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Identification of endothelial protein C receptor as a novel druggable agonistic target for reendothelialization promotion and thrombosis prevention of eluting stent

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ABSTRACT

The commercially available drug-eluting stent with limus (rapamycin, everolimus, etc.) or paclitaxel inhibits smooth muscle cell (SMC), reducing the in-stent restenosis, whereas damages endothelial cell (EC) and delays stent reendothelialization, increasing the risk of stent thrombosis (ST) and sudden cardiac death. Here we present a new strategy for promoting stent reendothelialization and preventing ST by exploring the application of precise molecular targets with EC specificity. Proteomics was used to investigate the molecular mechanism of EC injury caused by rapamycin. Endothelial protein C receptor (EPCR) was screened out as a crucial EC-specific effector. Limus and paclitaxel repressed the EPCR expression, while overexpression of EPCR protected EC from coating (eluting) drug-induced injury. Furthermore, the ligand activated protein C (APC), polypeptide TR47, and compound parmodulin 2, which activated the target EPCR, promoted EC functions and inhibited platelet or neutrophil adhesion, and enhanced rapamycin stent reendothelialization in the simulated stent environment and *in vitro*. *In vivo*, the APC/rapamycin-coating promoted reendothelialization rapidly and prevented ST more effectively than rapamycin-coating alone, in both traditional metal stents and biodegradable stents. Additionally, overexpression or activation of the target EPCR did not affect the cellular behavior of SMC or the inhibitory effect of rapamycin on SMC. In conclusion, EPCR is a promising therapeutical agonistic target for pro-reendothelialization and anti-thrombosis of eluting stent. Activation of EPCR protects against coating drugs-induced EC injury, inflammatory cell, or platelet adhesion onto the stent. The novel application formula for APC/rapamycin-combined eluting promotes stent reendothelialization and prevents ST.

1. Introduction

Coronary heart disease is the most common cause of death worldwide [1]. Cardiovascular deaths are expected to increase to 23.3 million by 2030 [2]. The implantation of a drug-eluting stent (DES) is becoming the most commonly used treatment for coronary heart disease

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[3]. Currently, two main types of commercially available coronary artery DES, i.e., paclitaxel and limus (such as rapamycin [also named sirolimus], everolimus, and zotarolimus) are used to reduce the risk of vascular restenosis. Limus inhibits the activation of mammalian target of rapamycin (mTOR), which is essential for the progression of the cell cycle from G1 to the S phase, thus preventing the proliferation of smooth muscle cells (SMC) [4]. Paclitaxel changes the interaction of microtubules with ß-Tubulin and induces cell cycle arrest, inhibiting SMC proliferation, migration, and intimal hyperplasia [5].

Previous studies [6,7] reported that the application of DES meaningfully reduced the incidence of in-stent restenosis (ISR) compared to the early bare metal stent (BMS) but significantly increased the risk of stent thrombosis (ST) in the late stage (1 month–1 year) and very late stage (>1year). ST is one of the most serious complications after coronary stent implantation, with a 0.6–3 % occurrence risk [8–10]and 16.7–33.8 % mortality [11,12] in the late stage. At 5 years, the recurrence rate increases to 15–20 % and the mortality rate to 5–45 % [13]. ST is mainly attributed to the non-selective inhibition of endothelial cell (EC) growth and, thereby, the delay of stent reendothelialization by the DES-coated drug, limus, or paclitaxel. Coating drugs interrupt the reendothelialization process, thus making the unwrapped stent constantly exposed and coronary thrombus formed. In recent years, it has been found that the application of biodegradable stents (BRS) possesses more conducive features to active vascular remodeling. Previously, we have developed a kind of BRS, XINSORB®, which has been widely used clinically for several years [14–16]. BRS achieving brilliant outcomes in many aspects. However, clinical studies have expressed a serious concern about whether bioresorbable vascular stents reduce the incidence of ST [17–19]. Apart from long-term antiplatelet therapy, there is no specific prevention and treatment method for ST in clinical practice [6,20]. However, antiplatelet therapy cannot effectively improve endothelial functions and fundamentally solve the problem of the delayed reendothelialization process [21]. Consequently, developing novel stents with outstanding anti-ST properties is of particular importance.

Excellent coating drug should inhibit SMC (preventing restenosis) and enhance EC function (promoting reendothelialization and preventing ST). Keeping this balance between the dual effects poses the biggest challenge for new stent development. Traditional DES only focuses on the former effect while ignoring the latter. One of the strategies is to explore new materials as coating drugs to inhibit SMC, including a series of rapamycin derivatives (such as deforolimus, biolimus A9, and pimecrolimus), statins [22], arsenic trioxide [23], CD146 antibody [24], microRNAs [25], exosomes [26], and similar. The other strategy is combining drugs that promote EC functions, obtaining a novel drug-eluting stent with multiple-layer coatings. These methods include anti-apoptotic and pro-survival molecules, like vascular endothlial growth factor (VEGF) [27], CD31 mimetic peptide [28], nitric oxide (NO) [29], and similar. While recent studies have made some progress in this field, a certain degree of non-specificity and blindness is difficult to avoid with this drug efficacy-based strategy rather than target-based research. More importantly, the failure to investigate the vascular SMC and EC as a whole is also indefensible. Accordingly, proposing an innovative strategy for cutting-edge research in this field is urgently needed.

Considering the differences in cellular behavior between the two types of cells involved in the design of coating drugs, SMC and EC, we searched for precise molecular targets from the perspective of cell specificity using the proteomics method. An innovative excitatory target, endothelial protein C receptor (EPCR), expressed explicitly in EC but not in SMC, was identified. Next, we proposed an original strategy of activating EPCR to protect EC induced by limus and paclitaxel, thus promoting the process of reendothelialization after stent implantation, which is expected to improve stent safety by reducing the ST risk.

2. Methods

2.1. Data availability

The list of materials (Table S1) and detailed methods are available within the article and in the online Data Supplements.

All procedures involving animals (mice and rabbits) conformed with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health and in accordance with the protocols approved by the Animal Ethics Committee of Guangzhou Medical University and the Animal Ethics Committee of Zhongshan Hospital of Fudan University. After treatments, C57BL6/J mice were euthanized by cervical dislocation under a chamber containing 2.5 % isoflurane in oxygen. New Zealand white rabbits were euthanized via ear vein application of an overdose of pentobarbital sodium (100 mg/kg). The blood obtained from healthy volunteers strictly adhered to the Helsinki Declaration and has been approved by the Ethics Committee of the Second Affiliated Hospital of Guangzhou Medical University (Ethics Number: 2022-KY-ks-01), and informed consent was obtained from the volunteers for all the experiments.

2.2. iTRAQ labeling proteomics

HCAECs were treated with 100 nm rapamycin for 48 h and then harvested. Proteomics analysis was supported by the FitGene BioTechnology Proteomics Platform. In brief, the prepared proteins were digested by trypsin and labeled using iTRAQ Reagent-8plex Multiplex Kit. Then the peptides were analyzed by LC-MS/MS and Proteinpilot 5.0 (AB Sciex) software was used to retrieve the separated peptides. This process was repeated three times, and the average was accepted as the final result.

2.3. In vivo stents implantation of rabbits

Healthy New Zealand White male rabbits (3.0–3.5 kg) were orally received 2 mg/kg of aspirin and 1.5 mg/kg of clopidogrel per day in their feed for 3 days and fasted for 12 h before stent implantation. Rabbits were anesthetized with pentobarbital sodium (30 mg/kg). The stent coated with different drugs mounted on a balloon catheter was inserted into the abdominal aorta through a 6F sheath from the right femoral artery incision. The balloon was inflated to 12–16 ATM and then deflated to maintain negative pressure, repeatedly for three times. Subsequently, the bleeder tube was slowly withdrawn, leaving the stent in place for imaging observation (GE Healthcare). Throughout the entire surgical process, rabbits were injected with 2–2.5 ml of heparin solution each time. All animals were injected with penicillin (5000 U/kg) within 3 days after surgery.

2.4. Statistical analysis

Unless otherwise specified, all data are expressed as mean ± Standard Error of the Mean (S.E.M.). The differences between groups were performed using Student's t-test for two groups or by one- or two-way analysis of variance (ANOVA) followed by Tukey-Kramer *post hoc* analysis when more than two groups were compared. All statistical analyses were performed using GraphPad Prism 8.0. $P < 0.05$ represented statistical significance.

