Targeting pericyte–endothelial cell crosstalk by circular RNA-cPWWP2A inhibition aggravates diabetes-induced microvascular dysfunction

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The crosstalk between vascular pericytes and endothelial cells (ECs) is critical for microvascular stabilization and remodeling; however, the crosstalk is often disrupted by diabetes, leading to severe and even lethal vascular damage. Circular RNAs are a class of endogenous RNAs that regulate several important physiological and pathological processes. Here we show that diabetes-related stress up-regulates cPWWP2A expression in pericytes but not in ECs. In vitro studies show that cPWWP2A directly regulates pericyte biology but indirectly regulates EC biology via exosomes carrying cPWWP2A. cPWWP2A acts as an endogenous miR-579 sponge to sequester and inhibit miR-579 activity, leading to increased expression of angiopoietin 1, occludin, and SIRT1. In vivo studies show that cPWWP2A overexpression or miR-579 inhibition alleviates diabetes mellitus-induced retinal vascular dysfunction. By contrast, inhibition of cPWWP2A-mediated signaling by silencing cPWWP2A or overexpressing miR-579 aggravates retinal vascular dysfunction. Collectively, this study unveils a mechanism by which pericytes and ECs communicate. Intervention of cPWWP2A or miR-579 expression may offer opportunities for treating diabetic microvascular complications.

circular RNA | endothelial cell | pericyte | retinal vascular dysfunction | microRNA sponge

Diabetes mellitus is a class of metabolic disorders characterized by hyperglycemia. Frequent and long-standing hyperglycemia can lead to a host of microvascular complications, including diabetic retinopathy (DR), diabetic nephropathy, diabetic neuropathy, and diabetic foot disorders (1). Retinal vasculature is known as the early and prevalent target in response to hyperglycemia injury. The onset of diabetic retinopathy is characterized by several morphologic changes, including loss of pericytes, thickening of basement membrane, increased vascular permeability, vascular occlusion, and microaneurysm. Retinal vascular cells mainly include pericytes and endothelial cells (ECs). Pericyte loss is the early pathologic feature of diabetic retinopathy, consistently present in the retinas of diabetic patients and animals (2, 3). Retinal ECs experience a phenotypic switch from a normal quiescent phenotype to an apoptotic and active phenotype (4). Retinal EC dysfunction can lead to increased vascular permeability, macula edema, and angiogenesis. Normal pericyte–EC crosstalk is important for the development and homeostasis of retinal vasculature. Abnormal pericyte–EC crosstalk can lead to retinal vascular leakage, obliteration, and formation of new vessels (5, 6). Thus, therapeutic intervention based on the regulation of pericyte–EC crosstalk would provide a method for the prevention and protection against diabetes-induced retinal vascular injury.

Currently, it still remains a great challenge to define the key regulators of gene expression during retinal vascular injury. Circular RNAs (circRNAs) are a class of abundant, stable, and ubiquitous RNA transcripts, which are formed by back splicing from the primary RNA transcripts (7). circRNAs often exhibit tissue-specific or developmentally specific expression pattern. They regulate gene expression by acting as microRNA (miRNA) sponges, RNA-binding protein sequestering agents, or transcriptional regulators (8, 9). Increasing evidence has shown that circRNAs play important roles in cell proliferation, apoptosis, mobility, and differentiation (7, 10, 11). circRNA dysregulation has been observed in several diseases, such as neurological diseases, cardiovascular diseases, cancers, and diabetes (12–15). Recently, several studies have highlighted the importance of circular RNAs in vascular EC dysfunction (16–19). However, the role of circRNAs in pericyte and pericyte–EC crosstalk remains unclear.

In this study, we reveal that diabetes-related stress could up-regulate cPWWP2A expression in pericytes. cPWWP2A could be transferred from pericytes to ECs through the exosomes. Dysregulated cPWWP2A leads to abnormal pericyte–EC crosstalk in vitro and retinal vascular dysfunction in vivo, suggesting that cPWWP2A is an important regulator in diabetes-induced retinal vascular pathogenesis.

Results

circRNA Expression Profiling Identifies cPWWP2A as a Potential Regulator of Diabetic Retinopathy. To investigate the potential involvement of circRNAs in diabetic retinopathy, total RNAs were isolated

Significance

The crosstalk between vascular endothelial cells (ECs) and pericytes in the microvascular is critical for vascular homeostasis and remodeling. However, the crosstalk between these two cells is often disrupted by diabetes, resulting in severe and even lethal vascular defects. Here, we show that diabetes-related stress up-regulates cPWWP2A expression in pericytes but not in ECs. Pericyte-derived cPWWP2A affects pericyte coverage and vascular integrity through interacting with miR-579 and its target genes, including angiopoietin 1/occludin/SIRT1. Our study thus reveals a critical role of cPWWP2A-mediated signaling in retinal microvascular dysfunction and implies potential therapeutic benefit with high-level expression of cPWWP2A or reduced miR-579 expression.

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from the retinas of leptin-receptor–deficient db/db mice or age-matched nondiabetic controls, respectively. circRNA microarray profiling was conducted using the Shanghai Biotechnology Company Mouse Circular RNA Microarray. A total of 844 circRNAs with gene annotations in circBase were differentially expressed between diabetic retinas and nondiabetic retinas, including 433 up-regulated circRNAs and 411 down-regulated circRNAs (selection criteria: fold change >3 and $P < 0.01$; *SI Appendix*, Table S1). We mainly focused on these circRNAs with the same genomic length and spliced length to rule out the possibility that these genes may undergo unannotated splicing regulation to generate unknown transcripts. We randomly selected 20 up-regulated and 20 down-regulated circRNAs to conduct hierarchical clustering analysis to obtain an overview comparison of circRNA profiling. The diabetic samples were clustered together into the same branch, whereas the nondiabetic samples were clustered into the other branch (Fig. 1*A*). We also conducted qRT-PCRs to verify the result of microarray. The expression patterns of 14 of 20 up-regulated and 16 of 20 down-regulated circRNAs were verified (*SI Appendix*, Table S2).

The top three up-regulated circRNAs were ranked: mmu_circ_0010536, mmu_circ_0004367, and mmu_circ_0000254. We then searched for the human homologous genes of these circRNAs. We only found the human homologous gene for mmu_circ_0000254, hsa_circ_0074837. The similarity of gene sequence was about 89% between human genome and mouse genome (*SI Appendix*, Fig. S1). The mmu_circ_0000254 was named as cPWWP2A in the subsequent study because its host gene is PWWP2A.

The sequence of cPWWP2A obtained by Sanger sequencing was consistent with the sequence of cPWWP2A annotated in circBase (Fig. 1*B*). cPWWP2A was resistant to RNase R digestion, whereas linear PWWP2A mRNA was easily degraded (Fig. 1*C*). qRT-PCR assays showed that retinal cPWWP2A expression was significantly higher in streptozotocin (STZ)-induced diabetic mice than that in nondiabetic controls (Fig. 1*D*). A similar event was observed in the retinas of db/db mice, a type 2 diabetic model, compared with their nondiabetic controls (Fig. 1*E*). By contrast, insulin treatment significantly decreased retinal cPWWP2A expression (Fig. 1 *D* and *E*). Clinical sample analysis showed that cPWWP2A expression in the fibrovascular membrane of a diabetic patient was significantly higher than that in the idiopathic epiretinal membrane of a nondiabetic control (Fig. 1*F* and *SI Appendix*, Table S3). Collectively, these results suggest that cPWWP2A is a potential regulator of diabetic retinopathy.

cPWWP2A Regulates Diabetes-Induced Retinal Vascular Dysfunction in Vivo. We first determined the effects of cPWWP2A silencing on diabetes-induced retinal vascular dysfunction in vivo. Three different adeno-associated viral shRNAs were designed for cPWWP2A silencing. cPWWP2A shRNA injection significantly decreased retinal cPWWP2A expression but did not alter cPWWP2A mRNA expression. We selected shRNA1 for cPWWP2A silencing because it had the best gene silencing efficiency. Moreover, cPWWP2A shRNA1 injection significantly decreased retinal cPWWP2A but not PWWP2A mRNA expression throughout the experiment (*SI Appendix*, Fig. S2).

