Targeting glycolysis in endothelial cells to prevent intraplaque neovascularization and atherogenesis in mice

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Cover Page

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Chapter 6

PFKFB3 gene deletion in endothelial cells inhibits intraplaque angiogenesis and lesion formation in a murine model of venous bypass grafting

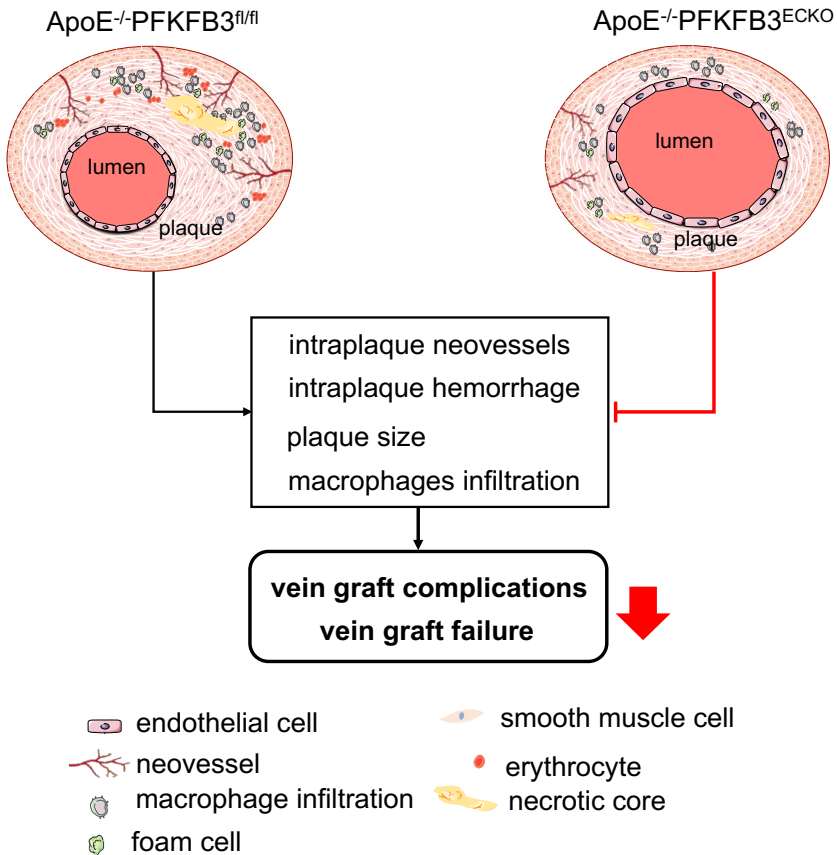**Perrotta P**, de Vries MR, De Meyer GRY, Quax P and Martinet W

Abstract

Objective: Vein grafting is a frequently used surgical intervention for cardiac revascularization. However, vein grafts display regions with intraplaque (IP) angiogenesis, which promotes atherogenesis and formation of unstable plaques. Graft neovessels are mainly composed of endothelial cells (ECs) that largely depend on glycolysis for migration and proliferation. In the present study, we aimed to investigate whether loss of the glycolytic flux enzyme phosphofructokinase-2/fructose-2,6-bisphosphatase 3 (PFKFB3) in ECs inhibits IP angiogenesis and as such prevents unstable plaque formation.

Approach and results: Apolipoprotein E deficient (ApoE$^{-/-}$) mice were backcrossed to a previously generated PFKFB3$^{fl/fl}$ Cdh5^{iCre} mouse strain. Animals were injected with either corn oil (ApoE$^{-/-}$PFKFB3$^{fl/fl}$) or tamoxifen (ApoE$^{-/-}$PFKFB3^{ECKO}), and were fed a western type diet for 4 weeks prior to vein grafting. Hereafter, mice received a western diet for an additional 28 days and were then sacrificed for graft assessment. Size and thickness of vein graft lesions were reduced respectively by 35% and 32% in ApoE$^{-/-}$PFKFB3^{ECKO} mice as compared to controls, whereas stenosis diminished by 23%. Moreover, vein graft lesions in ApoE$^{-/-}$PFKFB3^{ECKO} mice showed a significant reduction in macrophage infiltration (29%), number of neovessels (62%) and hemorrhages (86%). EC-specific PFKFB3 deletion did not show obvious adverse effects or changes in general metabolism.

To investigate the effect of an EC-specific deletion of PFKFB3 on native atherosclerosis, ApoE$^{-/-}$PFKFB3$^{fl/fl}$ and ApoE$^{-/-}$PFKFB3^{ECKO} were fed a western type diet for 12 weeks without the vein graft procedure. At the end of the experiment, brachiocephalic arteries were collected and analyzed. PFKFB3 EC-specific deletion also decreased plaque area and thickness of native lesions in atherosclerotic ApoE$^{-/-}$ mice.

Conclusions: EC-specific PFKFB3 gene deletion leads to a significant reduction in lesion size, IP angiogenesis and hemorrhagic complications in vein grafts. This study also shows that inhibition of endothelial glycolysis is a promising therapeutic strategy to slow down plaque progression in the context of accelerated atherosclerosis following vein graft surgical intervention.

