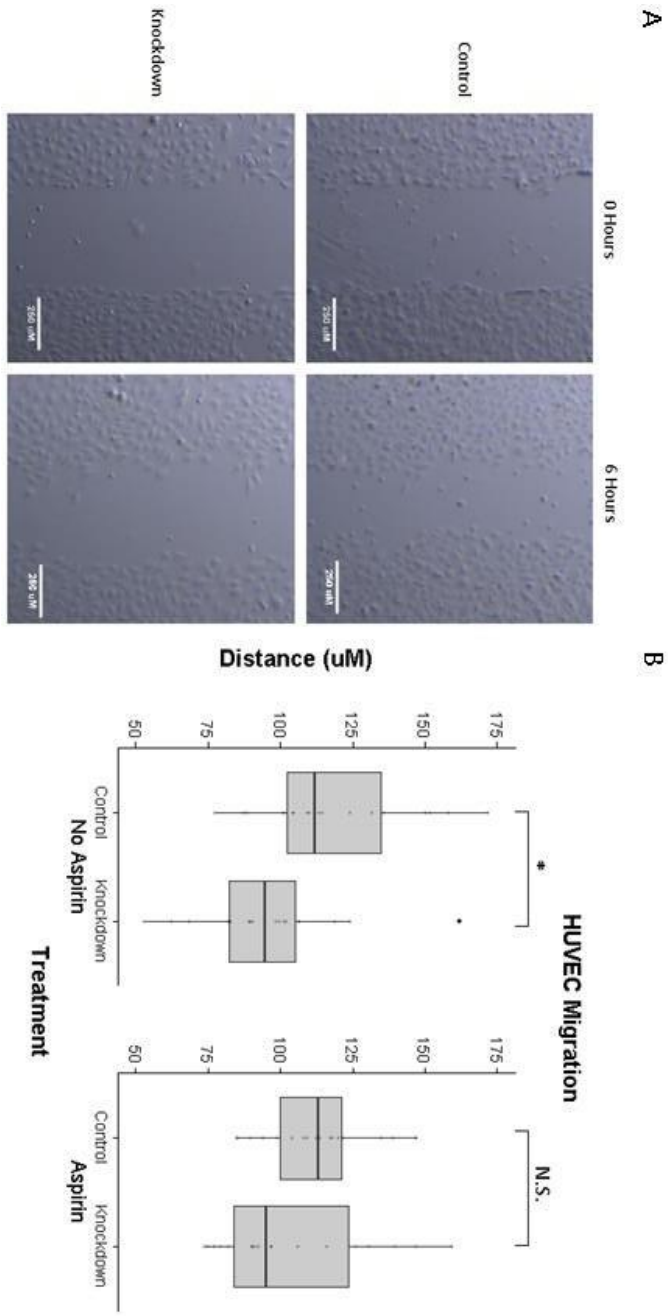

Figure 2. The impact of *PEAR1* knockdown on endothelial cell migration. A)

In untreated and aspirin-treated (1 mM for 24 hours) HUVECs, we evaluated the impact of shRNA-mediated knockdown of *PEAR1* on endothelial cell migration relative to cells infected with control shRNA (6 independent HUVEC lines). **B)** Phase-contrast microscopy images of human umbilical vein endothelial cells infected with lentiviral constructs expressing either *PEAR1*-targeting (shPEAR1-2058) or control shRNA in the absence or presence of aspirin. Images are shown at 20 X

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magnification. Abbreviations: PEAR1, platelet endothelial aggregation receptor 1; N.S., no significant difference. * P < 0.05