Immunofluorescent staining showed that cPWWP2A silencing significantly decreased pericyte coverage of retinal vasculature (Fig. 2 *A* and *B*). Loss or degeneration of pericyte could alter retinal vascular permeability (20). Evans blue assays showed that compared with diabetic retina, cPWWP2A silencing aggravated diabetes-induced retinal vascular leakage (Fig. 2 *C* and *D*). Pericyte loss, microaneurysms, and acellular microvascular are the important characteristics of diabetic retinas (3). Trypsin digests of retinal vasculature revealed that cPWWP2A silencing further increased the number of acellular vascular, pericyte loss, and microaneurysm in diabetic retinas (Fig. 2 *E*–*H*). ELISAs showed that cPWWP2A silencing aggravated diabetes-induced retinal inflammation as shown by increased expression of interleukin (IL)-2, IL6, TNF-α, VEGF, and MCP-1 (Fig. 2*I*).

We then investigated the effects of cPWWP2A overexpression on diabetes-induced retinal vascular dysfunction. cPWWP2A overexpression protected pericyte against diabetes-induced injury as shown by increased pericyte coverage, reduced retinal vascular leakage, decreased number of acellular vascular, decreased pericyte loss, and decreased microaneurysm (*SI Appendix*, Fig. S3). Collectively, these results suggest that cPWWP2A regulates retinal pericyte function and vascular integrity in vivo.

cPWWP2A Regulates Retinal Pericyte Function and Pericyte–EC Crosstalk in Vitro. Retinal vascular cells mainly include pericytes and ECs (21). Diabetes-related stresses, such as high glucose, oxidative stress, and inflammatory stimulus, significantly up-regulated cPWWP2A expression in pericytes (*SI Appendix*, Fig. S4 *A* and *C*) but did not alter cPWWP2A expression in ECs (*SI Appendix*, Fig. S4 *B* and *D*).

Pericyte loss is a hallmark of early diabetic retinopathy due to the impact of hyperglycemia on retinal vascular injury (3). cPWWP2A siRNA transfection significantly decreased cPWWP2A expression but did not alter PWWP2A expression (*SI Appendix*, Fig. S5). Pericytes were exposed to high glucose to mimic diabetic condition in vitro. Propidium iodide (PI) staining and caspase 3/7 activity assays showed that cPWWP2A silencing aggravated high glucose stress-induced pericyte apoptosis as shown by increased number of PI-positive cells (Fig. 3*A*) and caspase 3/7 activity (Fig. 3*B*).

Proliferative diabetic retinopathy usually involves destructive retinal angiogenesis. Normal pericyte function and pericyte–EC crosstalk is important for retinal vascular stability and the maturation of nascent vasculature (2). cPWWP2A silencing led to decreased expression of pericyte markers, including platelet-derived growth factor receptor (PDGF)-β, α-SMA, Desmin, and NG2 (Fig. 3*C*). Ki67 staining showed that cPWWP2A silencing decreased pericyte proliferation (Fig. 3*D*). In a coculture model of pericyte and human retinal vascular endothelial cells (HRVECs), cPWWP2A silencing reduced perictyes recruitment toward HRVECs (Fig. 3*E*). cPWWP2A silencing in pericyte also significantly increased endothelial permeability of macromolecules compared with the control group (Fig. 3*F*).

cPWWP2A Regulates Pericyte Function by Acting as a miRNA Sponge in Vitro. RNA-FISH assays showed that cPWWP2A was mainly expressed in the cytoplasm of pericytes (Fig. 4*A*). We speculated that cPWWP2A might act as a miRNA sponge to regulate gene expression. Ago2 protein is a core component of RNA-induced silencing complex (RISC) that binds miRNA complexes to target mRNAs (22). RNA immunoprecipitation (RIP) assays revealed that endogenous cPWWP2A was specifically pulled down by Ago2 antibody but not IgG (Fig. 4*B*). The entire cPWWP2A sequence was inserted into pGL3 luciferase reporter to create a LUC-cPWWP2A vector. We searched for cPWWP2A-interacting miRNAs by the TargetScan algorithm. Luciferase activity screening showed that miR-579 mimic transfection significantly decreased the activity of LUC-cPWWP2A (Fig. 4*C*). RNA-FISH assays demonstrated the colocalization between cPWWP2A and miR-579 in pericytes (Fig. 4*D*). The binding sites of miR-579 on cPWWP2A are shown in Fig. 4*E*.

cPWWP2A–miR-579–Angiopoietin 1/Occludin/SIRT1 Network Regulates Pericyte Function in Vitro. Next, we predicted the potential target genes of miR-579. Three candidate genes, including angiopoietin 1/occludin/SIRT1, attracted our interest due to their roles in maintaining vascular stability (23). The luciferase reporter gene was fused to the 3′-UTRs of angiopoietin 1/occludin/SIRT1, respectively. Luciferase reporter assay showed that overexpression of miR-579 decreased the luciferase activity for each of the putative target genes, whereas mutation of the putative miR-579 binding site within angiopoietin 1/occludin/SIRT1 prevented miR-579 overexpression-induced decrease in luciferase activity (Fig. 5*A*). We also showed that cPWWP2A silencing significantly reduced the expression of angiopoietin 1/occludin/SIRT1 in vitro (Fig. 5*B*).

We then investigated the role of miR-579 in pericyte biology and pericyte–endothelial cell crosstalk in vitro. miR-579 up-regulation