Graphic abstract

ApoE$^{-/-}$PFKFB3$^{fl/fl}$ — ApoE$^{-/-}$PFKFB3^{ECKO}

lumen, plaque, lumen, plaque

intraplaque neovessels
intraplaque hemorrhage
plaque size
macrophages infiltration

vein graft complications
vein graft failure

- endothelial cell
- neovessel
- macrophage infiltration
- foam cell
- smooth muscle cell
- erythrocyte
- necrotic core

Highlights

- PFKFB3 deletion in endothelial cells reduces intraplaque angiogenesis in a mouse model of venous bypass grafting.
- PFKFB3 deletion in endothelial cells reduces lesion size and macrophage infiltration in mouse vein grafts.
- PFKFB3 deletion in endothelial cells reduces hemorrhagic complications in mouse vein graft lesions.
- PFKFB3 deletion in endothelial cells does not affect general metabolism of adult mice.

Introduction

Atherosclerosis is a chronic inflammatory disease of the arterial wall and is one of the most important causes of cardiovascular disease including severe conditions such as coronary artery disease, myocardial infarction, heart failure and stroke. Vein bypass grafting is a surgical procedure that uses large saphenous veins to bypass occluded atherosclerotic arteries, thereby allowing revascularization of an ischemic region of the heart or limbs.[1] Unfortunately, at least 40% of patients suffer from bypass failure within eight years after the procedure due to negative vascular remodeling and intimal hyperplasia.[1-4] Furthermore, vein grafts often present accelerated atherosclerosis with formation of unstable plaques and increased risk of rupture.[5-7]

New capillaries can form inside vein grafts to fulfill an increased demand of oxygen and nourishment of the vessel wall. This event, which is further promoted by inflammatory conditions, leads to intraplaque (IP) angiogenesis and contributes to plaque instability in the vein graft.[8] Indeed, apolipoprotein E deficient (ApoE$^{-/-}$) mice undergoing a vein graft interposition of the carotid artery develop unstable plaques with extensive IP neovessels that are often dysfunctional or immature and contribute to lesion destabilization by enhancing leukocyte recruitment and accumulation of cholesterol and platelets.[9, 10]

Angiogenesis is an energy-intensive process that requires extensive metabolic functioning of endothelial cells (ECs) to support sprouting, migration and proliferation.[11] Recent studies have shown that ECs in neovessels generate more than 85% of their ATP by glycolysis.[12, 13] One of the rate-limiting checkpoints of glycolytic flux is the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate by 6-phosphofructo-1-kinase. Phosphofructokinase-2/fructose-2,6-bisphosphatase (PFKFB) enzymes synthesize fructose-2,6-bisphosphate, an allosteric activator of 6-phosphofructo-1-kinase and the most potent stimulator of glycolysis. Of all PFKFB iso-enzymes, PFKFB3 appears the major producer of intracellular fructose-2,6-bisphosphate in ECs. PFKFB3 is upregulated in ECs under inflammatory conditions and its pharmacological inhibition or gene silencing reduces pathological angiogenesis in response to injury and inflammation.[14] [15, 16] Previous findings have shown that inhibition of PFKFB3 leads to reduced EC migration and

proliferation in vitro. Additionally, sprout number and length of EC spheroids significantly decrease after knocking out PFKFB3.[17]

We have recently reported that the partial glycolysis inhibitor 3PO (3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one) reduces IP angiogenesis and plaque formation.[18] However, the specific role of endothelial PFKFB3 in the context of IP neovascularization and lesion progression remains to be investigated. Therefore, in the present study we used a vein graft procedure in EC-specific conditional PFKFB3 knockout mice on an ApoE$^{-/-}$ background to test whether endothelial PFKFB3 is an important driver of IP angiogenesis and atherosclerotic lesion progression.

Methods

All primary data that support the findings of this study are available from the corresponding author upon reasonable request.

Animals

EC-specific conditional PFKFB3 knockout mice (PFKFB3$^{fl/fl}$) were generated by crossbreeding PFKFB3$^{fl/fl}$ mice with VE-cadherin (PAC)-Cre^{ERT2} mice (Cdh5^{iCre}).[17] Resulting mice were crossbred with ApoE$^{-/-}$ mice to generate an ApoE$^{-/-}$ PFKFB3$^{fl/fl}$Cdh5^{iCre} strain. All mice were on C57BL/6N background. ApoE$^{-/-}$ PFKFB3$^{fl/fl}$Cdh5^{iCre} mice (male, 6 weeks old) were injected with tamoxifen (0.1g/kg body weight) for 5 consecutive days to induce PFKFB3 deletion in ECs, termed ApoE$^{-/-}$PFKFB3^{ECKO}. ApoE$^{-/-}$PFKFB3$^{fl/fl}$Cdh5^{iCre} control mice, further referred to as ApoE$^{-/-}$PFKFB3$^{fl/fl}$ mice, were injected with corn-oil using the same protocol. All animal procedures were conducted according to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Experiments were approved by the ethics committee of the University of Antwerp.