3. Results

3.1. Proteomic analysis of rapamycin-injured human coronary artery endothelial cells

In order to identify precise and specific molecular mechanisms through which rapamycin (sirolimus) delayed stent reendothelialization, we performed proteomics study of rapamycin-treated human

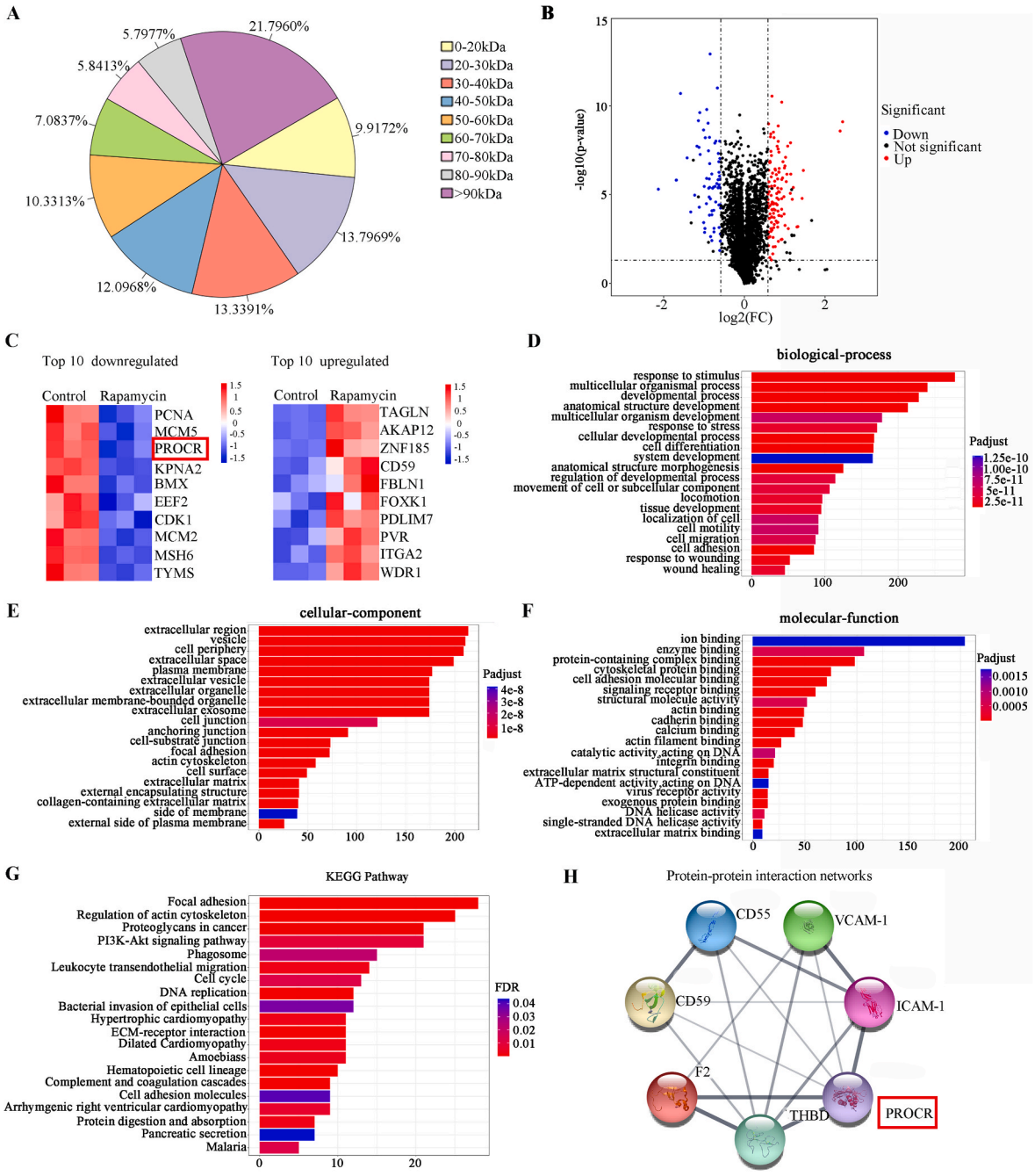

Fig. 1. Screening proteins of rapamycin-induced injury on HCAECs by iTRAQ-based proteomics. (*A*) Protein molecular weight statistics, the proportion of proteins with different molecular weights in the total protein, is shown in the pie chart. **(*B*)** Volcano plot showing 119 up-regulated and 68 down-regulated expressed proteins. **(*C*)** Heat map displaying of top 10 downregulated proteins and top 10 upregulated proteins. **(*D-F*)** GO analysis of differentially expressed proteins shows diverse biological processes, cell components, and molecular functions. **(*G*)** KEGG pathway analysis. **(*H*)** Differentially expressed proteins closely associated with EPCR (PROCR) by protein-protein interaction network analysis.

coronary artery endothelial cells (HCAECs) using isobaric Tags for Relative and Absolute Quantification (iTRAQ) labeling quantitative technology combined with liquid chromatography-mass spectrometry (LC-MS). A total of 4678 proteins were identified and quantified (Table S2). 90.1 % of the proteins were identified with the molecular weight ≥20 kDa (Fig. 1A), and >90 % of them were identified by at least two peptides (Table S2). Among them, 187 proteins were recognized as differentially expressed proteins with a change fold ≥1.5 when compared to the control group, including 119 up-regulated and 68 down-regulated proteins (Fig. 1B–C, **S1,** and Table S3). Gene Ontology (GO) analysis showed that these proteins were significantly enriched in biological processes, cellular components, and molecular functions of the cells, which were related to the cellular functions, including cell adhesion, anatomical structure porphogenesis, extracellular space, integrin binding, and so on (Fig. 1D–F). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed that these differentially expressed proteins are involved in DNA replication, extracellular matrix-receptor interaction, leukocyte transdermal migration, regulation of actin cytoskeleton, cell cycle, phosphoinositide-3 kinase (PI3K)-Akt signaling pathways, etc (Fig. 1G–Table S4). Moreover, protein-protein interaction network analysis showed the connection degree between these proteins (Fig. S2).

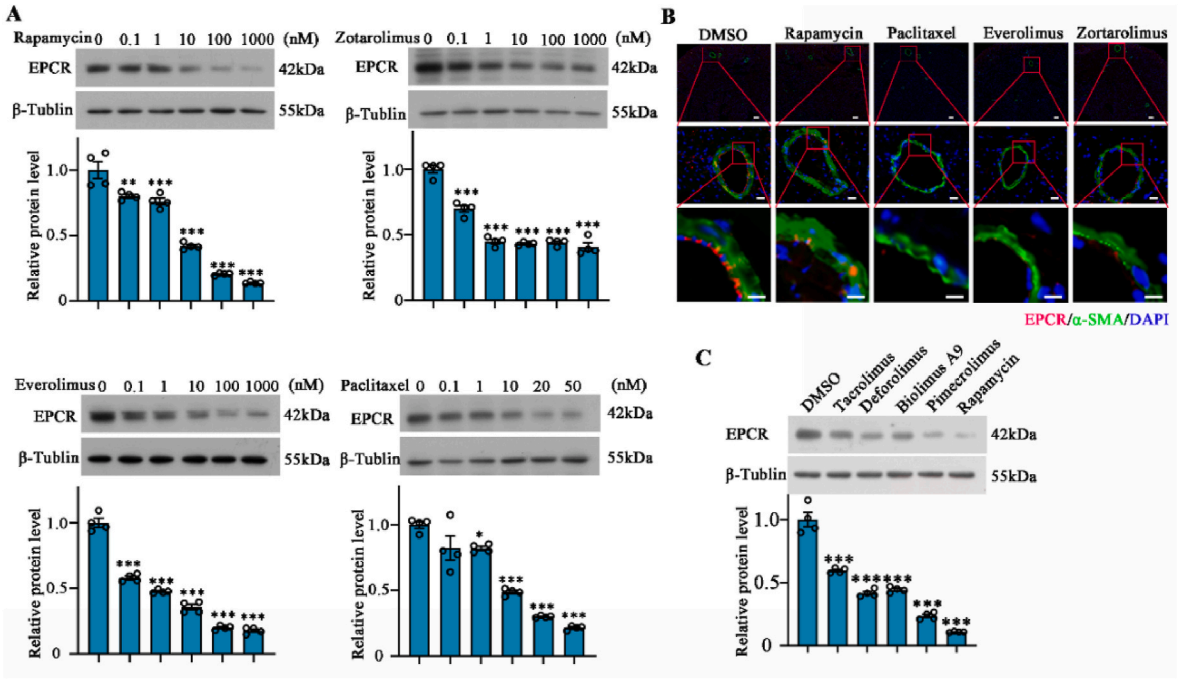

Fig. 2. EPCR is downregulated by limus and paclitaxel in HCAECs and in cardiac vessels of mice. (*A*) Rapamycin, zotarolimus, everolimus and paclitaxel inhibited the protein expression of EPCR in a dose-dependent manner. HCAECs were respectively treated with different concentrations of indicated limus or paclitaxel for 48 h, and then immunoblotting was performed. **(*B*)** EPCR protein level was reduced in limus or paclitaxel-treated mice myocardial vessels. α-SMA: alpha-smooth muscle actin. Scale bars: upper panel, 100 μm; middle panel, 20 μm; lower panel, 10 μm. **(*C*)** The level of EPCR proteins in HCAECs treated with pimecrolimus (20 μM), tacrolimus (100 nM), deforolimus (100 nM), and biolimus A9 (5 μM) was decreased. n = 3–6 biological replicates. All data are presented as mean ± standard error mean. Data in panels A and C were analyzed by one-way ANOVA with Tukey-Kramer *post hoc* test. $^{**}P < 0.01$, and $^{***}P < 0.001$ for indicated comparisons.