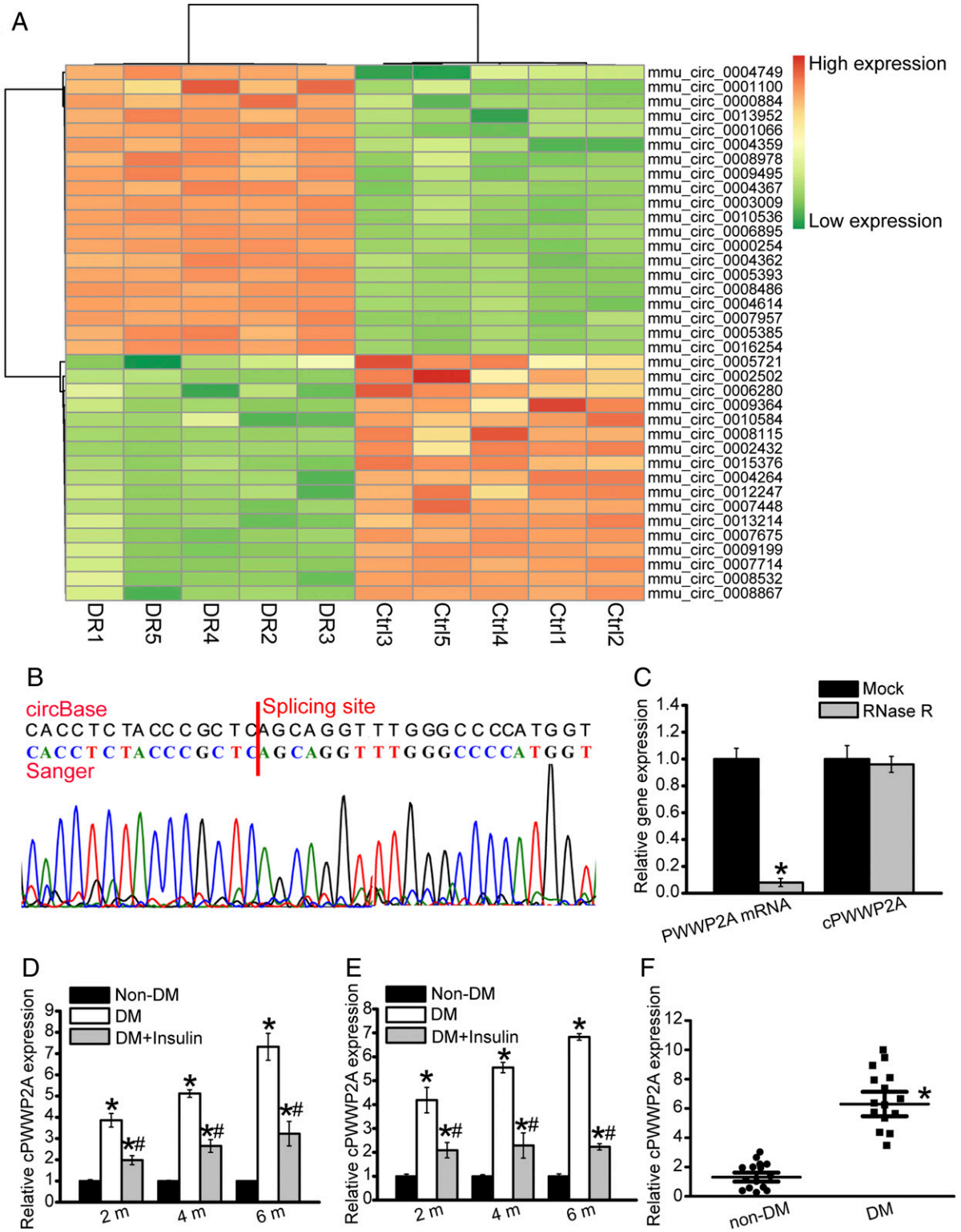

Fig. 1. circRNA expression profiling identifies cPWWP2A as a potential regulator of diabetic retinopathy. (*A*) Hierarchical cluster analysis was conducted to obtain the overview of circRNA expression profiles between nondiabetic retinas and diabetic retinas. (*B*) Sanger sequencing was conducted to detect retinal cPWWP2A expression. The obtained cPWWP2A sequence (*Lower* part) was consistent with the sequence of cPWWP2A annotated in circBase (*Upper* part). (*C*) Retinal RNAs were digested with RNase R followed by qRT-PCR detection of cPWWP2A expression. PWWP2A mRNA was detected as the RNase R-sensitive control ($n = 4$, Student's t test). (*D* and *E*) qRT-PCRs were conducted to detect cPWWP2A expression in STZ-induced diabetic retinas, db/db diabetic retinas, and their nondiabetic controls with or without insulin treatment ($n = 4$, two-way ANOVA followed by Bonferroni's test, $^{*}P < 0.05$ versus nondiabetic, $^{\#}P < 0.05$ diabetic versus diabetic plus insulin). (*F*) qRT-PCR assays were conducted to detect cPWWP2A expression in the fibrovascular membranes of diabetic patients and idiopathic epiretinal membranes of nondiabetic patients ($^{*}P < 0.05$ versus nondiabetic, two-tailed Student's t test).

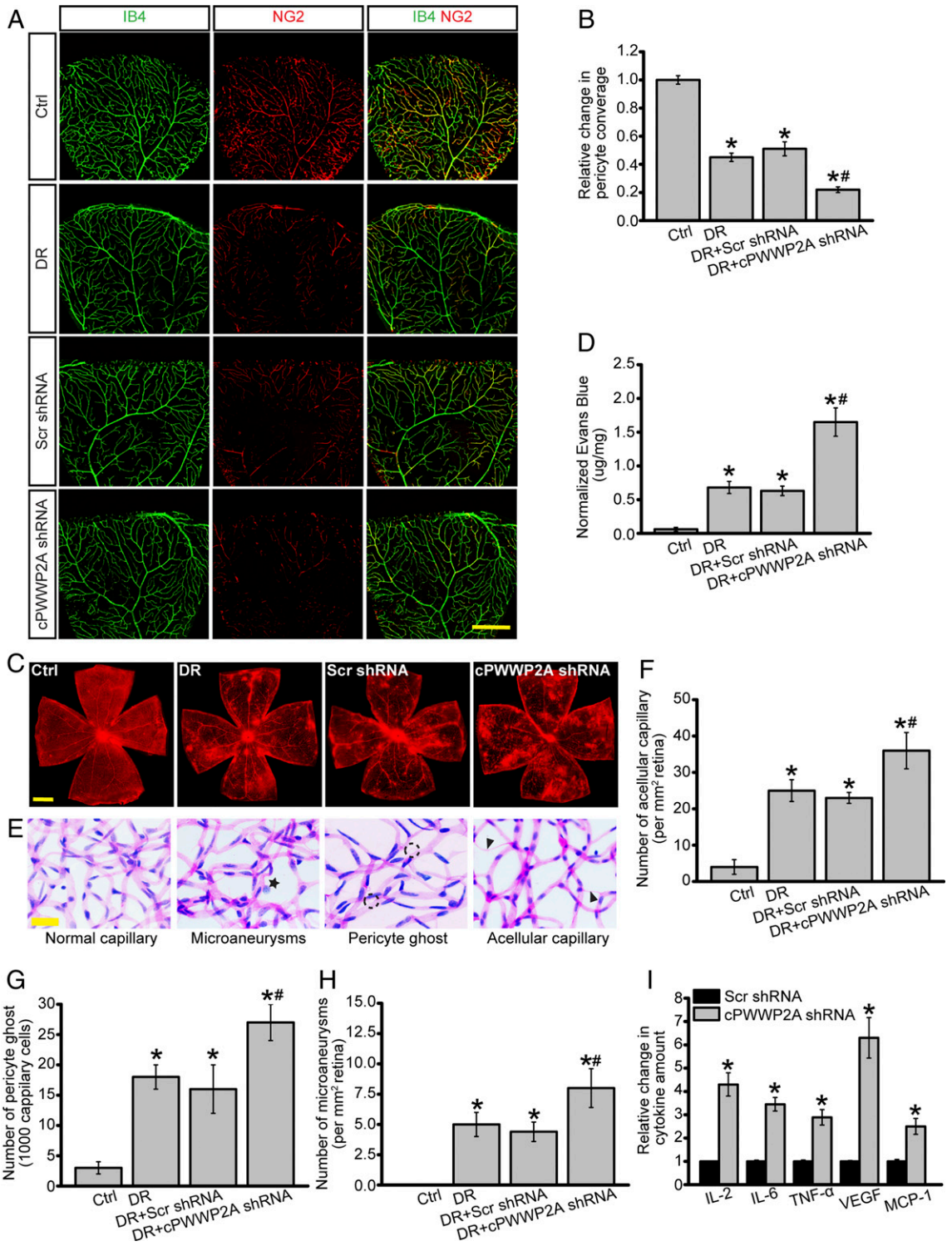

Fig. 2. cPWWP2A regulates diabetes-induced retinal vascular dysfunction in vivo. (*A* and *B*) Flat-mounted retinas were double stained by IB4 (in green, vascular) and NG2 (in red, pericytes) to visualize pericytes covering retinal vasculature in nondiabetic C57BL/6 mice (Ctrl), diabetic mice without or with intravitreous injection of scrambled (Scr) shRNA or cPWWP2A shRNA ($n = 5$). (Scale bar, 200 μm.) To visualize a whole leaf of retinal vasculature, a tile-scanning technique was used whereby multiple overlapping (10–20% overlap) images were acquired using a 10× lens with identical gain settings. The composite images were constructed by arraying the individual images in Photoshop. The representative composite images and statistical result are shown. (*C* and *D*) The nondiabetic or diabetic mice were infused with Evans blue dye for 2 h. The tile-scanning images of the entire retinal vasculature were taken using a 4× lens with identical gain settings. The representative images of flat-mounted retinas along with the statistical result of Evans blue leakage are shown ($n = 5$). (Scale bar, 500 μm.) The red fluorescent indicates Evans blue signaling. (*E*–*H*) Retinal trypsin digestion was conducted to detect the number of acellular vascular, pericyte ghost, and microaneurysms. The arrows indicate acellular vascular; dashed circle indicates pericyte ghost; and star indicates microaneurysm. (Scale bar, 100 μm.) (*I*) ELISAs were conducted to detect the amount of IL-2, IL-6, TNF-α, VEGF, and MCP-1 in retinal lysates ($n = 5$; $^*P < 0.05$ Scr shRNA versus cPWWP2A shRNA group). The significant difference was evaluated by Mann–Whitney's *u* test or Kruskal–Wallis's test followed by post hoc Bonferroni's test. $^*P < 0.05$ versus Ctrl group; $^{\#}P < 0.05$ DR + Scr shRNA versus DR + cPWWP2A shRNA.