Vein graft surgery

ApoE-/-PFKFB3ECKO and ApoE-/-PFKFB3fl/fl mice were fed a western-type diet (Altromin, C1000 diet supplemented with 20% milkfat and 0.15% cholesterol, #100171) for 4 weeks (Supplemental Figure I). Next, vein graft surgery was performed as described.[5, 7] Briefly, thoracal caval veins from donor ApoE-/-PFKFB3ECKO or ApoE-/-PFKFB3fl/fl mice were harvested. In the first group, ApoE-/-PFKFB3fl/fl recipient mice received the caval veins from ApoE-/-PFKFB3fl/fl donor mice; in the second group, ApoE-/-PFKFB3ECKO mice received the caval veins from ApoE-/-PFKFB3ECKO mice. For each experiment, the right carotid artery of recipient mice was dissected and cut in the middle. On both the proximal and distal artery end, a nylon cuff was sleeved and fixated with hemostatic clamps. The artery was everted around the cuffs and ligated with 8.0 sutures. Next, the caval veins were positioned over both cuffs, and ligated. Before surgery, mice were anesthetized with midazolam (5 mg/kg body weight, i.p., Roche), medetomidine (0.5 mg/kg body weight, i.p., Orion) and Fentanyl (0.05 mg/kg body weight, i.p., Janssen). After the procedure, mice were antagonized with atipamezole (2.5 mg/kg body weight, i.p., Orion) and fluminazenil (0.5 mg/kg body weight, i.p., Fresenius Kabi). Buprenorphine (0.1 mg/kg body weight, i.p., MSD Animal Health) was given after surgery to relieve pain. Animals were sacrificed under the aforementioned anesthesia 28 days after the graft procedure, followed by 2 minutes of in vivo perfusion-fixation.

Native Atherosclerosis study

In another series of experiments, atherosclerotic plaques in the brachiocephalic arteries of ApoE^{-/-} PFKFB3^{fl/fl} and ApoE^{-/-} PFKFB3[ECKO] mice (8 weeks old) were examined after feeding western-type diet (WD) for 12 weeks (Supplemental Figure IB).

Histology

After euthanasia, vein graft segments were collected, fixed in 4% paraformaldehyde (PFA) for 24 hours, dehydrated overnight in 60% isopropanol and subsequently

embedded in paraffin. Cross sections of vein graft segments were stained with hematoxylin and eosin to evaluate lumen and lesion area, plaque thickness and percentage of vein stenosis. Neovessels were detected inside vein graft lesions via standard immunohistochemistry using anti-CD31 antibody (endothelial cells; ab124432, Abcam). Anti-TER-119 (550565, BD Biosciences) was used to determine plaque hemorrhages and anti-α-smooth muscle actin (α-SMA) (A2547, Sigma-Aldrich) was used to determine vascular smooth muscle cell (VSMC) coverage of neovessels. Anti-MAC3 (550292, Pharmingen), a Masson's Trichrome stain and anti-vascular cell adhesion molecule-1 (VCAM) (ab134047, Abcam) were used to stain macrophages, collagen and VCAM positive ECs, respectively.

Metabolic parameters

To determine whether ApoE$^{-/-}$PFKFB3^{ECKO} mice exhibit an alteration in glucose metabolism and to characterize the metabolic phenotype, a glucose tolerance test (GTT) and insulin tolerance test (ITT) were done.

To perform GTT, mice were fasted for 16 hours, injected with a single dose of glucose (1 g/kg body weight, i.p.) and then glucose levels in peripheral blood (from tail) were determined after fixed time intervals (0-30-60-120 minutes) using hand-held glucometer (OneTouch Ultra, range 20-600 mg/dL; Lifescan). For ITT, a single insulin dose was injected (Novorapid, 1 U/kg body weight, i.p.) in mice and blood glucose levels were monitored as in GTT. Liver enzymes, total cholesterol and triglycerides, were analyzed with an automated Vista 1500 System (Siemens Healthcare Diagnostics). Insulin and β-hydroxybutyrate in plasma samples were determined with a mouse insulin ELISA kit (80-INSMS-E01, ALPCO) and β-hydroxybutyrate assay kit (ab83390, Abcam), respectively.

Aortic sprouting

An aortic ring assay was performed as previously described.[19] In brief, murine thoracic aortas were dissected, cleaned under sterile conditions, transferred to 10 cm culture dishes, and cut into 0.5 mm thick rings with a sterile scalpel. After overnight starvation in serum-free Opti-MEM at 37°C, ring segments were transferred into wells of a 96-well plate coated with 50 µL of a freshly prepared collagen type I solution (1 mg/mL). The aortic rings remained in Opti-MEM (supplemented with 2.5% fetal bovine serum and antibiotics) in the presence or absence of vascular endothelial growth factor (40 ng/mL, R&D Systems). Medium was

replaced every 2 days. On day 6, rings were fixed with 4% paraformaldehyde and stained with von Willebrand factor antibody (PC054, Binding Site), that was added overnight prior to fluorescence microscopy imaging. The number of sprouts was counted for each ring and sprout numbers per ring were averaged for each group and graphed.

Mouse lung EC isolation

Primary mouse ECs were isolated as previously described.[20] Briefly, 4 lungs were harvested, finely minced with scissors and digested with 1.5 mg/ml collagenase Type I (Sigma-Aldrich #C0130) at 37°C for 45 min (under gentle agitation). The digested cell suspension was filtered on a 70 µM sterile cell strainer, and spun at 400g for 10 min. The pellet was resuspended in 2 ml of 0.1% bovine serum albumin and 50 µl magnetic dynabeads (ThermoFisher #11035) precoated overnight with anti-mouse CD31 (BD Pharmingen #553370) for EC-positive selection. After 20 min at room temperature under slow rotation, the bead-bound cells were recovered with a magnetic separator and washed five times with DMEM containing 10% fetal bovine serum. Cells were finally resuspended in 10 ml of complete DMEM medium (DMEM containing 20% fetal bovine serum, endothelial cell growth supplement and antibiotics) and seeded onto gelatin-precoated 10 cm plates.