Among these differentially expressed proteins, we noted an interesting protein molecule EPCR (PROCR), which was reduced to 0.311-fold (the third highest degree of reduction) in rapamycin-induced HCAECs (Table S3). Protein-protein interaction network analysis indicated that proteins including thrombomodulin (THBD), prothrombin (F2), CD151, CD59, intercellular cell adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) were closely related to EPCR, implying the regulation roles of EPCR in blood clotting, cell adhesion, and inflammatory response (Figs. 1H and S2).

3.2. Limus and paclitaxel repress EPCR expression in HCAECs

We firstly validated that rapamycin (sirolimus) inhibited the expression of EPCR levels in a concentration-dependent manner in HCAECs (Fig. 2A). Consequently, we speculated whether other regularly used stent-coating drugs also had similar effects. HCAECs were treated with different concentrations of zotarolimus, everolimus, or paclitaxel, respectively, showing that these coating drugs all inhibited the protein expression of EPCR in a dose-dependent manner (Fig. 2A). Immunofluorescence staining assay further confirmed that the EPCR expressions in mice myocardial vessels were lessened by limus and paclitaxel treatment *in vivo*, compared to the control group (Fig. 2B). In addition, we observed that other limus drugs, including tacrolimus, deforolimus, biolimus A9, and pimecrolimus, similarly inhibited the protein levels of EPCR in HCAECs (Fig. 2C).

Next, we explored the mechanisms by which limus and paclitaxel repressed the expression of EPCR. We found that limus inhibited the mRNA level of EPCR (Fig. S3A), whereas 0.1–50 nM paclitaxel made no effect on it (Figs. S3A and B). On the other hand, limus and paclitaxel promoted EPCR cleavage to soluble EPCR (sEPCR) and shedding into the culture media (Fig. S3C). A disintegrin and metalloproteinase domains 10/17 (ADAM 10/17) had been reported severed as mediators of EPCR cleavage [30,31]. Here we confirmed that this process was mediated by ADAM10/17, since inhibitor GW280264X restrained the conversion of sEPCR from EPCR (Fig. S3D). Furthermore, ELISA of culture media confirmed that limus and paclitaxel promoted the secretion of sEPCR (Fig. S3E). Therefore, these results indicate that coating drugs downregulate the expression of endothelial EPCR by inhibiting its mRNA transcription, or/and promoting protein cleavage and shedding.

3.3. Overexpression of EPCR protects EC from limus- or paclitaxel-induced injury, inflammatory cell adhesion, and platelet adhesion

It has been well established that EPCR is highly and widely expressed in EC, having a vital role in maintaining normal endothelial functions through the biased protease-activated receptor 1 (PAR1)-Ras related C3 botulinum toxin substrate 1 (RAC1)-p21-activated kinase (PAK)/Akt-endothelial nitric oxide synthases (eNOS) pathways [32,33] (diagramed in Fig. 3A). In order to confirm its functions in EC, we investigated the effect of downregulation of EPCR expression using the RNA interference technique. siRNA 1# and siRNA 2# -mediated downregulation of EPCR inhibited the Akt-eNOS and RAC1-PAK2 signaling pathways in EC (Fig. S4A). Downregulation of EPCR also decreased the cell viability (Fig. S4B), cell proliferation (Fig. S4C), and migration (Fig. S4D), as well as impaired tube formation ability (Fig. S4E) of EC. In addition, si-EPCR promoted the protein expression of ICAM-1 and VCAM-1 in EC (Fig. S4F), amplified the secretion of soluble ICAM-1 (sICAM-1) and soluble VCAM-1 (sVCAM-1) (Fig. S4G), augmented neutrophil adhesion (Fig. S4H), and destroyed the barrier function (Fig. S4I) of EC. Overall, these results demonstrate that the downregulation of EPCR weakens cell proliferation, migration, tubular structure formation, and barrier function of EC, and promotes inflammatory cell adhesion, which is consistent with previous research reports [33,34].

Furthermore, we examined the impact of overexpression of EPCR on limus- or paclitaxel-induced EC injury. Using adenovirus as a vector, we detected that overexpression of EPCR (Fig. 3B) reactivated the RAC1-