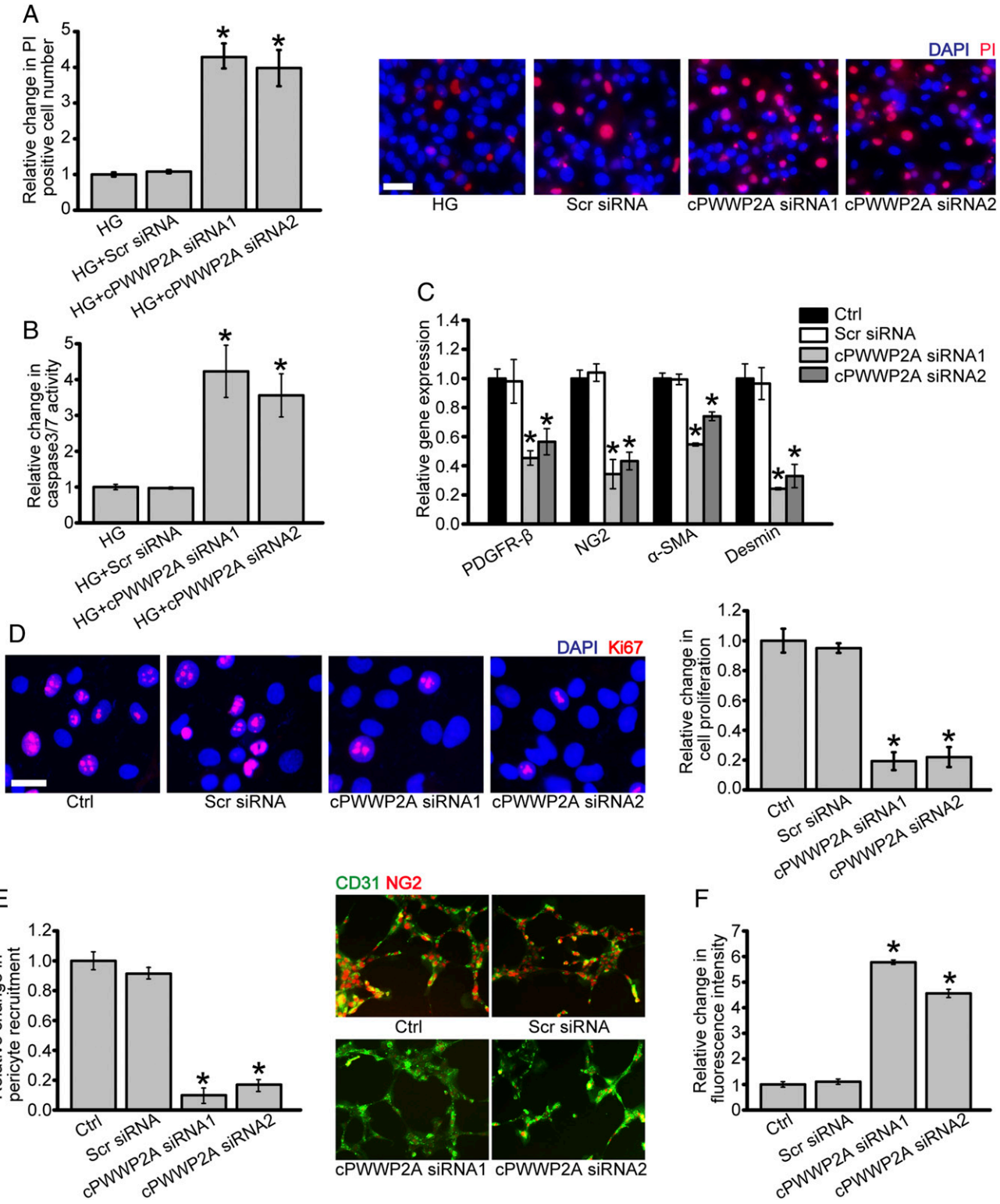

Fig. 3. cPWWP2A regulates retinal pericyte function and pericyte–EC crosstalk in vitro. (*A* and *B*) Pericytes were transfected with scrambled (Scr) siRNA, cPWWP2A siRNA1 or -2, or left untreated (Ctrl), and then exposed to 30 mM glucose for 48 h. Apoptotic cells were determined by PI staining (*A*, $n = 4$) (Scale bar, 50 μm.) and caspase 3/7 activity (*B*, $n = 4$). (*C* and *D*) Pericytes were transfected with Scr siRNA, cPWWP2A siRNA1 or -2, or left untreated (Ctrl) for 48 h. qRT-PCRs were conducted to detect the expression of pericyte markers (*C*, $n = 4$; $^*P < 0.05$ versus Ctrl group). Cell proliferation was determined by Ki67 staining. (*D*, $n = 4$; $^*P < 0.05$ versus Ctrl group). (Scale bar, 20 μm.) (*E*) Wild-type, cPWWP2A siRNA1, cPWWP2A siRNA2, or Scr siRNA-transfected pericytes were synchronized by serum starvation for 24 h, and then cocultured with HRVECs for 6 h. They were stained with NG2 and CD31 to label pericytes (in red) and HRVECs (in green) to detect the number of recruited pericytes on HRVECs ($n = 4$; $^*P < 0.05$ versus Ctrl group). (Scale bar, 100 μm.) (*F*) Pericytes were cocultured with HRVECs to assess endothelial barrier function. The passage of macromolecular 70-kDa FITC–dextran was determined ($n = 4$; $^*P < 0.05$ versus Ctrl group). The significant difference was determined by one-way ANOVA followed by Bonferroni's post hoc test.

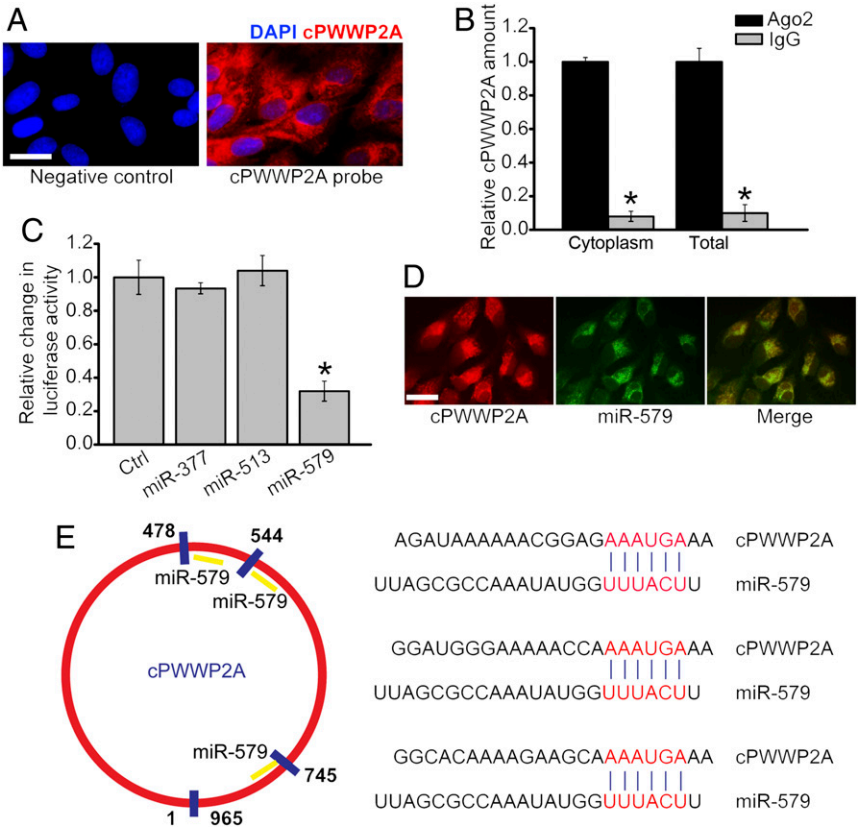

Fig. 4. cPWWP2A regulates pericyte function by acting as a miRNA sponge in vitro. (*A*) RNA-FISH assays were conducted to detect cPWWP2A expression in pericytes. Nuclei were stained with DAPI. (Scale bar, 20 μm.) (*B*) The cytoplasm and total cellular fractions were isolated from pericytes and immunoprecipitated using Ago2 or IgG antibody. cPWWP2A amount in the immunoprecipitate was detected by qRT-PCRs ($n = 4$, $^*P < 0.05$). (*C*) The entire sequence of cPWWP2A was cloned into the pGL3 luciferase reporter to construct the LUC-cPWWP2A vector. Pericytes were cotransfected LUC-cPWWP2A with different miRNA mimics. Luciferase activity was detected by the dual luciferase assay 48 h after transfection ($n = 4$, $^*P < 0.05$ versus Ctrl group). (*D*) RNA-FISH assays were conducted to detect the expression of cPWWP2A (red) and miR-579 (green) in pericytes. (Scale bar, 20 μm.) (*E*) The schematic image shows the putative binding sites of miR-579 on cPWWP2A transcript.

aggravated high glucose-induced pericyte apoptosis as shown by increased PI-positive cells and increased caspase 3/7 activity (Fig. 5 *C* and *D*). miR-579 up-regulation decreased the expression of pericyte markers, including PDGF-β, α-SMA, Desmin, and NG2 (Fig. 5*E*) and decreased the proliferation of pericytes (Fig. 5*F*). Moreover, miR-579 up-regulation reduced pericyte recruitment toward HRVECs (Fig. 5*G*). By contrast, cPWWP2A overexpression partially reversed miR-579–mediated effects on pericyte biology (Fig. 5 *C*–*G*).

cPWWP2A–miR-579–Angiopoietin 1/Occludin/SIRT1 Network Regulates Retinal Vascular Dysfunction in Vivo. We then investigated the role of miR-579 in retinal vascular dysfunction in vivo. Injection of miR-579 agomir led to decreased expression of angiopoietin 1, occludin, and SIRT1 in mouse retinas (Fig. 6*A*), which is consistent with the effect of cPWWP2A silencing on angiopoietin 1, occludin, and SIRT1 expression (Fig. 6*B*). Injection of miR-579 agomir significantly decreased the number of pericyte coverage of retinal vasculature (Fig. 6 *C* and *D*). Trypsin digests of retinal vasculature showed that injection of miR-579 agomir increased the number of acellular capillary, pericyte ghost, and microaneurysm in diabetic retinas (Fig. 6 *E*–*G*). Injection of miR-579 agomir also aggravated diabetes mellitus-induced retinal vascular leakage (Fig. 6 *H* and *I*). By contrast, injection of miR-579 antagomir increased the number of pericyte coverage of retinal vasculature and decreased the number of acellular vascular, pericyte ghost, and microaneurysm in diabetic retinas. Moreover, injection of miR-579 antagomir alleviated diabetes mellitus-induced retinal vascular leakage (*SI Appendix*, Fig. S6).