Western blot analyses

Cells were lysed in an appropriate volume of Laemmli sample buffer (Bio-Rad) containing β-mercaptoethanol (Sigma-Aldrich) and boiled for 5 min. Protein samples were then loaded onto pre-casted Bolt 4-12% Tris-Bis gels (Invitrogen) and after electrophoresis transferred to Immobilon-FL PVDF membranes (Millipore) according to standard procedures. Membranes were blocked for 1 hour with Odyssey blocking buffer (LI-COR Biosciences) diluted 1:5 with PBS. After blocking, membranes were probed overnight at 4°C with primary antibodies diluted in Odyssey blocking buffer, followed by 1 hour incubation with IRDye-labeled secondary antibodies at room temperature. Antibody detection was achieved using an Odyssey SA infrared imaging system (LI-COR Biosciences). The intensity of the protein bands was quantified using Image Studio software. The following primary antibodies were used: anti-β-actin (ab8226, Abcam) and anti-PFKFB3 (ab181861, Abcam). IRDye-labelled secondary antibodies (goat anti-mouse IgG, 926-68070, and goat anti-rabbit IgG, 926-32211) were purchased from LI-COR Biosciences.

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Statistics

All data are expressed as mean ± SEM. Statistical analyses were performed using Graph Prism software (version 8). Statistical tests are specified in the figure legends. Differences were considered significant at P<0.05.

Results

ECs of ApoE$^{-/-}$PFKFB3^{ECKO} mice are PFKFB3 deficient

To assess the efficiency of PFKFB3 deletion after tamoxifen injection, lung ECs from ApoE$^{-/-}$PFKFB3$^{fl/fl}$ and ApoE$^{-/-}$PFKFB3^{ECKO} mice were isolated and examined by western blotting. PFKFB3 protein levels were reduced by more than 80% in ApoE$^{-/-}$PFKFB3^{ECKO} mice (Supplemental Figure II A). Similar findings were observed after co-staining of thoracic aorta segments with anti-PFKFB3 and anti-von Willebrand factor antibodies (Supplemental Figure II B).

ECs of ApoE$^{-/-}$PFKFB3^{ECKO} mice show impaired sprouting in an ex vivo mouse aortic ring assay

Previous findings have shown that inhibition of PFKFB3 leads to a reduction of EC migration and proliferation in vitro.[17] In line with these results, we found that vascular endothelial growth factor-induced sprouting in aortic rings of ApoE$^{-/-}$PFKFB3^{ECKO} mice was 65% less as compared to ApoE$^{-/-}$PFKFB3$^{fl/fl}$ control mice (Supplemental Figure III). This observation indicates a direct effect of endothelial PFKFB3 on angiogenesis.

PFKFB3 deficiency in ECs does not cause metabolic changes in adult mice

To evaluate whether EC-specific PFKFB3 gene deletion affects general metabolism, we analyzed plasma samples of ApoE$^{-/-}$PFKFB3$^{fl/fl}$ and ApoE$^{-/-}$PFKFB3^{ECKO} mice after 4 weeks western-type diet. No differences were observed in liver enzymes (γ-glutamyltransferase, alanine transaminase, alkaline phosphatase) and insulin (Table 1), indicating that PFKFB3 deletion in ECs has no obvious systemic side effects.

Moreover, there were no differences in glucose and insulin tolerance tests after 12 weeks western-type diet (Supplemental Figure IV), indicating that glucose absorption and insulin receptor sensitivity is normal in both strains. Also, body weight and cholesterol levels were not different between both groups of mice (Table 1). ApoE$^{-/-}$PFKFB3^{ECKO} mice displayed a trend toward reduced levels of plasma triglycerides as compared to controls, though this effect was not statistically significant (P=0.3698). In addition, levels of the ketone-body β-hydroxybutyrate were not changed in ApoE$^{-/-}$PFKFB3^{ECKO} mice as compared to ApoE$^{-/-}$PFKFB3$^{fl/fl}$ mice (Table 1). These observations suggest that endothelial PFKFB3 deletion does not induce a metabolic switch from glucose to fatty acid–derived ketones and does not cause major side effects in ApoE$^{-/-}$ mice.

Table 1. Metabolic parameters of ApoE$^{-/-}$PFKFB3$^{fl/fl}$ and ApoE$^{-/-}$PFKFB3^{ECKO} mice

Metabolic parameters	ApoE$^{-/-}$PFKFB3$^{fl/fl}$	ApoE$^{-/-}$PFKFB3^{ECKO}
Liver enzymes		
- γ-glutamyltransferase (U/L) - Alanine transaminase (U/L) - Alkaline phosphatase (U/L)	5.5 ± 0.6 39 ± 11 132 ± 13	5 ± 0.4 34 ± 6 154 ± 13
Fasting blood glucose (mg/dL)	116 ± 4	100 ± 8
Non-fasting blood glucose (mg/dL)	152 ± 9	138 ± 10
Insulin (ng/ml)	0.2 ± 0.1	0.2 ± 0.1
Total cholesterol (mg/dL)	306 ± 2	317 ± 1
Body weight (g)	20 ± 0.4	19 ± 0.3
Triglycerides (mg/dL)	79 ± 23	57 ± 6
β-hydroxybutyrate (µM)	1.9 ± 0.2	2.0 ± 0.1

Data between ApoE$^{-/-}$PFKFB3^{ECKO} and ApoE$^{-/-}$PFKFB3$^{fl/fl}$ mice are not significantly different (Independent Sample t-test, n=8-11).