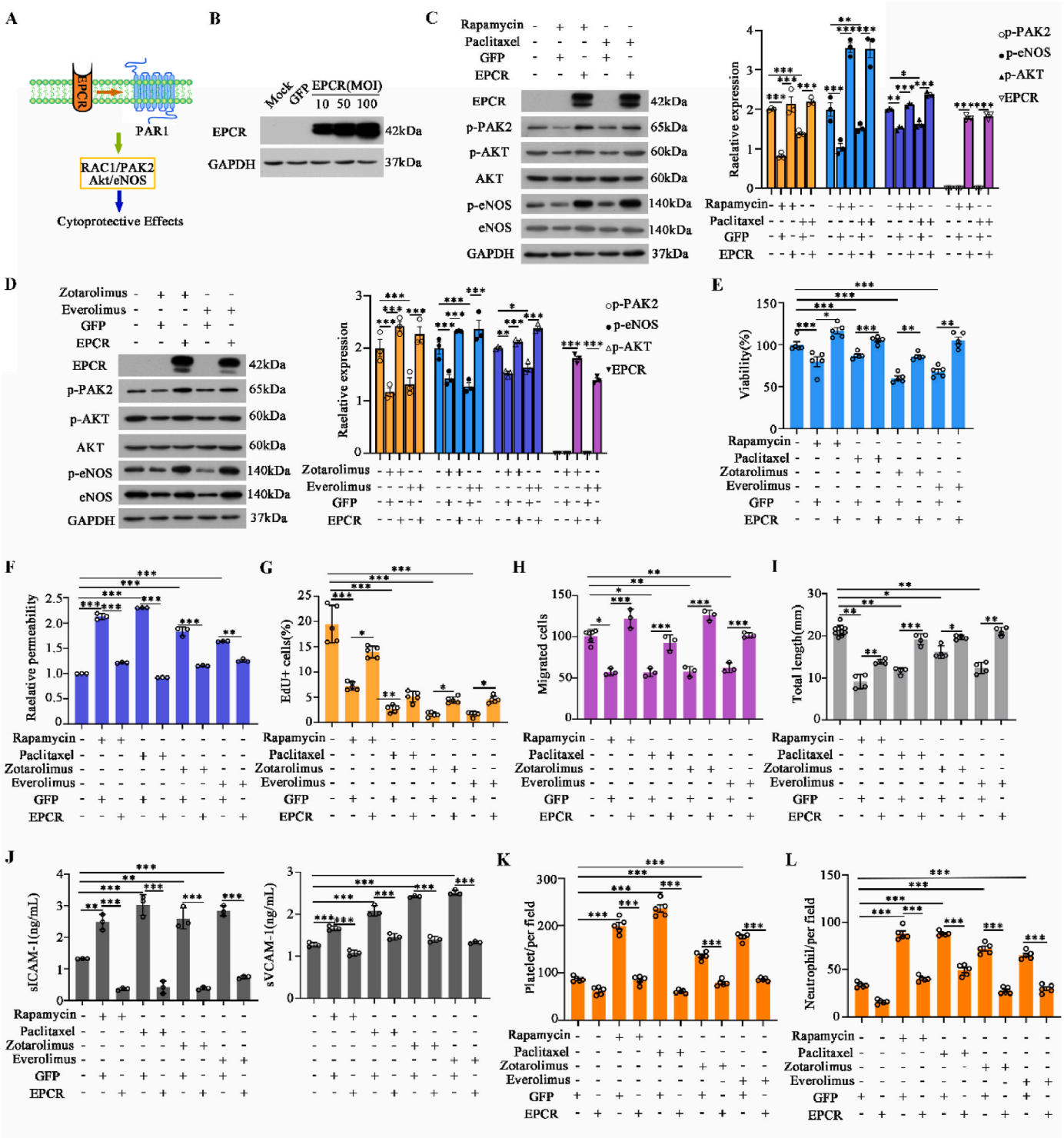

Fig. 3. Overexpression of EPCR protects HCAECs from limus- or paclitaxel-induced injuries by activating biased PAR1-RAC1-PAK/Akt-eNOS pathways. (*A*) Pattern diagram of EPCR-biased PAR1-RAC1-PAK2/Akt-eNOS pathways. **(*B*)** Adenovirus-mediated overexpression of EPCR with 10, 50, or 100 multiplicity of infection (MOI). **(*C*, *D*)** Overexpression of EPCR promoted the activation of biased PAR1-RAC1-PAK/Akt-eNOS pathways. EPCR increased eNOS, AKT and PAK1/2 phosphorylation in HCAECs demaged by limus and paclitaxel. **(*E*)** EC viability detected by CCK-8 assay. **(*F*)** EC barrier function detected by Transwell inserts and Evans blue dye. n = 3. **(*G-I*)** Overexpression of EPCR improved the positive EdU cells, migrated cells (Transwell) and total tuble length in EPCR-infected HCAECs under limus or paclitaxel stimulation. **(*J*)** Overexpression of EPCR inhibited the release of sICAM-1 and sVCMA-1 detected by ELISA, and decreased the EC adhesion of platelets **(*K*)** and neutrophils **(*L*)**, influenced by limus (100 nM) or paclitaxel (20 nM) treatment. All results are presented as the means ± standard error mean, n = 3–10 biological replicates. C-L, MOI 50. Data in panels C–L were determined by two-way repeated measures ANOVA of variance with Tukey's *post hoc* test. **P* < 0.05, ***P* < 0.01 or ****P* < 0.001 for indicated comparisons.

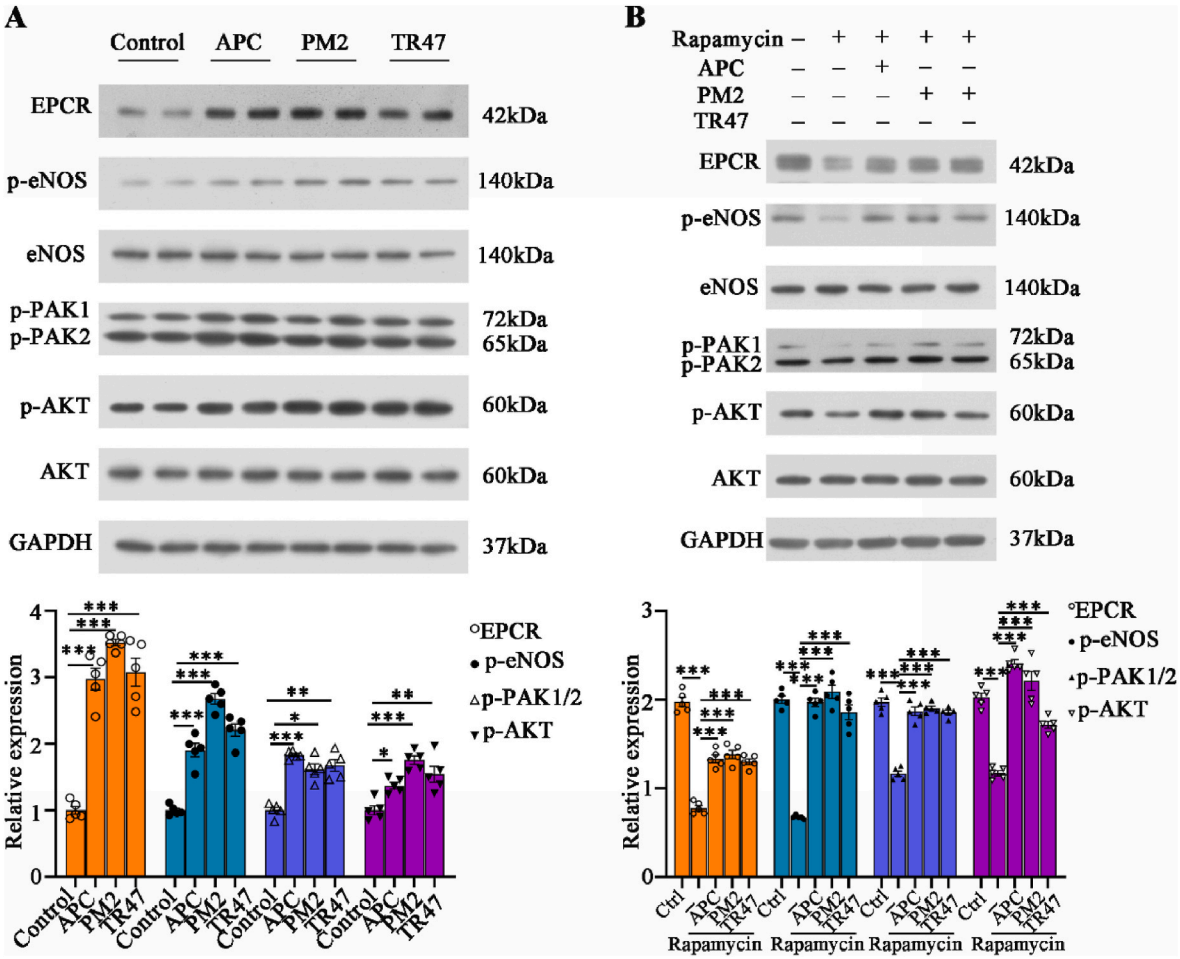

Fig. 4. EPCR-biased PAR1 pathway is protectively activated by APC, TR47, and parmodulin 2. (***A***) APC, TR47, and parmodulin 2 induced the expression of EPCR and activated EPCR-biased PAR1-RAC1-PAK/Akt-eNOS pathways in HCAECs. (***B***) APC, TR47, and parmodulin 2 rescued the rapamycin-induced damage effect in HCAECs. HCAECs were exposed to APC, TR47, or parmodulin 2 without or with rapamycin. Then immunoblotting analyses were performed with indicated antibodies. PM2: parmodulin 2. All results are presented as the mean ± standard error mean, n = 5 biological replicates. Statistical significance was determined by one-way ANOVA with Tukey's *post hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ for indicated comparisons.

PAK/Akt-eNOS pathways (Fig. 3C and D), restored cell viability (Fig. 3E), and rescued barrier function from damage (Fig. 3F). Additionally, the overexpression of EPCR reversed the inhibition of cell proliferation (Figs. 3G and S5A), migration (Fig. 3H–S5B), and tube formation ability (Fig. 3I–S5C) of HCAECs, which was damaged by limus and paclitaxel. Moreover, upregulation of EPCR decreased the release of adhesion molecules sICAM-1 and sVCAM-1 (Fig. 3J) as well as the EC adhesion of platelets (Fig. 3K–S5D) and neutrophils (Fig. 3L–S5E). Thus, overexpression of EPCR protects EC against functional damage and barrier dysfunction, inflammatory cells adhesion, and platelets adhesion induced by limus and paclitaxel drugs.

3.4. Activation of EPCR by APC, TR47, and parmodulin 2

Principally, upregulating the expression of EPCR, stimulating the activity of residual EPCR, and activating the downstream of EPCR-biased PAR1 signal pathway are three ways to reactivate the EPCR pathway for EC protection against damage induced by limus and paclitaxel coating drugs. Activated protein C (APC) [32,33,35], peptide TR47 [36], and compound parmodulin 2 [37,38] are found to activate EPCR. APC is the natural ligand of receptor EPCR, which has an anti-coagulant and direct cytoprotective role in EC [32,33,35]. TR47 is a peptide extracted from the PAR1 protein, which can stimulate protective EPCR-biased PAR1 signaling in EC. Herein, we confirmed that APC, TR47, and parmodulin 2 activated the downstream biased PAR1 pathway of EPCR (Fig. 4A and B). More importantly, APC, TR47, and parmodulin 2 increased the expression level of EPCR in HCAECs regardless of rapamycin treatment (Fig. 4A and B). Furthermore, the EPCR protein level was reduced in the myocardial vessels of rapamycin-treated mice, which was reversed by APC treatment (Fig. S6). Therefore, the ligand APC, the peptide TR47, and the small molecule compound parmodulin 2, three agonists with diverse pharmacological mechanism, exert dual effects of activating the residual EPCR pathway and inducing EPCR expression, thereby activating the EPCR-biased PAR1-RAC1-PAK/Akt-eNOS pathways.