We further determined whether miR-579/cPWWP2A crosstalk is involved in retinal vascular dysfunction in vivo. Compared with the DR group, cPWWP2A silencing led to decreased pericyte coverage, increased retinal vascular leakage, acellular microvascular, pericyte ghosts, and microaneurysms. cPWWP2A silencing led to a released miR-579 amount sponged by cPWWP2A. Injection of exogenous miR-579 antagomir abolished the function of released miR-579, which rescued the effects of cPWWP2A silencing on retinal vascular dysfunction, suggesting that exogenous miR-579 intervention could overwhelm miRNA sponge function of cPWWP2A (*SI Appendix*, Fig. S7).

Transfer of cPWWP2A Between Pericyte and EC Regulates Pericyte–EC Crosstalk. Primary pericytes and ECs were sorted from nondiabetic retina and diabetic retina, respectively. cPWWP2A expression in diabetic retina was significantly higher than that in nondiabetic retina both in pericytes and ECs (Fig. 7*A*). However, high glucose only increased cPWWP2A expression in the primary pericytes but not ECs in vitro (Fig. 7*B*). These results suggest that cPWWP2A was likely to be acquired in a paracrine manner and but not produced by ECs. In situ hybridization and fluorescent staining revealed that cPWWP2A was mainly expressed in pericytes and surrounding ECs in nondiabetic retina. Diabetes led to a marked reduction in NG2 signaling, indicating the loss of pericytes. However, cPWWP2A was highly expressed in the ECs in diabetic retinas (Fig. 7*C*). High glucose significantly increased cPWWP2A expression in the medium of pericytes in vitro (*SI Appendix*, Fig. S8*A*). Incubation of ECs with the medium from high glucose-treated pericytes led to increased expression of SIRT1 and occludin in ECs (*SI Appendix*, Fig. S8*B*). cPWWP2A expression from the plasma of diabetic mice was significantly higher than that of nondiabetic mice (*SI Appendix*, Fig. S8*C*). Incubation of ECs with the medium from high glucose-treated pericytes significantly increased the migration and tube formation ability of endothelial cells (*SI Appendix*, Fig. S8 *D* and *E*), suggesting that pericytes regulate EC function through paracrine cPWWP2A-mediated signaling.

We then determine the possible pericyte-secreted constituents responsible for endothelial cell function. Pretreatment with proteinase K, but not DNase I or RNase A led to decreased cPWWP2A expression in pericyte medium (*SI Appendix*, Fig. S9*A*), suggesting that proteinase K pretreatment may affect the biogenesis of circular RNAs. The ability of pericyte medium to increase

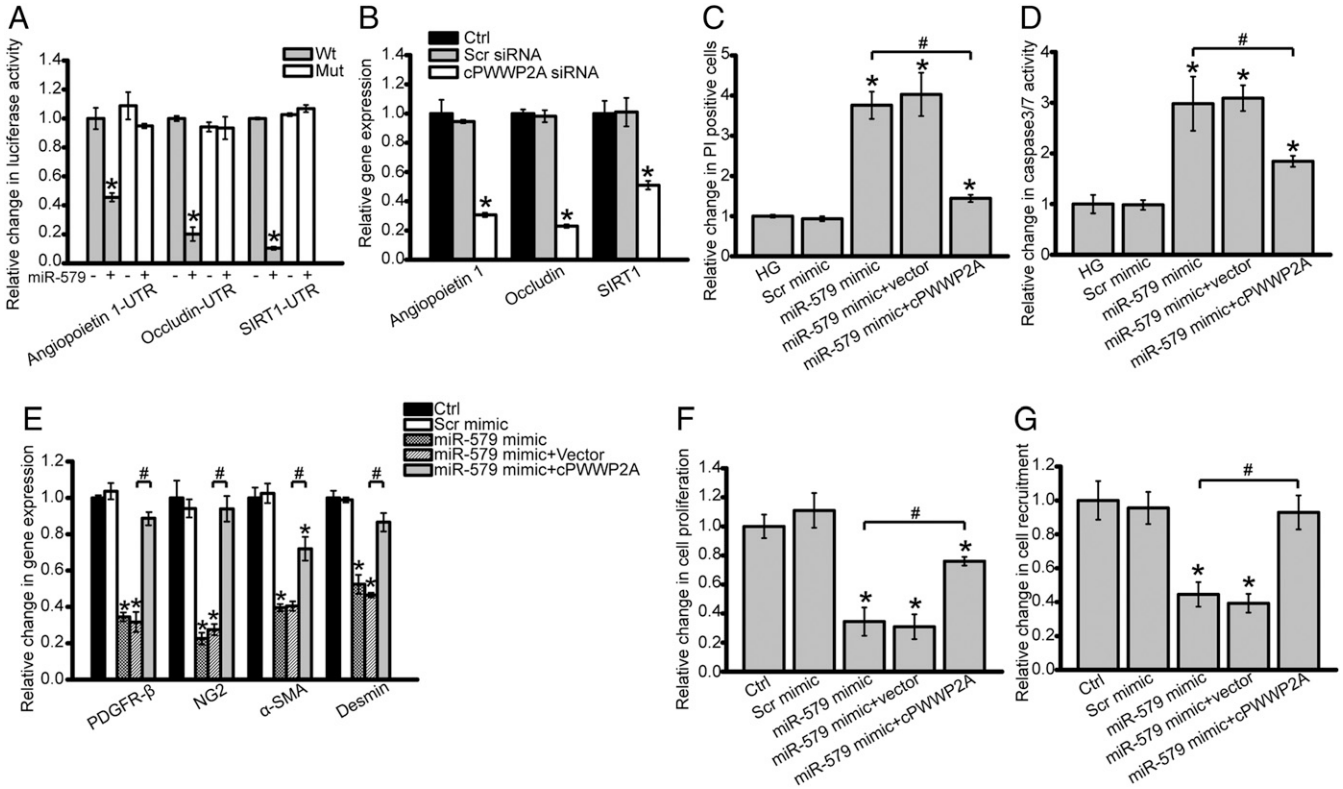

Fig. 5. The cPWWP2A–miR-579–angiopoietin 1/occludin/SIRT1 network regulates pericyte function and pericyte–EC crosstalk in vitro. (*A*) Pericytes were cotransfected wild-type (Wt) or mutant (Mut) angiopoietin 1–UTR, occludin–UTR, SIRT1–UTR with or without miR-579 mimic. Luciferase activity was detected 48 h after transfection ($n = 4$, $^{*}P < 0.05$ versus Wt group without miR-579 mimic group). (*B*) Pericytes were transfected with cPWWP2A siRNA, scrambled (Scr) siRNA, or left untreated (Ctrl). Twenty-four hours after transfection, qRT-PCRs were conducted to detect the expression of angiopoietin 1, occludin, and SIRT1 ($n = 4$). (*C–G*) Pericytes were treated as shown for 48 h. Apoptotic cells were determined by PI staining ($n = 4$, *C*) or caspase 3/7 activity ($n = 4$, *D*). qRT-PCRs were conducted to detect the expression of pericyte markers, including PDGFR-β, NG2, α-SMA, and Desmin ($n = 4$, *E*). Cell proliferation was detected by Ki67 staining ($n = 4$, *F*). Pericytes were stained with NG2 and CD31 to label pericytes and HRVECs, and the number of recruited pericytes on HRVECs determined ($n = 4$, *G*). $^{*}P < 0.05$ versus Ctrl group or HG group; $^{\#}P < 0.05$ miR-579 mimic group versus miR-579 mimic + cPWWP2A group. The significant difference was evaluated by one-way ANOVA followed by Bonferroni's post hoc test.

cPWWP2A abundance in ECs was abolished by proteinase K but unaffected by DNase and RNaseA treatment (*SI Appendix*, Fig. S9*B*), suggesting that circRNAs or RNA–protein complexes but not DNA components were transmitted from pericytes to endothelial cells. In addition, the ability of pericyte medium to increase tube formation and EC migration was partially abolished by proteinase K but not affected by DNase and RNase A treatment (*SI Appendix*, Fig. S9 *C* and *D*).