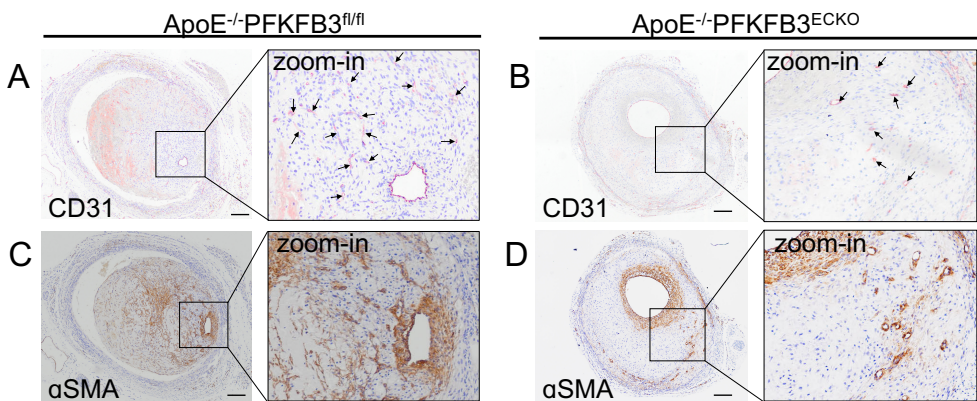## PFKFB3 deficiency in ECs inhibits neovascularization in vein graft lesions

Both ApoE$^{-/-}$PFKFB3$^{fl/fl}$ and ApoE$^{-/-}$PFKFB3^{ECKO} mice displayed an intact endothelium 28 days after vein graft surgery (black arrows Figure 1A-B; Figure 2A-B). Circular oriented VSMCs were seen in ApoE$^{-/-}$PFKFB3$^{fl/fl}$ and ApoE$^{-/-}$PFKFB3^{ECKO} vein graft lesions, close to the lumen, suggesting a cap-like organization (white arrows, Figure 1A-B). Foam cells, a small necrotic core and cholesterol crystals were found particularly in vein grafts of ApoE$^{-/-}$PFKFB3$^{fl/fl}$ mice near the luminal side (asterisks, Figure 1A-B). Furthermore, neovessels were found through the vein graft wall, predominantly in ApoE$^{-/-}$PFKFB3$^{fl/fl}$ mice. The newly formed vessels were often leaky as extravasated erythrocytes were found near and outside these microvessels (Figure 1 A-B).
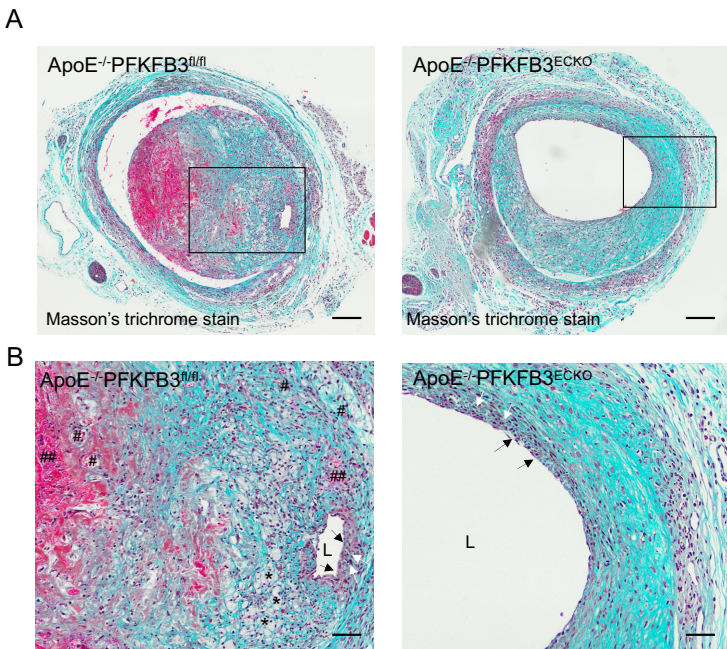
Figure 1. PFKFB3 deletion in endothelial cells evokes a more stable vein graft lesion phenotype. (A) Masson's Trichrome staining of representative vein grafts. Scale bar = 200 μm. (B) Boxed area of panel A showing an almost intact endothelium exposed to the lumen (L) (black arrows), a fibrous cap with numerous vascular smooth muscle cells (white arrows),

small foam cells (asterisks), an area with extravasate erythrocytes (##) and neovascularization (#). Scale bar = 100 µm.