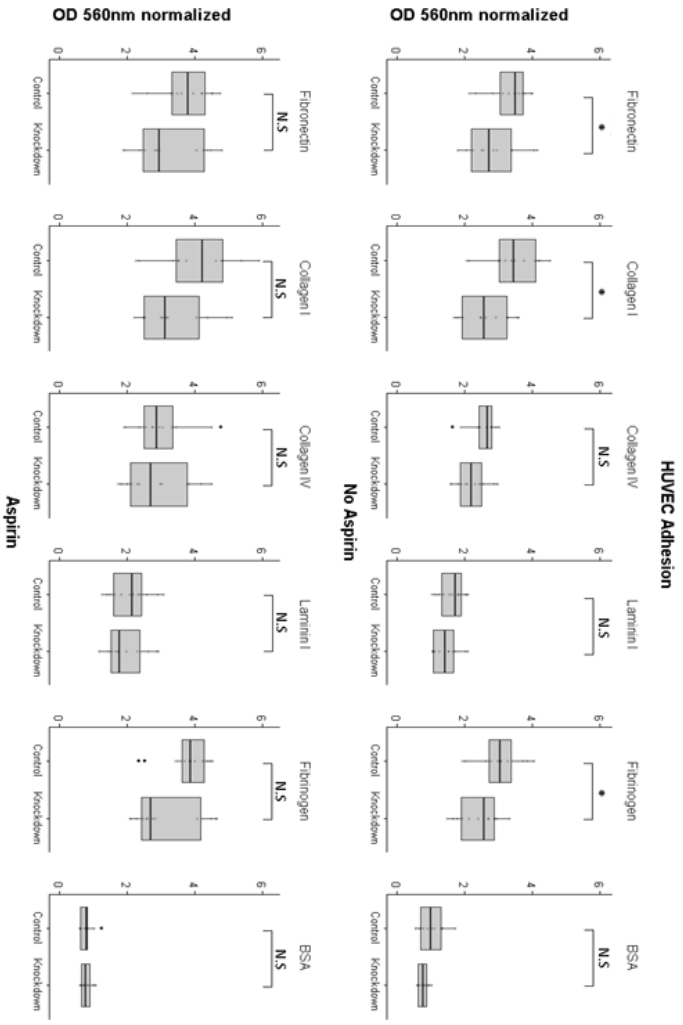

Figure 3. The influence of *PEAR1* knockdown on cellular adhesion to extracellular matrix proteins. In untreated and aspirin-treated (1 mM for 24 hours) human umbilical vein endothelial cells, we tested the influence of shRNA-mediated knockdown of *PEAR1* on adhesion to the extracellular proteins fibronectin, collagen I, collagen IV, laminin I, fibrinogen, as well as BSA relative to cells infected with control shRNA (6 independent HUVEC lines). Adhesion to each extracellular matrix protein was quantified using OD560 nm as described in the Methods section and normalized to BSA binding in untreated control HUVECs. Abbreviations: BSA, bovine serum albumin; PEAR1, platelet endothelial aggregation receptor 1; N.S., no significant difference. * P < 0.05

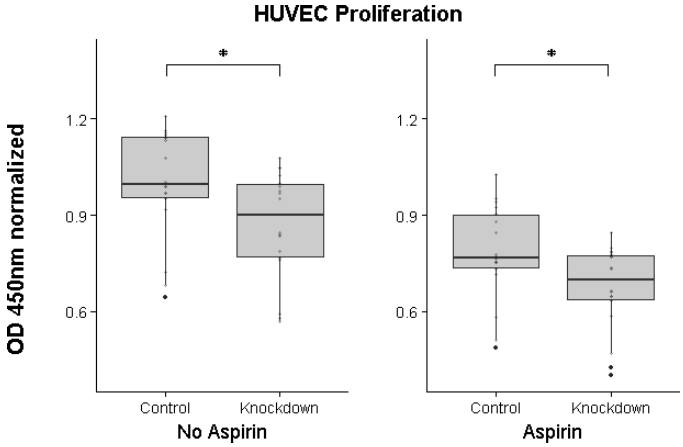

Figure 4. *PEAR1* knockdown results in reduced proliferation in untreated and ASA-treated endothelial cells.