Extracellular vesicles may derive from the remnants of apoptotic cells or actively exported exosomes. Inhibition of exosome generation with GW4869 decreased the transfer of cPWWP2A from pericytes to ECs, whereas apoptosis inhibition did not affect cPWWP2A transfer (Fig. 7*D*), suggesting that cPWWP2A transfer is mediated by exosomes rather than apoptotic bodies. We then investigated whether exosome-delivered cPWWP2A from pericytes is involved in regulating EC function. We used the CRISPR/Cas9 system to construct cPWWP2A knockout ECs, which could not express cPWWP2A. We could rule out the possibility of other factors potentially increasing the production of cPWWP2A within ECs. High glucose-treated pericyte medium increased the ability of proliferation, migration, and tube formation ability of cPWWP2A$^{-/-}$ ECs. By contrast, cPWWP2A antisense transcript addition abolished high glucose-treated pericyte medium-mediated cPWWP2A$^{-/-}$ EC functions (Fig. 7 *E–G*).

Discussion

Retinal microvasculature comprises organized layers of pericytes and ECs. Aberrations in pericyte–EC crosstalk would lead to vascular dysfunction (3). Here we show that diabetes-related stress up-regulates cPWWP2A expression in pericytes but not in ECs. cPWWP2A could be transferred from pericytes to ECs through exosomes carrying cPWWP2A. cPWWP2A regulates pericyte–EC crosstalk in vitro and diabetes-induced retinal vascular dysfunction in vivo. cPWWP2A acts as an endogenous miR-579 sponge to inhibit miR-579 activity. Intervention of cPWWP2A or miR-579 expression would become therapeutic strategies for diabetic microvascular complications.

Retinal microvasculature mainly include pericytes and ECs (3). Hyperglycemia leads to increased cPWWP2A expression in pericytes. Pericytes have been shown to play key roles in regulating vascular stability and remodeling. At the early stage of DR, pericyte loss is shown as the earliest histopathological hallmark (2). cPWWP2A overexpression could protect pericytes against high glucose-induced injury in vitro. At the proliferative stage of DR, retinal neovascularization not only involves the proliferation, migration, and maturation of ECs, but also involves the proliferation and recruitment of pericytes. However, asynchronized or insufficient proliferation and recruitment of pericytes affects the maturation of new blood vessels, leading to fragile and leaky vessels (1, 5). Aberration in cPWWP2A expression impairs pericyte biology, including cell proliferation, recruitment, and differentiation, which could affect the maturation of new blood vessels. Thus, it is not surprising that cPWWP2A is involved in diabetes-induced retinal vascular dysfunction by regulating pericyte biology. ECs usually undergo a phenotypic switch from a normal quiescent phenotype to an apoptotic and active phenotype in response to hyperglycemia stress (1, 24). The medium from pericytes affects the proliferation, migration, and tube formation

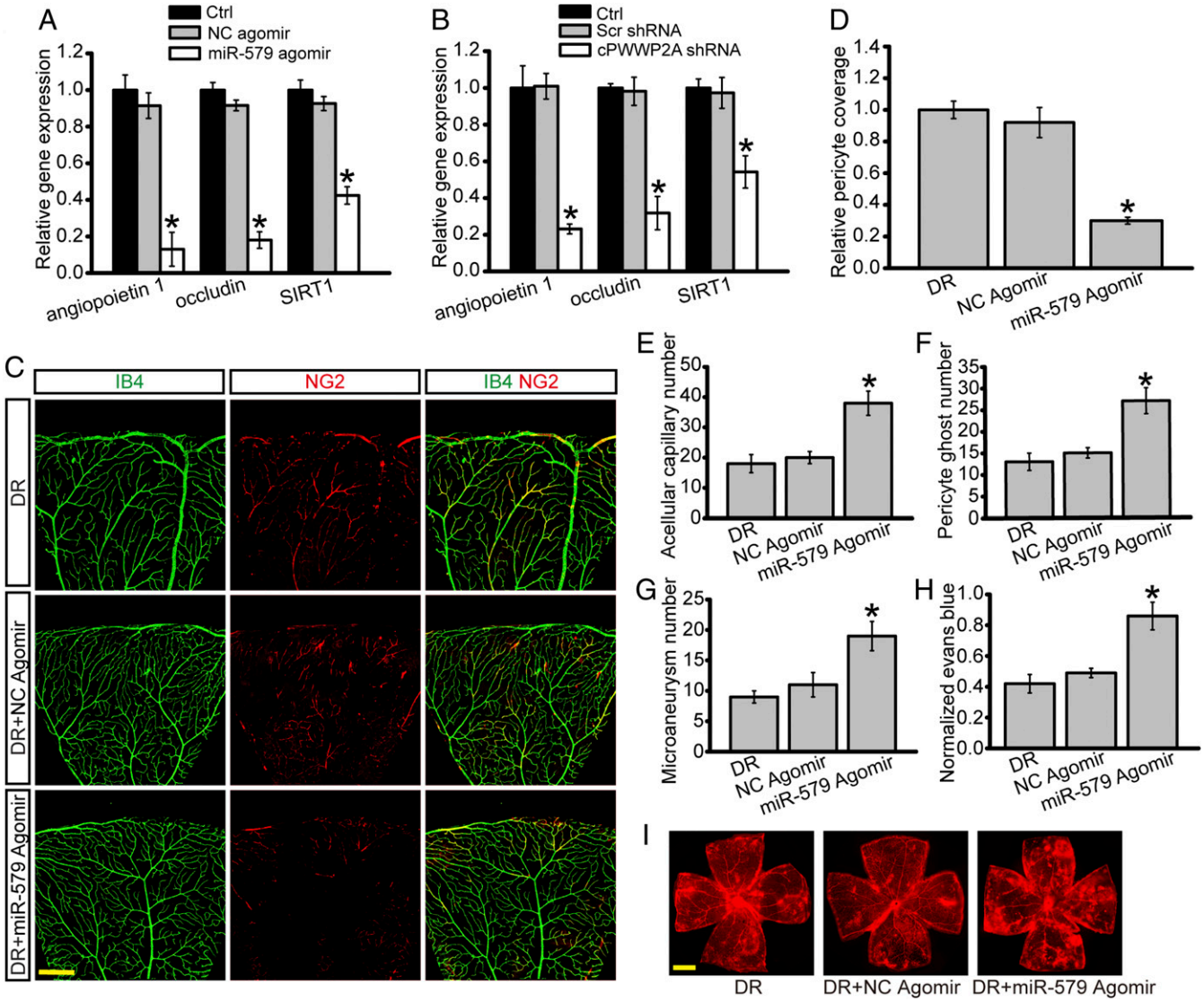

Fig. 6. The cPWWP2A–miR-579–angiopoietin 1/occludin/SIRT1 network regulates retinal vascular dysfunction in vivo. (*A* and *B*) Diabetic C57BL/6 mice (2 mo old, male) received an intravitreous injection of miR-579 agomir, negative control (NC) agomir, cPWWP2A shRNA, scrambled (Scr) shRNA, or left untreated (Ctrl) for 1 mo. qRT-PCRs were conducted to detect angiopoietin 1, occludin, and SIRT1 expression ($n = 4$, $^*P < 0.05$ versus Ctrl group). (*C–I*) Diabetic C57BL/6 mice (2 mo old, male) received an intravitreous injection of miR-579 agomir, NC agomir, or left untreated (DR) once monthly for a total of 4 mo. "DR" indicates diabetic mice without virus injection. Flat-mounted retinas were double-stained with IB4 and NG2 to visualize pericytes covering retinal vasculature. Tile-scanning images of a leaf of retinal vasculature were taken using a 10× lens. The composite images were constructed by arraying the individual images in Photoshop. The representative composite images and statistical result are shown. (Scale bar in *C* and *D*, 200 μm.) Retinal trypsin digestion was performed to detect the number of acellular vascular, pericyte ghost, and microaneurysms (*E–G*). They were infused with Evans blue dye for 2 h. Tile-scanning images of the entire retinas were taken using a 4× lens. The representative composite images of flat-mounted retinas along with the quantification results of Evans blue leakage are shown. The red fluorescent is Evans blue signaling (*H* and *I*, $n = 5$). (Scale bar, 500 μm.) The significant difference was evaluated by Kruskal–Wallis's test followed by post hoc Bonferroni's test.

ability of ECs. High glucose-induced cPWWP2A could be transferred from pericytes to ECs. Intervention of cPWWP2A in pericytes would affect the angiogenic effects of ECs, suggesting that cPWWP2A-mediated signaling also plays an important role in regulating pericyte–EC crosstalk.