As shown by a CD31 staining, the number of microvessels per lesion was reduced by 62% in ApoE^{-/-}PFKFB3[ECKO] vs ApoE^{-/-}PFKFB3^{fl/fl} mice (Figure 2A-2B, 2E). IP microvessels, were further characterized with α-SMA staining to detect the presence of a VSMC layer around the microvessels (Figure 2C-D). VSMC coverage was observed in vein grafts from both ApoE^{-/-}PFKFB3[ECKO] and ApoE^{-/-}PFKFB3^{fl/fl} mice (Figure 2F). There was not statistically difference between the two groups (p=0.05), although a trend of higher VSMC coverage in ApoE^{-/-}PFKFB3[ECKO] mice was observed. The organization of the microvessel network was further assessed by immunofluorescence confocal microscopy (Figure 3). Staining of graft lesions with CD31 antibody revealed IP microvessels that were often covered by VSMCs as demonstrated by α-SMA staining (Figure 3). These findings suggest that IP microvessels are able to reach a significant level of structural and multicellular complexity. Anti-TER-119 staining showed more erythrocyte infiltration into the lesions of ApoE-/-PFKFB3^{fl/fl} mice as compared to lesions of ApoE^{-/-}PFKFB3[ECKO] mice (Figure 4A-C), suggesting increased IP vessel leakage in ApoE-/-PFKFB3^{fl/fl} mice. A possible mechanism behind this effect may be due to tightening EC junctions following PFKFB3 deletion as recently demonstrated. [15]
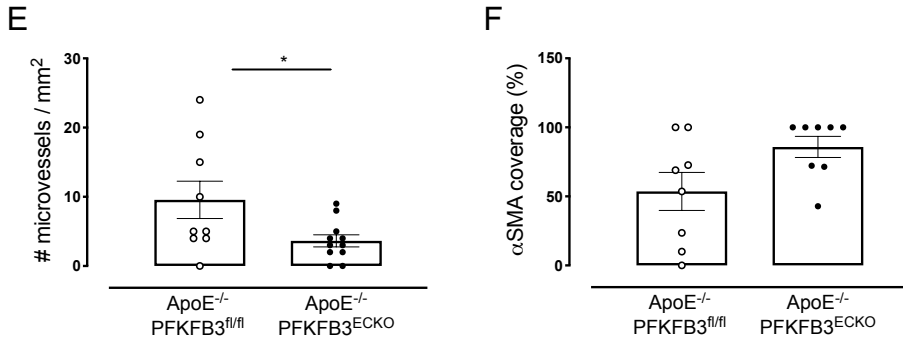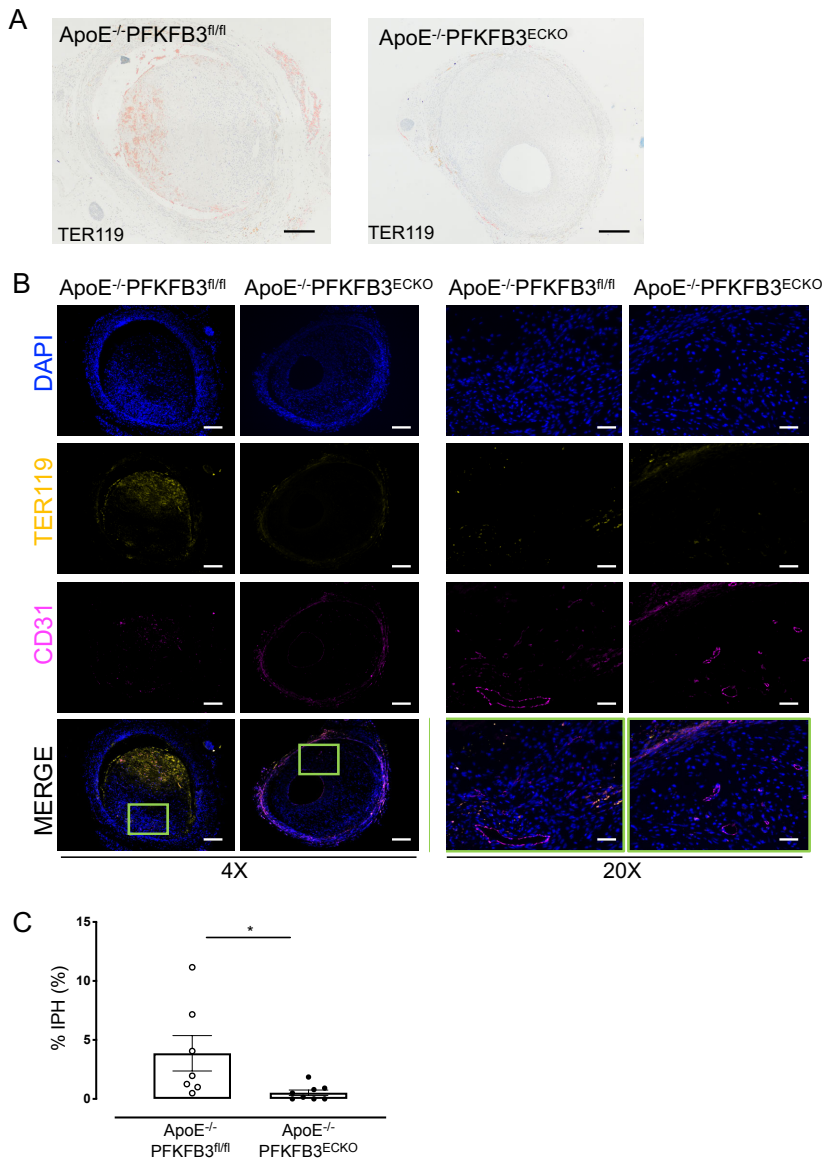