3.5. APC, TR47, and parmodulin 2 promote endothelialization and inhibit platelets or neutrophils adhesion in a simulated stent environment

316L stainless steel (316L SS) is one of the most widely used stent materials due to its excellent corrosion resistance and suitable mechanical properties. We investigated the effects of APC, TR47, and parmodulin 2 on HCAECs in a simulated stent environment using Poly (lactic-*co*-glycolic acid) (PLGA)-drug coating 316L SS plate. PLGA wrapped-rapamycin, -APC/rapamycin, -parmodulin 2/rapamycin, or -TR47/rapamycin, were coated onto the surface of 316L SS plate, respectively. The rapamycin inhibited the adhesion, cell viability, proliferation, and migration of HCAECs in this simulated stent environment (Fig. 5A–D). Interestingly, APC/rapamycin, TR47/rapamycin, or parmodulin 2/rapamycin rescued these above EC functions that were

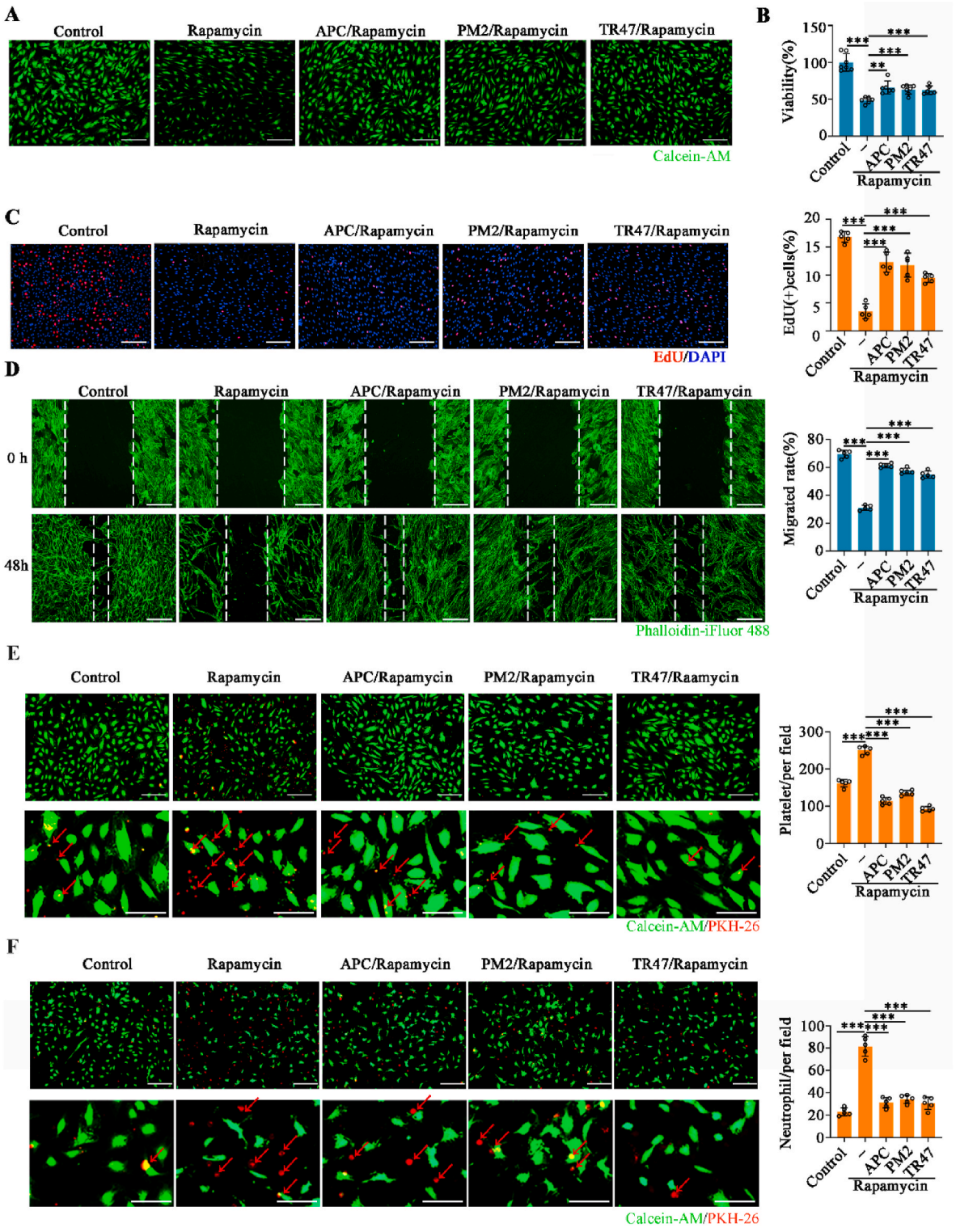

Fig. 5. The effect of APC, TR47 and parmodulin 2 in a simulated stent environment and *in vitro*. PLGA wrapped-rapamycin, - APC/rapamycin, - TR47/rapamycin, or - parmodulin 2/rapamycin were coated onto the surface of 316L stainless steel plate, respectively. (*A*) APC, TR47, and parmodulin 2 increased HCAECs attachment in the simulated stent environment. Calcein-AM stained HCAECs were observed by fluorescence microscope. PM2: parmodulin 2. (*B-D*) APC, TR47, and parmodulin 2 protectively increased the decline of cell viability (*B*), proliferation (*C*), and migration (*D*) induced by rapamycin in HCAECs. HCAECs were treated with rapamycin or APC, TR47 and parmodulin 2 for 48 h, and then CCK-8 assay, EdU incorporation analyses, and scratch wound assay were performed. (*E-F*) APC, TR47, and parmodulin 2 protectively inhibited the platelets (*E*) or neutrophil adhesion (*F*) to HCAECs. Green: calcein-AM labeled HCAECs. Red: PKH-26 labeled platelets or neutrophils. upper panel, 200 μm; lower panel, 100 μm. n = 5-6 biological replicates. Data are represented as mean±standard error mean. Statistical significance was determined by one-way ANOVA with Tukey's *post hoc* test. $^{**}P < 0.01$ and $^{***}P < 0.001$ for indicated comparisons.

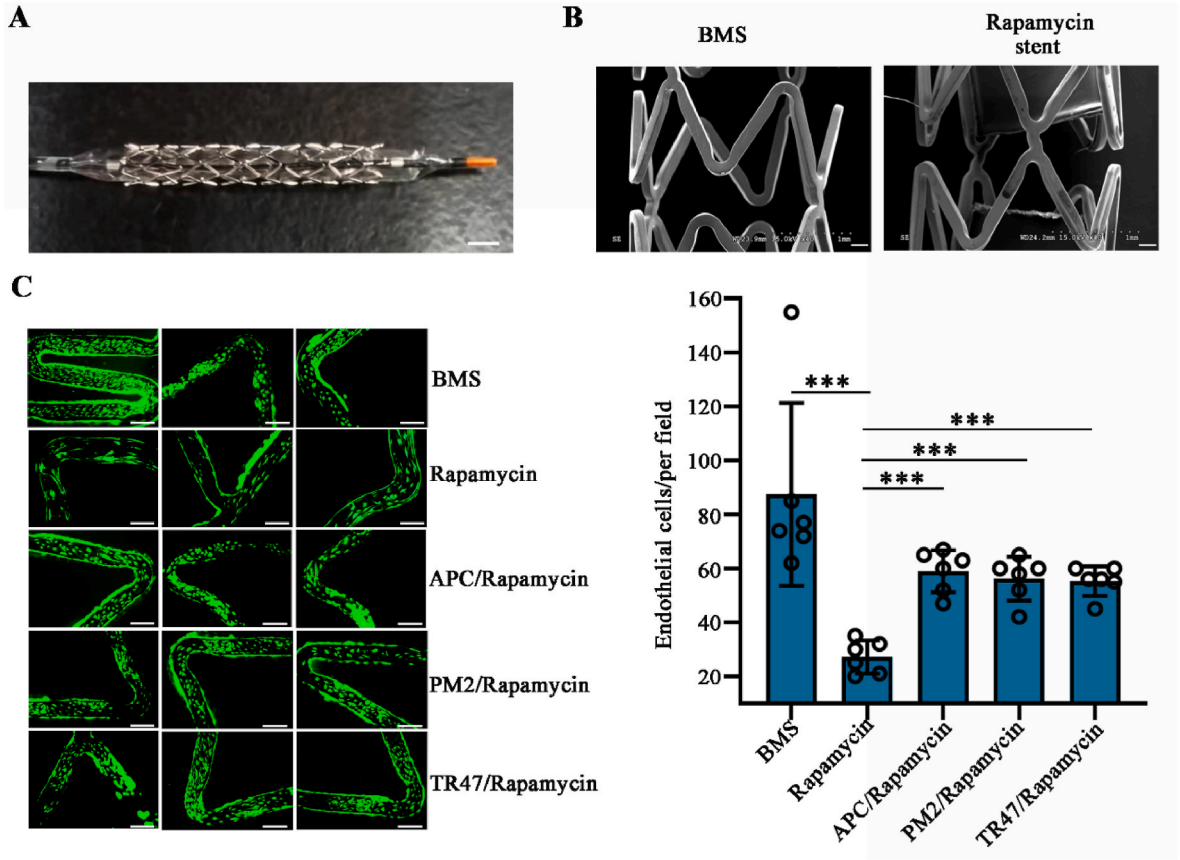

Fig. 6. APC-, TR47-or parmodulin 2-coating stents promote endothelialization *in vitro*. (***A***) Macro shape of a vascular metal stent. Scale bar, 2 mm. (***B***) Representative SEM images of bare metal stent (BMS) and coated rapamycin stent. (***C***) Calcein-AM staining of HCAECs on the stent surface. Three days after being seeded on various coatings of stents *in vitro*, EC were stained with 5 μM calcein-AM (green) and detected by fluorescence microscopy. n = 6. Scale bars, 200 μm. Right panel, quantification of fluorescence EC on stent surface per field. PM2: parmodulin 2. Data were represented as mean ± standard error mean. Statistical significance was determined by one-way ANOVA with Tukey's *post hoc* test. ***$P < 0.001$ for indicated comparisons.