In untreated and aspirin-treated (1 mM for 24 hours) human umbilical vein endothelial cells, we tested the influence of shRNA-mediated knockdown of *PEAR1* on cellular proliferation in 6 independent cell lines. Proliferation was quantified using OD450 nm as described in the Methods section and normalized to the proliferation of untreated control HUVECs. Abbreviations: ASA, acetylsalicylic acid; PEAR1, platelet endothelial aggregation receptor 1; N.S., no significant difference. * P < 0.05

Table 1. Effect of ASA in control HUVECs (n=6)

	No Aspirin	Aspirin	P-value
Migration	118.47 ± 26.40	113.46 ± 19.41	0.34
Fibronectin	0.38 ± 0.07	0.42 ± 0.09	0.29
Collagen I	0.39 ± 0.09	0.46 ± 0.13	0.09
Collagen IV	0.29 ± 0.05	0.34 ± 0.10	0.25
Laminin I	0.19 ± 0.04	0.24 ± 0.07	0.02
Fibrinogen	0.34 ± 0.07	0.43 ± 0.08	0.06
BSA	0.11 ± 0.04	0.09 ± 0.02	0.08
Proliferation	1.52 ± 0.26	1.19 ± 0.22	3.52×10^{-6}

Values are listed as mean ± standard deviation. Migration distance measured as μM. Extracellular matrix adhesion measured as OD 560nM. Proliferation measured as OD 450nM.

Table 2. Effect of ASA in *PEAR1* knockdown HUVECs (n=6)

	No Aspirin	Aspirin	P-value
Migration	95.36 ± 24.67	103.74 ± 26.41	0.29
Fibronectin	0.32 ± 0.09	0.37 ± 0.12	0.42
Collagen I	0.30 ± 0.08	0.38 ± 0.11	0.11
Collagen IV	0.25 ± 0.05	0.33 ± 0.11	0.12
Laminin I	0.16 ± 0.04	0.22 ± 0.07	0.03
Fibrinogen	0.27 ± 0.07	0.35 ± 0.11	0.13
BSA	0.09 ± 0.02	0.09 ± 0.02	0.48
Proliferation	1.32 ± 0.25	1.02 ± 0.20	1.53×10^{-5}

Values are listed as mean ± standard deviation. Migration distance measured as μM. Extracellular matrix adhesion measured as OD 560nM. Proliferation measured as OD 450nM. Evaluation of PEAR1 rs12041331 on endothelial function

In addition to investigating the effect of *PEAR1* knockdown on endothelial cell migration, adhesion, and proliferation, we also assessed whether the well-described *PEAR1* rs12041331 variant significantly influenced these

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phenotypes as well as protein expression. In 9 primary HUVEC lines that were selected based on rs12041331 genotype (4 G/G, 3 G/A, and 2 A/A), PEAR1 protein expression was moderately reduced in A- allele carriers, though this difference was not significant (P=0.45, Figure 5).

Genotypic differences in endothelial cell migration were assessed at 6 hours post- insert removal. In contrast to the results obtained after shRNA-mediated knockdown of *PEAR1*, no difference in endothelial migration was observed between genotype groups prior to ASA treatment (P = 0.28, 138.3 ± 47.5 μm for the 4 G/G cell lines, 120.5 ± 20.9

μm for the 3 G/A cell lines, and 121.343 ± 19.3 μm for the 2 A/A cell lines). Similarly, no difference in endothelial cell migration was observed by *PEAR1* rs12041331 genotype after ASA exposure (P = 0.99, 118.1 ± 40.3 μm for the 4 G/G cell lines, 115.7 ± 34.9 μm for the 3 G/A cell lines, and 118.7 ± 10.5 μm for the 2 A/A cell lines)

Discussion

The endothelial monolayer that lines the interior of blood vessels serves as a protective barrier between the blood and surrounding tissue and is critical for maintaining cardiovascular health20. The major functions of the endothelium include regulating hemostatic balance, vascular tone, wound healing, angiogenesis, and inflammation20.

During wound repair and angiogenesis, endothelial cells must be able to migrate fromthe vasculature, adhere to the extracellular matrix, and proliferate to form new vasculature31. Risk factors such as high lipid levels and tobacco use20,26, as well as genetic variation in genes involved in essential endothelial processes compromise the integrity of the endothelium and result in increased risk of cardiovascular diseases. Given the results of previous investigations by our group and others53,108,160, we hypothesized that PEAR1 was an important determinant of endothelial cell function and that changes in *PEAR1* expression would influence endothelial-related processes important in cardiovascular- relatedhealth.