Figure 2. **PFKFB3 deficiency in endothelial cells inhibits intraplaque neovascularization in vein graft lesions but does not alter αSMA coverage of microvessels.** (A-B) Representative vein graft lesions stained with CD31 antibody. (C-D) Representative vein graft lesions stained with anti-αSMA. (E) Quantification of microvessels in vein grafts of ApoE$^{-/-}$ PFKFB3$^{fl/fl}$ and ApoE$^{-/-}$PFKFB3^{ECKO} mice. (F) Quantification of α-SMA coverage of microvessels in vein grafts of ApoE$^{-/-}$PFKFB3$^{fl/fl}$ and ApoE$^{-/-}$PFKFB3^{ECKO} mice. (Independent samples t test; n=8-11). Microvessels are marked by arrows. Scale bar= 200 µm
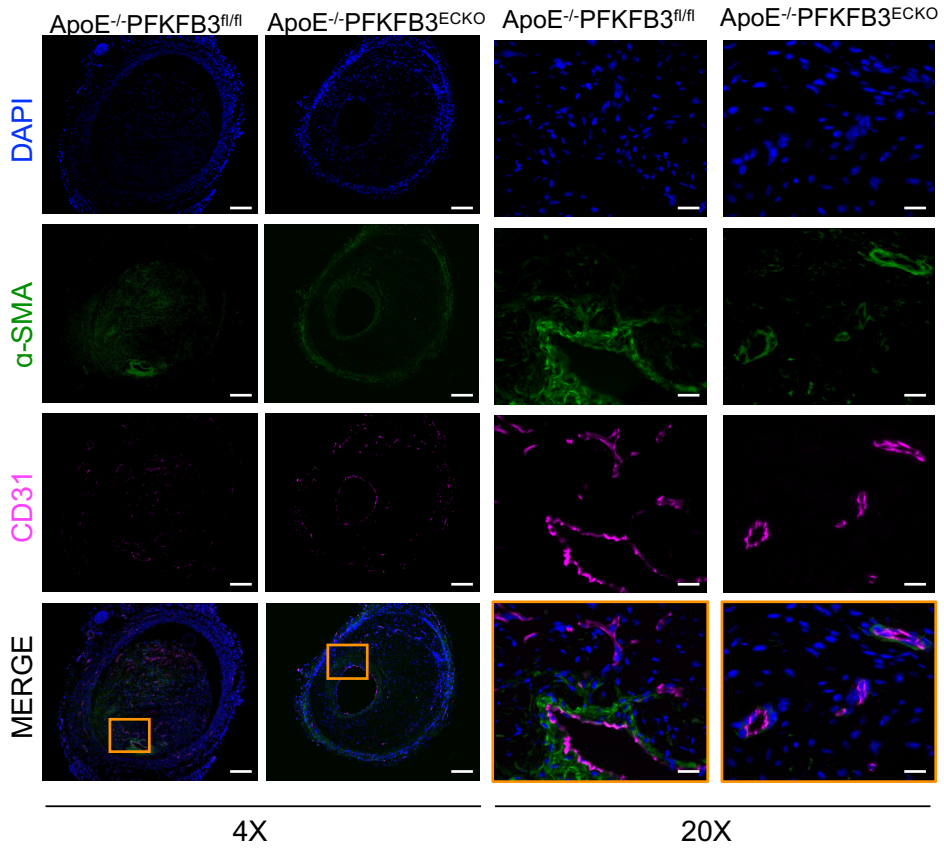
Figure 3. Examples of atherosclerotic lesions in vein grafts of ApoE$^{-/-}$PFKFB3$^{fl/fl}$ and ApoE$^{-/-}$PFKFB3^{ECKO} mice stained with anti-CD31, α-SMA and DAPI. An overlay of the three stainings is also shown as well as a magnification (20x) of the boxed areas showing mature and immature vessels. Scale bar = 500 μm (4x) or 100 μm (20x).
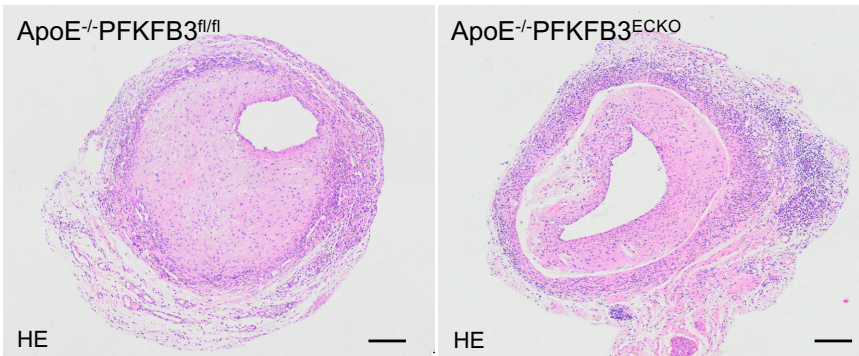
Figure 4. PFKFB3 deficiency in endothelial cells reduces intraplaque hemorrhages (IPH) in vein graft lesions. (A) Representative vein graft lesions stained with anti-TER-119. Scale bar= 200 μm. (B) Examples of atherosclerotic lesions in vein grafts of ApoE^{-/-}PFKFB3^{fl/fl} and ApoE^{-/-}PFKFB3[ECKO] mice stained with anti-CD31, anti-TER-119 and DAPI. An overlay of the three stainings is also shown as well as a magnification (20x) of the boxed areas showing erythrocyte extravasation. Scale bar = 500 μm (4x) or 100 μm (20x). (C) Quantification of IPH. *P<0.05 versus ApoE^{-/-}PFKFB3^{fl/fl} (Independent samples t-test; n=6-8).
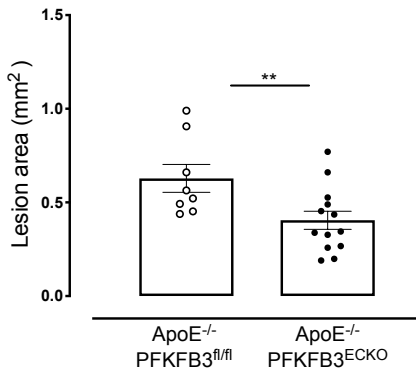
PFKFB3 deficiency in ECs reduces lesion size of vein grafts