damaged by rapamycin in the steel plate (Fig. 5A–D), including the adhesion, cell viability (increased by 66.07 ± 3.62 %, 62.36 ± 2.35 % and 63.02 ± 2.31 %, respectively), proliferation (increased by 12.03 ± 0.80 %, 9.461 ± 0.35 % and 11.78 ± 0.97 %, respectively), and migration (increased by 61.38 ± 0.62 %, 54.86 ± 1.13 % and 57.52 ± 0.89 %, respectively). Meanwhile, rapamycin coating promoted the adhesion of platelets and neutrophils to EC, while APC/rapamycin, TR47/rapamycin, or parmodulin 2/rapamycin coating exerted the opposite effect (Fig. 5E and F). The above data reveal that the combined use of APC, TR47, or parmodulin 2 rescues the rapamycin-induced EC injury, promotes endothelialization, and inhibits platelets or neutrophils adhesion in a simulated stent environment of PLGA-drug coating 316L SS plate.

3.6. APC, TR47, and parmodulin 2 promote rapamycin stent endothelialization in vitro

Next, using PLGA as the drug carrier, we fabricated various novel coated metal stents for experimental study (Fig. 6A): rapamycin, APC/rapamycin, TR47/rapamycin, and parmodulin 2/rapamycin stents. The morphology of the stent after release was shown under scanning electron microscopy (SEM) (Fig. 6B). Our results showed that rapamycin stents largely reduced the endothelialization than BMS, while APC/rapamycin (increased 59.0 ± 3.2 cells/per field), parmodulin 2/rapamycin (increased 56.2 ± 3.3 cells/per field), and TR47/rapamycin (increased 55.3 ± 2.2 cells/per field) stents had better endothelialization effect than stents coated with rapamycin alone (Fig. 6C). These results demonstrate that APC, TR47, and parmodulin 2 promote endothelialization of rapamycin-eluting stent *in vitro*.

3.7. APC-coating prevents stent thrombosis and promotes reendothelialization in both metal stent and biodegradable stent in vivo

We chose metal and biodegradable stents coated with APC, the natural ligand of EPCR, for further *in vivo* study respectively. We first evaluated APC/rapamycin stents using the *in vitro* platelet adhesion test and ex vivo arteriovenous shunt assay. After 2 h of ex vivo circulation, the BMS and rapamycin stents surface formed more thrombus with a network of fibers and activated platelets, while the APC/rapamycin stents surface only had a few resting, non-activated platelets adhered (Figs. S7A–C). The thrombus weight in the APC/rapamycin group was significantly lower than the rapamycin group (Fig. S7D), indicating excellent anticoagulant properties of APC. The APC/rapamycin coating significantly reduced platelet adhesion and activation compared to rapamycin alone, with platelets exhibiting a resting morphology (Fig. S7E). In conclusion, the APC/rapamycin coating demonstrated excellent antithrombotic effects for preventing stent thrombosis.

APC-coating DES was used for rabbit abdominal aortic implantation, one of the most commonly used models. The ultrastructure of the stent artery lumen in each group was evaluated under SEM after 28 days. Compared with rapamycin-eluting stents, the surfaces of APC/rapamycin stents were adhered by EC, forming a uniform cobblestone-like cell layer, which were almost entirely covered by EC (Fig. S8A). The main axis direction of the cell layer was consistent with the blood flow, and the platelet adhesion was significantly reduced (golden arrow) (Fig. S8A), indicating the absence of thrombosis after APC/rapamycin

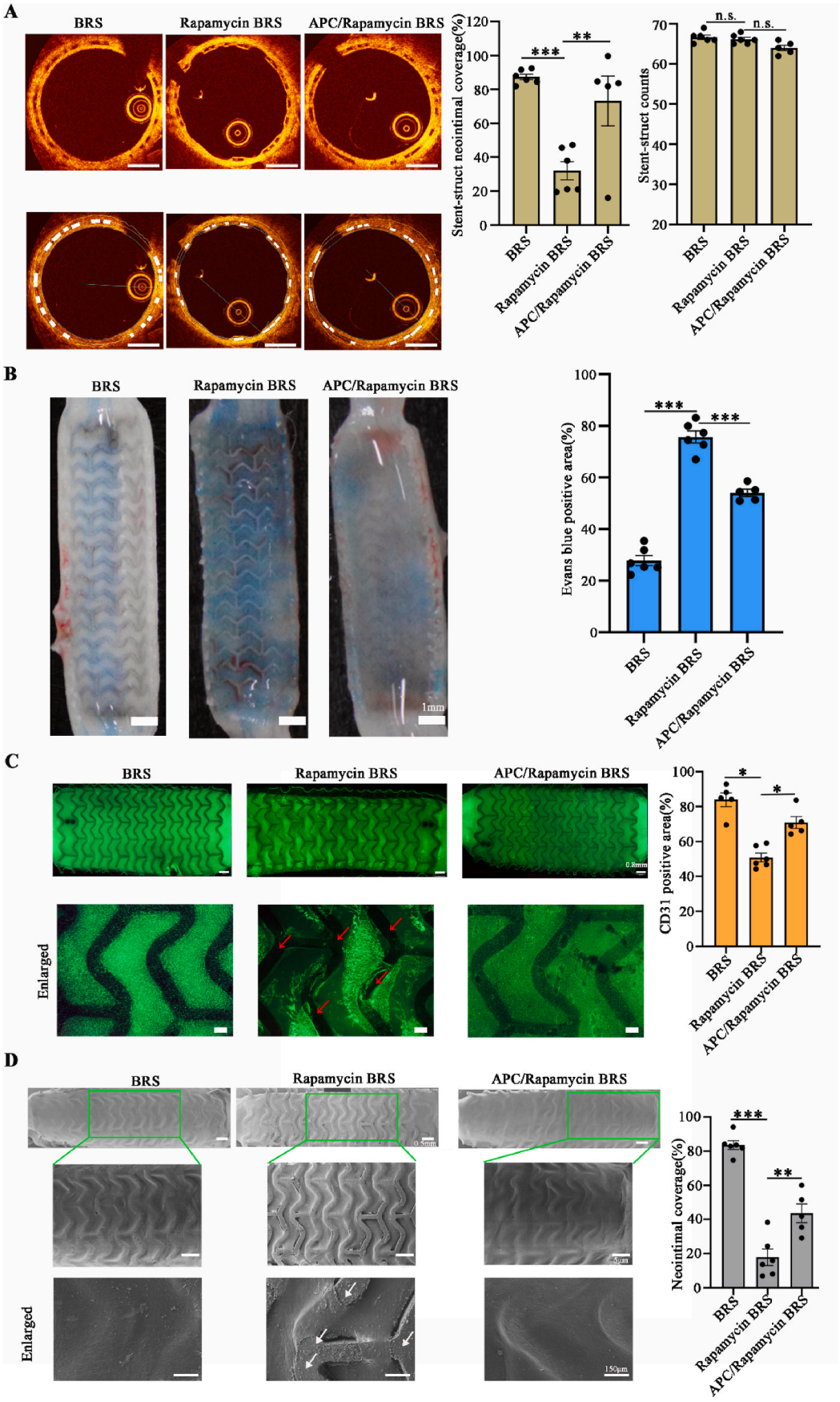

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Fig. 7. ***In vivo*** **reendothelialization effect evaluation of the APC/rapamycin biodegradable stents.** ***(A)*** *In vivo* evaluation of neointimal endothelial coverage 45 days after stent implantation using optical coherence tomography. Scale bar, 1 mm. ***(B)*** Evans blue dye unveiled the vascular injury of rapamycin BRS stented arteries. Scale bar, 1 mm. ***(C)*** CD31 positive fluorescence intensity (green) of stented arteries. The red arrows indicate the stent segments not fully reendothelialized in the rapamycin BRS group. Scale bar: upper panel, 0.8 mm; lower panel, 200 μm. ***(D)*** Representative SEM images showed the reendotheliazation in the rabbit abdomen aorta. The white arrow indicates incomplete endothelialization and adhered platelets. Control BRS, n = 11; Rapamycin BRS, n = 10; APC/Rapamycin BRS, n = 6. Scale bar: upper panel, 0.5 mm; middle panel, 5 μm; lower panel, 150 μm. Data were represented as mean ± standard error mean. Statistical significance was determined by one-way ANOVA with Tukey's *post hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ for indicated comparisons. n.s., not significant.

stents implantation resulting from accelerated reendothelialization of stents.