Retinal microvasculature is the early and prevalent target injured by hyperglycemia (25, 26). In diabetic retinas, cPWWP2A silencing decreases pericyte coverage in retinal vasculature and aggravates retinal vascular dysfunction as shown by increased vascular cell loss, acellular vascular, and aneurysm formation. Impaired vascular function would increase vascular permeability and inflammation (1, 27). cPWWP2A silencing also aggravates diabetes-induced retinal vascular leakage and inflammation. By contrast, cPWWP2A overxpression alleviates diabetes-induced retinal vascular dysfunction. Collectively, cPWWP2A expression is tightly associated with retinal pericyte biology and vascular homeostasis. We speculate that cPWWP2A up-regulation is shown as a compensatory and beneficial response to combat against hyperglycemia stress. cPWWP2A up-regulation increases the proliferation and recruitment of pericytes, protects pericytes against diabetes-related stresses, and retards the development of retinal vascular dysfunction.

Previous studies have revealed that functional circRNAs fulfill regulatory functions by acting as cytoplasm miRNA sponges, RNA-binding protein sequestering agents, or nuclear transcriptional regulators (7, 9). cPWWP2A is mainly expressed in the cytoplasm of pericytes and specifically pulled down by Ago2, providing the possibility that cPWWP2A acts as the miRNA sponge. In vitro studies and in vivo studies show that intervention of miR-579 sponged by cPWWP2A affects pericyte biology, pericyte–EC crosstalk, and diabetes-induced retinal vascular

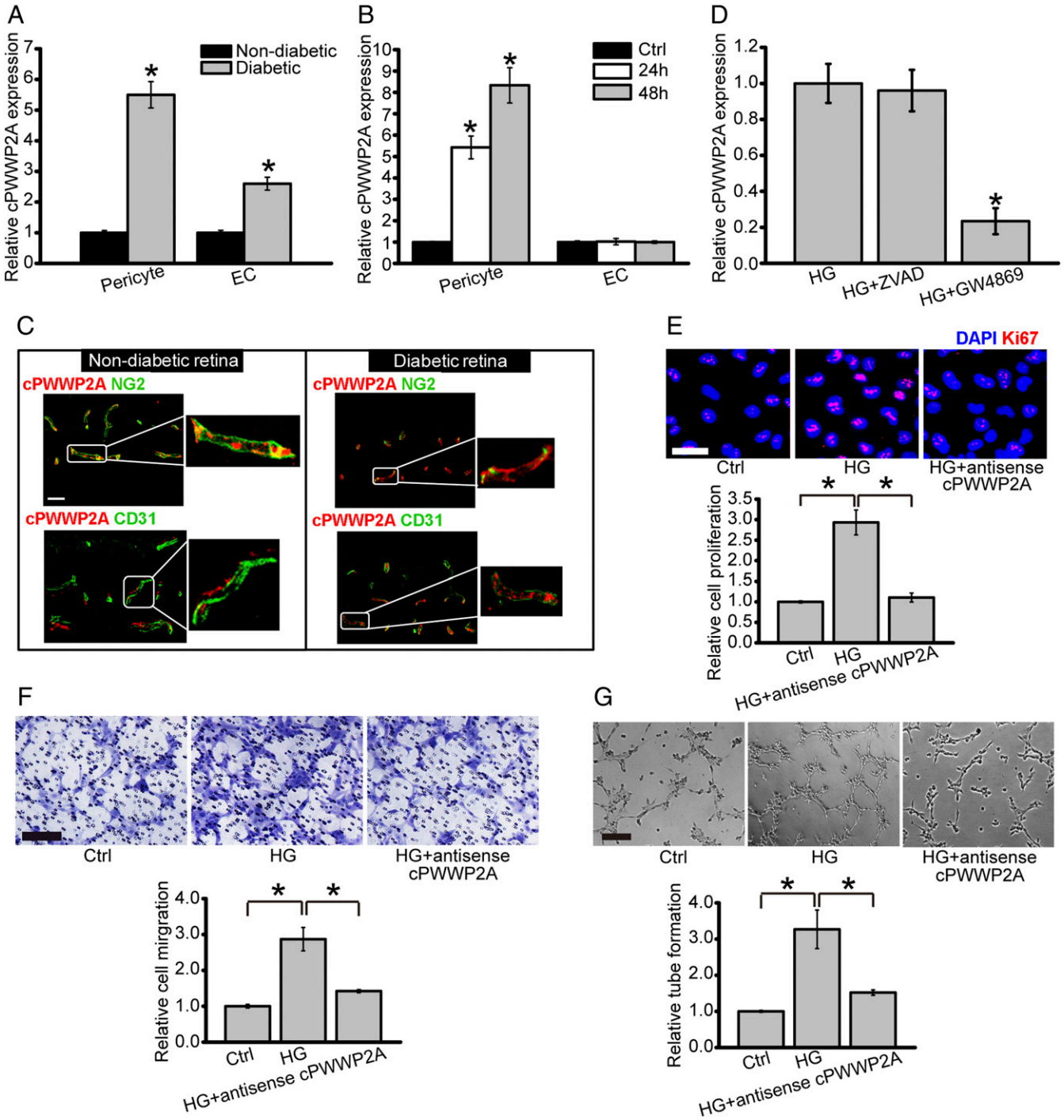

Fig. 7. Transfer of cPWWP2A between pericyte and EC regulates pericyte–EC crosstalk. (*A*) Pericytes and ECs were sorted from nondiabetic and diabetic retinas (after 3-mo diabetes induction). cPWWP2A expression in pericytes and ECs was determined by qRT-PCRs ($n = 5$ per group; $^*P < 0.05$ versus nondiabetic group). (*B*) Pericytes and ECs were sorted from nondiabetic retinas, and then were exposed to 30 mM glucose for 24 h and 48 h, or left untreated (Ctrl). cPWWP2A expression in pericytes and ECs was determined by qRT-PCRs ($n = 5$ per group). (*C*) Representative CD31 staining (green) or NG2 staining (green) with FISH detection of cPWWP2A expression (red) in nondiabetic and diabetic retinas. (Scale bar, 100 μm.) (*D*) The medium of high glucose (HG)-treated pericytes was pretreated with or without apoptosis inhibitor Z-VAD-FMK (ZVAD) or N-SMase inhibitor GW4869. ECs were then incubated with the vesicles isolated from the above-mentioned medium. cPWWP2A expression in ECs was determined by qRT-PCRs ($n = 4$, $^*P < 0.05$ versus HG). (*E–G*) Pericytes were incubated with the medium containing 5 mM glucose (Ctrl) or 30 mM glucose (HG) for 24 h. Pericyte medium with or without cPWWP2A antisense transcript was then incubated with cPWWP2A$^{-/-}$ ECs. EC proliferation was detected at 48 h postincubation using Ki67 staining (*E*). EC migration and tube formation was detected at 8 h postincubation using a Transwell assay (*F*) and growth factor-reduced Matrigel (*G*). The significant difference was evaluated by one-way ANOVA followed by Bonferroni's post hoc test.

dysfunction. Moreover, exogenous miR-579 intervention could overwhelm the miRNA sponge function of cPWWP2A. Thus, intervention of cPWWP2A or cPWWP2A-mediated downstream signaling is a promising therapeutic strategy for ameliorating DR phenotypes. Given cPWWP2A has been overexpressed in DR, inhibition of miR-579, the downstream member of cPWWP2A signaling, would have greater translational significance for treating diabetes-related retinal vascular dysfunction.