PEAR1 is a recently identified 150 kDa transmembrane protein involved in a number of diverse biological processes including phagocytosis, thrombosis, and vasculogenesis1,10, and has been previously implicated in risk of myocardial infarction8. While PEAR1 is well-characterized as an important determinant of growing platelet aggregates through induction of secondary glycoprotein IIb/IIIa activation1, the function of PEAR1 in the endothelium, where expression is 6-fold higher than in platelets15, is less understood, though recent investigations suggest a significant role in angiogenesis and wound healing3,18. In this investigation, we extend on previous studiesby showing that PEAR1 knockdown significantly impacts endothelial cell migration, adhesion, and proliferation.

Through the use of shRNA-mediated knockdown of *PEAR1* in primary HUVECs, which resulted in a 50% reduction in protein expression, we observed an approximate 20% reduction in endothelial cell migration compared to cells treated with a control shRNA. In addition, given the known role of PEAR1 as a contact-contact receptor that propagates thrombus formation through secondary stabilization of aggregation-based pathways, we also evaluated the influence of PEAR1 knockdown on endothelial cell binding capacity to several extracellular matrix proteins critical in cellular adhesion. We observed that reduction in PEAR1 expression impacted the ability of endothelial cells to bind to collagen I by approximately 25%. Interestingly, we also observed that HUVEC binding capacity to fibrinogen and fibronectin was reduced upon *PEAR1* knockdown (P =

0.04 and 0.04, respectively). No difference in cellular adhesion to collagen IV or laminin I was observed upon *PEAR1* knockdown. Finally, we evaluated the influence of PEAR1 on the ability of HUVECs to proliferate and observed an approximate 15% reduction in proliferation upon *PEAR1* knockdown compared to control cells.

While gene knockdown is a useful method to elucidate novel functions of genes with unknown or poorly understood features, the impact of naturally occurring genetic variation in those genes can better define the

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importance of these functions *in vivo* and is often more clinically relevant. Therefore, we complemented our *PEAR1* knockdown experiments by evaluating the effect of rs12041331, the most widely investigated polymorphism in *PEAR1*, on HUVEC migration, adhesion and proliferation. Previous investigations have consistently shown that rs12041331 genotype significantly influences

platelet and endothelial-related functions of PEAR18,53,61,104,145,156 and is associated with cardiovascular disease risk8. Moreover, rs12041331 was recently shown to modify *PEAR1* expression through disruption of a DNA methylation site, presumably leading to differences in transcription factor binding capacity160. Though there was a trend for reduced PEAR1 protein expression with the minor allele of rs12041331, this difference was not statistically significant. We did not find increasing copies of the rs12041331 minor allele to be significantly associated with reduced adhesion to collagen I, collagen IV, fibrinogen, laminin I, or fibronectin. This stands in contrast to the results obtained after shRNA-mediated knockdown of *PEAR1*, and is likely due to the high variability in PEAR1 expression observed in A/A cell lines. Further, no difference in endothelial cell migration or proliferation was observed by rs12041331 genotype.

In this study, we show that *PEAR1* knockdown modifies basic processes of endothelial function. *PEAR1* expression was strongly correlated with HUVEC migration,adhesion, and proliferation prior to aspirin exposure. However, after ASA treatment, only HUVEC proliferation was impacted by *PEAR1* knockdown. Endothelial dysfunction is characterized by an increased adhesive capacity of the endothelium, as well as a decreased repair capacity for wound healing, both of which contribute to increased thrombotic risk65,172,173. While the discordance in the scant published studies on PEAR1 endothelial function makes it difficult to speculate how deleterious genetic variation in *PEAR1* might impact endothelial health *in vivo*, the observed association with extracellular matrix adhesion after *PEAR1* knockdown makes biological sense in consideration of the known role for PEAR1 as a platelet contact receptor. Future investigations of PEAR1 signaling as well as evaluations of endothelial biomarkers in at- risk patients with known *PEAR1* genotype will provide further clarity to the mechanisms by which PEAR1 functions and offer insight into the influence of PEAR1 in the context of cardiovascular disease. Additionally, variation in *PEAR1* may augment the effectiveness of aspirin, which has implications for at-risk patients in need of antiplatelet therapy. Further research is needed to characterize PEAR1 in the endothelium, as well as further clarify a potential interaction between *PEAR1* variation and aspirin response.

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