Biodegradable stent (BRS) is made of biodegradable or absorbable materials which have good histocompatibility and biodegradability. Due to its more conducive properties, BRS is regarded as the future direction for cardiac stent development and application. We used BRS to fabricate various stents (customized in XINSORB® described in the Supplemental Method Section in detail), including control BRS, rapamycin BRS, and APC/rapamycin BRS, which were implanted into the abdominal aorta of rabbits to evaluate the effect of EPCR activated coating stent on vascular reendothelialization. Stents were implanted under the guide of real-time angiography (Fig. S9). After 45 days, all stents placed into the rabbits were underwent optical coherence tomography (OCT) examination to evaluate *in vivo* vascular reendothelialization, finding that the stent-struct neointimal coverage area of the APC/rapamycin BRS group (73.22 ± 14.65 %) was significantly improved compared to the rapamycin BRS group (32.10 ± 5.31 %) (Fig. 7A). Using Evans Blue dye analysis, we verified that the rapamycin BRS group had severe vascular damage area and delayed reendothelialization compared to the control BRS group (75.82 % *vs.* 27.84 %, Fig. 7B). Comparatively, the APC/rapamycin BRS presented a much better reendothelialization effect (54.12 % *vs.* 75.82 %, Fig. 7B). We also used CD31 immunofluorescence to evaluate EC coverage, finding that the density of positive fluorescence endothelium in the APC/rapamycin BRS group was significantly higher compared to the rapamycin BRS group (70.90 % *vs.* 50.89 %), suggesting a pro-angiogenesis role of EPCR activation by APC (Fig. 7C). Further SEM analysis revealed that many EC adhered to the APC/rapamycin (43.62 ± 5.53 %) or control BRS (83.62 ± 2.54 %) surface, forming a uniform cobblestone-like cell layer, with the main axis direction being consistent with blood flow (Fig. 7D). In contrast, the rapamycin BRS was still exposed with incomplete endothelialization (17.90 ± 4.83 %), and a large number of platelets adhered (Fig. 7D). These data demonstrate that APC-coating promotes stent reendothelialization after implantation *in vivo*.

3.8. Outcomes of overexpression or activation of EPCR on SMC cellular behaviors and rapamycin effect

After stent implantation, pathological vascular SMC from the damaged vascular wall undergo inappropriate proliferation, resulting in ISR. Inhibition of SMC proliferation and migration by limus or paclitaxel is crucial for inhibiting ISR. Subsequently, we examined the outcomes of EPCR activation on SMC cellular behaviors and the inhibitory effect of limus. In APC-coating DES experiment *in vivo* (Fig. S8B), BMS had more intimal hyperplasia than rapamycin and APC/rapamycin stents, while there was no significant difference between rapamycin and APC/rapamycin stents after implantation, suggesting that the activation of EPCR (by APC) did not affect the antiproliferative effects of rapamycin on SMC.

Previous studies have well established that EPCR is specifically expressed in EC, while scarcely expressed in SMC (reviewed in Refs. [33,34,39]and references [40,41]). Herein, we also confirmed that EPCR was highly expressed in HCAECs, human aortic endothelial cells (HAECs), human umbilical vein endothelial cells (HUVECs), and human cardiac microvascular endothelial cells (HCMECs), while SMC such as human aortic smooth muscle cells (HASMCs) and human coronary artery smooth muscle cells (HCASMCs) could be hardly detected (Fig. 8A). Next, we explored the effect of EPCR on SMC, finding that overexpression of EPCR or activation of EPCR by APC did not affect the cell viability (Fig. 8B and C), proliferation (Fig. 8D and E), and migration (Fig. 8F–I) of SMC, nor the inhibitory effect of rapamycin on SMC (Fig. 8C–E, G, I). Furthermore, we detected the protein changes in the contractile phenotype (α-smooth muscle actin [α-SMA] and smooth muscle 22α [SM22α]) and synthetic phenotype (Osteopontin) of HCASMCs after EPCR overexpression or APC stimulus with or without rapamycin treatment. The results showed that EPCR overexpression or APC treatment did not affect the above cell phenotype in smooth muscles (Fig. S10A). In addition, APC alone or APC treated with rapamycin did not change the contractile phenotype markers (α-SMA and SM22α) and proliferative phenotype marker (OPN) (Fig. S10B) of smooth muscles. APC also did not change the effect of rapamycin (Fig. S10B). Therefore, activation of the target EPCR does not affect the SMC cellular behaviors or the inhibitory effect of rapamycin on SMC.

4. Discussion

There are two strategies for identifying cell-specific molecular targets to design novel vessel stents based on the differences between SMC and EC. The first is to find molecular targets specifically expressed in SMC but not in EC, thereby effectively suppressing SMC without affecting EC. The second is to activate molecular targets specifically expressed in EC but not in SMC, thereby promoting EC functions and the endothelialization process without affecting the effectiveness of eluting drugs. In the present study, we used the second strategy (the research strategy and process are illustrated in Fig. S11).

Finding a protective intervention for EC injury induced by limus and paclitaxel, which promotes reendothelialization and effectively prevents ST represents an urgent problem in the vascular eluting stent. In the present study, we attempted to solve this issue from the perspective of specific molecular mechanisms in EC. We found an imperative target for promoting the stent reendothelialization process and prevention of ST, which has potential significance for future research and clinical application of novel vascular stent.

4.1. Limus and paclitaxel promote thrombosis by downregulating EPCR expression

As a foreign object in the body, the implanted stent with coating drugs (such as limus and paclitaxel), inevitably causes EC damage and loss, activates surrounding platelets and inflammatory cells, and then triggers thrombosis. Therefore, the process rate of stent reendothelialization determines the magnitude of ST risk. However, the exact and specific mechanisms underlying coating drugs-induced EC injury have not yet been fully elucidated.

EPCR, a vital effector molecule for EC, maintains its basic functions, such as proliferation, migration, tubule formation, and barrier function through the APC-EPCR-biased PAR1-RAC1-PAK/Akt-eNOS pathways (reviewed in Ref. [42]). APC-EPCR also has a crucial role in EC survival and angiogenesis (reviewed in Refs. [33,43]). In the current study, we confirmed the EPCR-mediated EC functions through several techniques, including siRNA (Fig. S4), overexpression (Fig. 3 and S5), and agonists (Figs. 4 and 5).

EPCR is the central component of the APC anticoagulant system (reviewed in Refs. [33,42]. Fetal mice lacking the EPCR gene die early in the embryo due to thrombosis suggesting the role of EPCR in maintaining a normal thrombin balance [44]. Human subjects research has