Angiopoietin 1 (Ang-1) is a major regulator of blood vessel formation. It is secreted by pericytes and activates the tyrosine kinase receptor Tie-2 expressed by ECs (28). This signal could stabilize the tube of newly formed blood vessels. Occludin is a transmembrane tight junction protein that contributes to diverse cellular functions, such as control of barrier properties, cell migration, and proliferation (29). Loss of SIRT1 function blocks sprouting angiogenesis and branching morphogenesis of endothelial cells (30). Based on the above-mentioned evidence, we know that angiopoietin 1/occludin/SIRT1 is involved in regulating pericyte or EC biology during vascular remodeling. cPWWP2A overexpression becomes a sink for miR-579 and releases the repressive effect of miR-579 on cPWWP2A. Thus, it is not surprising that the cPWWP2A–miR-579–angiopoietin 1/occludin/SIRT1 network regulates retinal vascular dysfunction in vivo.

In conclusion, this study reveals a mechanism underlying diabetes mellitus-induced microvascular dysfunction. This mechanism involves the coordinated regulation of cPWWP2A, miR-579, and angiopoietin 1/occludin/SIRT1 in pericytes, which also leads to altered EC biology after cPWWP2A transfer from pericytes to ECs by exosomes.

Materials and Methods

Ethics Statement. The animal experiments were approved by the guidelines of the Institutional Animal Care and Use Committee of Eye and Ears, Nose, and Throat Hospital and Nanjing Medical University. All mice were housed in a specific pathogen-free facility with free access to diet and water in a 12-h day/night cycle (lights on at 08:00 and off at 20:00).

Streptozotocin-Induced Diabetic Mice. C57BL/6 mice (2 mo old, male) were fasted for 6 h before STZ (Sigma-Aldrich) injection. Diabetic mice were developed by i.p. injection of STZ (50 mg/kg) for 5 consecutive days. The control group received an i.p. injection of vehicle (citrate buffer control). Tail vein blood glucose was measured 2 d and 1 wk after the last injection. Diabetes mellitus was confirmed by the fasting blood glucose level of more than 250 mg/dL.

Cell Culture. HRVECs (ACBRI-181) and human retinal pericytes (ACBRI-183) were obtained from Cell Systems. HRVECs were cultured in EGM2-MV medium (Lonza) supplemented with 10% FBS (Gibco). Pericytes were cultured in DMEM supplemented with 10% FBS. All cultures were incubated at 37 °C, in 5% CO_2, and 95% relative humidity. Cells at passages 5–8 at ∼90% confluence were used.

Preparation of Pericyte Medium and DNase, RNase, and Proteinase Treatment. Pericytes were cultured in DMEM supplemented with 10% FBS. After the required treatment, the media were collected and centrifuged at 500 × *g*, 1,000 × *g*, and 1,500 × *g* for 10 min to remove cells and debris, respectively. For DNase and RNase treatment, the supernatant was incubated with DNase I (1 unit/mL; Invitrogen) or RNase A (10 μg/mL; Invitrogen) at 37 °C for 15 min. For proteinase K treatment, recombinant, PCR-grade, DNase/RNase-free proteinase K (Roche) was dissolved in RNase-free water. The supernatant was incubated with proteinase K (50 μg/mL) at 60 °C for 15 min and then at 95 °C for 5 min to inactivate proteinase K.

Statistical Analysis. All data were tested for normality using D'Agostino–Pearson's omnibus normality test and homogeneity of variances using Levene's test. For normally distributed data with equal variance, the difference was evaluated by two-tailed Student's *t* test (two-group comparisons), one-way or two-way analysis of variance (ANOVA) followed by post hoc Bonferroni's test (multigroup comparisons) as appropriate. For nonnormally distributed data or data with unequal variances, the difference was evaluated by nonparametric Mann–Whitney's *u* test (two-group comparisons) or Kruskal–Wallis's test followed by post hoc Bonferroni's test (multigroup comparisons). $P < 0.05$ was considered statistically significant.

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1. Fowler MJ (2008) Microvascular and macrovascular complications of diabetes. *Clin Diabetes* 26:77–82.
2. Hammes HP, Feng Y, Pfister F, Brownlee M (2011) Diabetic retinopathy: Targeting vasoregression. *Diabetes* 60:9–16.
3. Hammes HP, et al. (2002) Pericytes and the pathogenesis of diabetic retinopathy. *Diabetes* 51:3107–3112.
4. Watson EC, Grant ZL, Coultas L (2017) Endothelial cell apoptosis in angiogenesis and vessel regression. *Cell Mol Life Sci* 74:4387–4403.
5. Gaengel K, Genové G, Armulik A, Betsholtz C (2009) Endothelial-mural cell signaling in vascular development and angiogenesis. *Arterioscler Thromb Vasc Biol* 29:630–638.
6. Armulik A, Abramsson A, Betsholtz C (2005) Endothelial/pericyte interactions. *Circ Res* 97:512–523.
7. Lasda E, Parker R (2014) Circular RNAs: Diversity of form and function. *RNA* 20:1829–1842.
8. Ebbesen KK, Kjems J, Hansen TB (2016) Circular RNAs: Identification, biogenesis and function. *Biochim Biophys Acta* 1859:163–168.
9. Salzman J (2016) Circular RNA expression: Its potential regulation and function. *Trends Genet* 32:309–316.
10. Du WW, et al. (2016) Foxo3 circular RNA retards cell cycle progression via forming ternary complexes with p21 and CDK2. *Nucleic Acids Res* 44:2846–2858.
11. Legnini I, et al. (2017) Circ-ZNF609 is a circular RNA that can be translated and functions in myogenesis. *Mol Cell* 66:22–37.e9.
12. Piwecka M, et al. (2017) Loss of a mammalian circular RNA locus causes miRNA deregulation and affects brain function. *Science* 357:eaam8526.
13. Zhao ZJ, Shen J (2017) Circular RNA participates in the carcinogenesis and the malignant behavior of cancer. *RNA Biol* 14:514–521.
14. Wang K, et al. (2016) A circular RNA protects the heart from pathological hypertrophy and heart failure by targeting miR-223. *Eur Heart J* 37:2602–2611.
15. Stoll L, et al. (2018) Circular RNAs as novel regulators of β-cell functions in normal and disease conditions. *Mol Metab* 9:69–83.
16. Dang RY, Liu FL, Li Y (2017) Circular RNA hsa_circ_0010729 regulates vascular endothelial cell proliferation and apoptosis by targeting the miR-186/HIF-1α axis. *Biochem Biophys Res Commun* 490:104–110.
17. Liu C, et al. (2017) Silencing of circular RNA-ZNF609 ameliorates vascular endothelial dysfunction. *Theranostics* 7:2863–2877.
18. Shan K, et al. (2017) Circular noncoding RNA HIPK3 mediates retinal vascular dysfunction in diabetes mellitus. *Circulation* 136:1629–1642.
19. Boeckel JN, et al. (2015) Identification and characterization of hypoxia-regulated endothelial circular RNA. *Circ Res* 117:884–890.
20. Witmer AN, Vrensen GF, Van Noorden CJ, Schlingemann RO (2003) Vascular endothelial growth factors and angiogenesis in eye disease. *Prog Retin Eye Res* 22:1–29.
21. Motiejünaite R, Kazlauskas A (2008) Pericytes and ocular diseases. *Exp Eye Res* 86:171–177.
22. Winter J, Jung S, Keller S, Gregory RI, Diederichs S (2009) Many roads to maturity: MicroRNA biogenesis pathways and their regulation. *Nat Cell Biol* 11:228–234.
23. Carmeliet P, Jain RK (2011) Molecular mechanisms and clinical applications of angiogenesis. *Nature* 473:298–307.
24. Caporali A, et al. (2017) Contribution of pericyte paracrine regulation of the endothelium to angiogenesis. *Pharmacol Ther* 171:56–64.
25. Ferland-McCollough D, Slater S, Richard J, Reni C, Mangialardi G (2017) Pericytes, an overlooked player in vascular pathobiology. *Pharmacol Ther* 171:30–42.
26. Giacco F, Brownlee M (2010) Oxidative stress and diabetic complications. *Circ Res* 107:1058–1070.
27. Caporali A, et al. (2015) p75(NTR)-dependent activation of NF-κB regulates microRNA-503 transcription and pericyte-endothelial crosstalk in diabetes after limb ischaemia. *Nat Commun* 6:8024.
28. Fiedler U, Augustin HG (2006) Angiopoietins: A link between angiogenesis and inflammation. *Trends Immunol* 27:552–558.
29. Murakami T, Felinski EA, Antonetti DA (2009) Occludin phosphorylation and ubiquitination regulate tight junction trafficking and vascular endothelial growth factor-induced permeability. *J Biol Chem* 284:21036–21046.
30. Potente M, et al. (2007) SIRT1 controls endothelial angiogenic functions during vascular growth. *Genes Dev* 21:2644–2658.