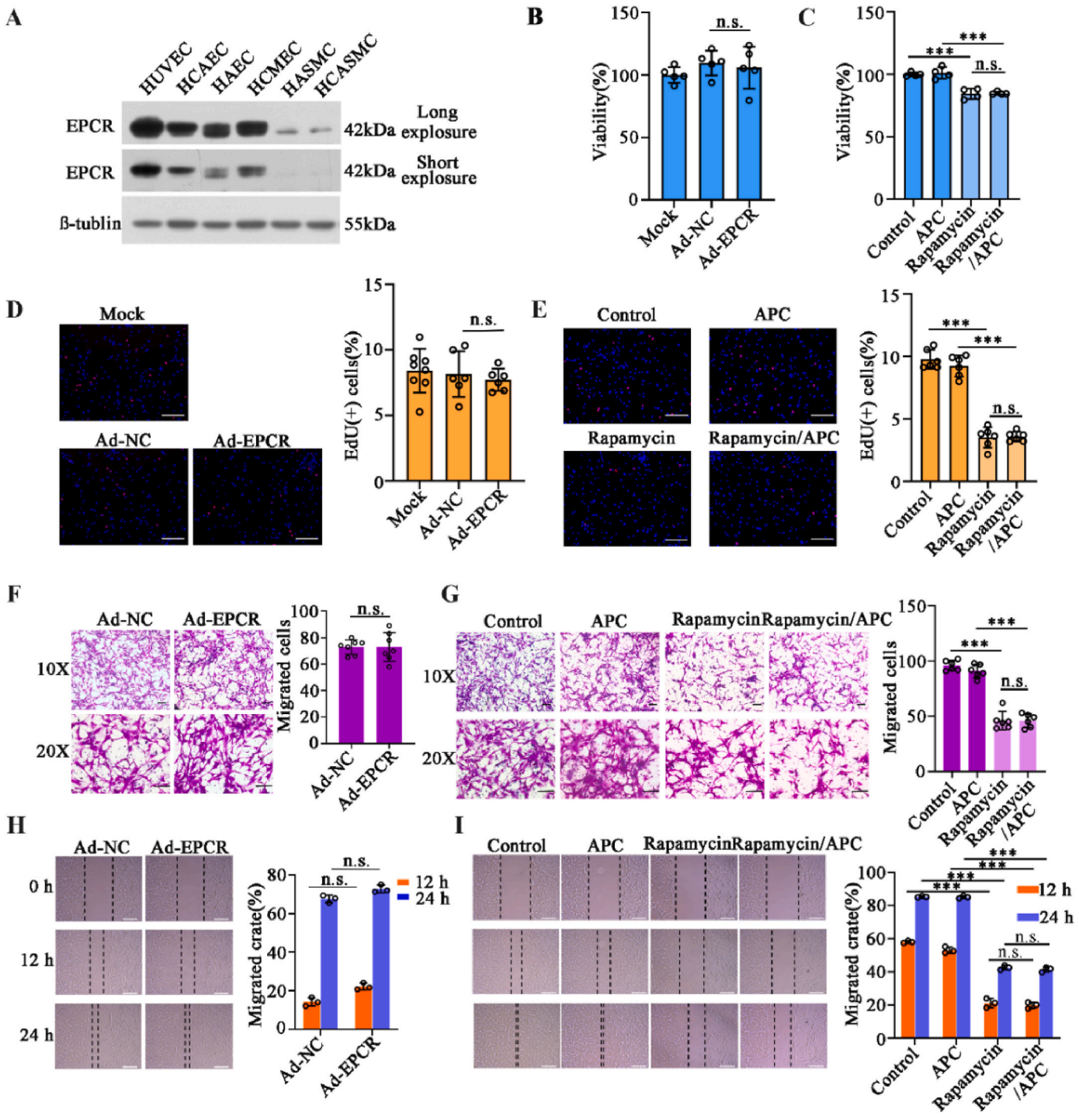

Fig. 8. Overexpression or activation of EPCR does not affect the cellular behavior of SMC or the inhibitory effect of rapamycin. (*A*) Immunoblotting analysis of EPCR expression levels in HUVECs, HCAECs, HAECs, HCMECs, HASMCs, and HCASMCs. Note that EPCR is extraordinarily higher expressed in diverse EC than SMC. **(*B*, *C*)** CCK-8 cell viability assay of HCASMCs handled with indicated treatment. n = 5. **(*D*, *E*)** Cell proliferation was analyzed by EdU incorporation of HCASMCs treated with Ad-NC, Ad-EPCR, rapamycin, or APC/rapamycin. Scale bar: 200 μm. n = 5–6. **(*F*, *G*)** Transwell inserts analysis of HCASMCs under indicated treatments. Scale bar: 100 μm. **(*H*, *I*)** Scratch assay of HCASMCs with Ad-NC, Ad-EPCR, rapamycin, or APC/rapamycin treatment. Scale bar: 100 μm. n = 3–8. ***$P <$ 0.001 for indicated comparisons. n.s., not significant. Data were represented as mean ± standard error mean. ***$P < 0.001$ for indicated comparisons. Statistical significance was determined by one-way ANOVA with Tukey's *post hoc* test (A-B, D, F), and two-way repeated measures ANOVA of variance with Tukey's *post hoc* test (C, E, G, H–I).

also found that defect in the EPCR gene leads to an increased risk of thrombosis [45]. Surprisingly, in COVID-19 patients, the expression of EPCR in pulmonary EC was found to be dramatically downregulated, resulting in irregular EC functions, severe thrombosis, and hypercoagulability in the lungs [46,47]. Whereas APC could improve their symptoms in COVID-19 patients.

Considering the role of EPCR in anticoagulation, the downregulation of EPCR expression by limus and paclitaxel drugs may be a primary motive for the increased risk of ST. This study found that limus and paclitaxel significantly suppressed EPCR expression (Figs. 1 and 2). On

the other hand, both overexpression and activation of EPCR (by APC, TR47, and parmodulin 2) rescued limus or paclitaxel-induced EC injuries and platelets adhesion (Figs. 3 and 5). Moreover, EPCR ligand (APC) coating stent reduced platelets adhesion and thrombosis (Figs. 5 and 7, S8). These results demonstrate that limus and paclitaxel promote thrombosis by downregulating EPCR expression. Therefore, overexpression or activation of EPCR largely contributes to preventing ST.

4.2. EPCR is a novel potential agonistic target for the interference of ST

Considering its unique and forceful role, EPCR can indeed be an innovative potential agonistic target for ST intervention: (**1**) As mentioned earlier, EPCR is the central component of the APC anticoagulant system, guiding the APC activity and preventing thrombosis. (**2**) Activation of the EPCR pathway also reduces inflammation by downregulating inflammatory mediators and vascular adhesion molecules, thereby reducing the adhesion and penetration of immune cells and limiting the damage to vascular tissue [48]. In the present study, we confirmed that either overexpression of EPCR (Fig. 3J–L, S5D-E) or activation of EPCR (by APC, peptide TR47, or parmodulin 2) reduced the expression and secretion of adhesion molecules and the EC adhesion of inflammatory cells and platelets (Fig. 5E–F, S8A, 7D). (**3**) As mentioned above, EPCR is a crucial effector for maintaining endothelial functions, such as proliferation, migration, tubule formation, and barrier function. EPCR promotes endothelialization and angiogenesis and improves EC survival by activating the anti-apoptotic pathway. (**4**) EPCR is specifically expressed in EC, while scarcely in SMC, as previously described. Thus, there is a possibility that activating EPCR only promotes EC functions without affecting SMC cellular behavior and the effects of coating drugs on SMC (Fig. 8). (**5**) EPCR is a cell surface receptor. This property makes it possess the naturalness to be a convenient drug target.

4.3. Stent combined coated with APC is expected to become a potential and practical way to prevent ST

As the ligand and natural agonist of EPCR, APC has similar functions to EPCR: anticoagulant, anti-inflammation, anti-apoptosis, stabilizing endothelial barrier, and promoting angiogenesis (reviewed in Ref. [33]). APC is an attractive candidate for treating coagulation related diseases, such as sepsis [49]. Further studies have shown that short-term coronary artery exposure to APC alone reduced inflammatory response, neointimal hyperplasia, restenosis [50], and stent thrombosis [51].

Limus has a detrimental effect on EC, leading to a risk of thrombosis and, ultimately, myocardial infarction and death. By activating EPCR, APC can potentially oppose this complication. Herein, we proposed a formula to combine limus and APC to complement their properties and achieve both anti-restenosis and anti-ST effects. This study confirmed that APC significantly attenuated the EC injury and the neutrophil and platelet adhesion by coating limus drugs (Fig. 3, S5, 5). Moreover, combined APC coating stent verified that APC could promote the process of reendothelialization *in vitro* and *in vivo* (Figs. 6 and 7, S8). Therefore, APC and other EPCR agonists may be potential drugs for preventing and treating ST.

In summary, this novel formula based on a combination of limus and APC exerts a dual effect of inhibiting SMC proliferation and promoting EC reendothelialization, and is expected to gain a strong potential practical value in improving the stent safety and effectiveness by reducing the risk of both IRS and ST (Graphical Abstract illustrated in Fig. S12).

Data availability

All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials. The additional data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Second Affiliated Hospital of Guangzhou Medical University (Ethics Number: 2022-KY-ks-01). All animal experimentation conducted at the Guangzhou Medical University and the Animal Ethics Committee of Zhongshan Hospital of Fudan University received prior approval from the Experimental Animal Ethics Committee (Ethics Number of mice: A2019-014, Ethics Number of rabbits: SH2022-06009).

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CRediT authorship contribution statement

Jing Chen: Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Changyi Zhou:** Software, Methodology, Investigation, Formal analysis, Data curation. **Weilun Fang:** Methodology, Investigation, Data curation. **Jiasheng Yin:** Software, Methodology, Investigation, Funding acquisition. **Jian Shi:** Methodology, Investigation. **Junbo Ge:** Supervision, Software, Funding acquisition. **Li Shen:** Writing – review & editing, Validation, Supervision, Software, Resources, Funding acquisition. **Shi-Ming Liu:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Funding acquisition. **Shao-Jun Liu:** Writing – review & editing, Validation, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

S.J.L., S.M.L., J.C., and W.F. are inventors of “Novel application of endothelial protein C receptor” (China Patent no. ZL 202010260514.6). The other authors declare no conflict interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2024.07.028.

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