Twenty-eight days after vein graft surgery, the lesion area in the graft was decreased by 36% in ApoE$^{-/-}$PFKFB3^{ECKO} mice as compared to ApoE$^{-/-}$PFKFB3$^{fl/fl}$ mice (Figure 5A-B), suggesting a significant role of endothelial PFKFB3 in lesion formation and/or progression. Vein graft stenosis was reduced by 23% and lesion thickness was reduced by 33% in vein grafts of ApoE$^{-/-}$PFKFB3^{ECKO} mice (Figure 5C-D).

A

B

C

D

Plaque thickness (mm)

*

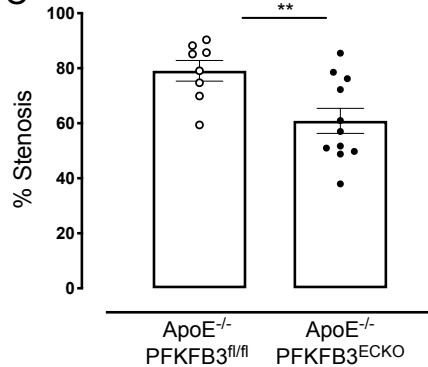ApoE$^{-/-}$
PFKFB3$^{fl/fl}$
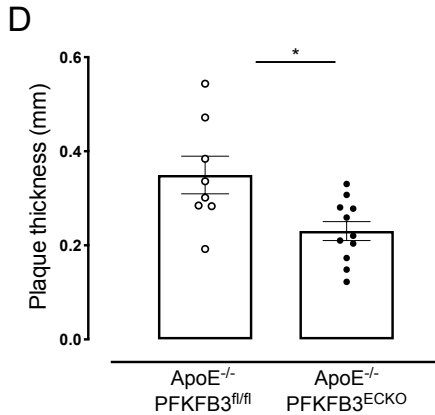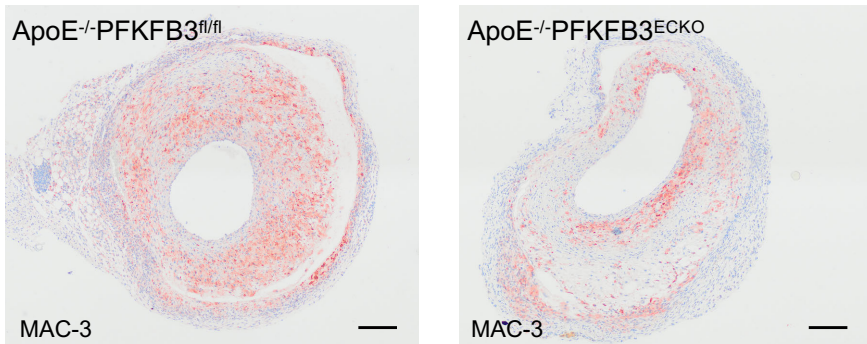
ApoE$^{-/-}$
PFKFB3^{ECKO}

Figure 5. **PFKFB3 deficiency in endothelial cells reduces the area, percentage stenosis and thickness of vein graft lesions.** Scale bar= 200 µm. (A) Representative cross sections of H&E-stained vein grafts from ApoE-/-PFKFB3fl/fl and ApoE-/-PFKFB3ECKO mice. Scale bar= 200 µm. (B-D) Quantification of lesion area, percentage stenosis and thickness of vein graft lesions. *P<0.05, **P<0.01 versus ApoE-/-PFKFB3fl/fl (Independent samples t-test; n=8-11).
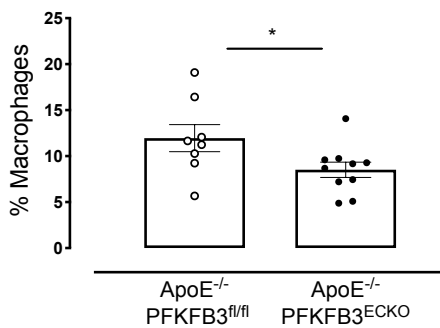
PFKFB3 deficiency in ECs decreases macrophage infiltration

Macrophage accumulation was mainly observed underneath the luminal EC or around the endothelium of microvessels in vein graft lesions of ApoE$^{-/-}$PFKFB3^{ECKO} mice. In vein graft lesions of ApoE$^{-/-}$PFKFB3$^{fl/fl}$ mice, macrophages appeared much more diffuse in the vascular wall (Figure 6A). Quantification of the macrophage infiltration showed a significant decrease in ApoE$^{-/-}$PFKFB3^{ECKO} mice (Figure 6B). Analysis of VCAM-1 expression at the luminal side of the vein graft did not reveal significant differences in ApoE$^{-/-}$PFKFB3^{ECKO} mice as compared to ApoE$^{-/-}$PFKFB3$^{fl/fl}$ mice (Figure 7 A-C).

A

ApoE$^{-/-}$PFKFB3$^{fl/fl}$

MAC-3

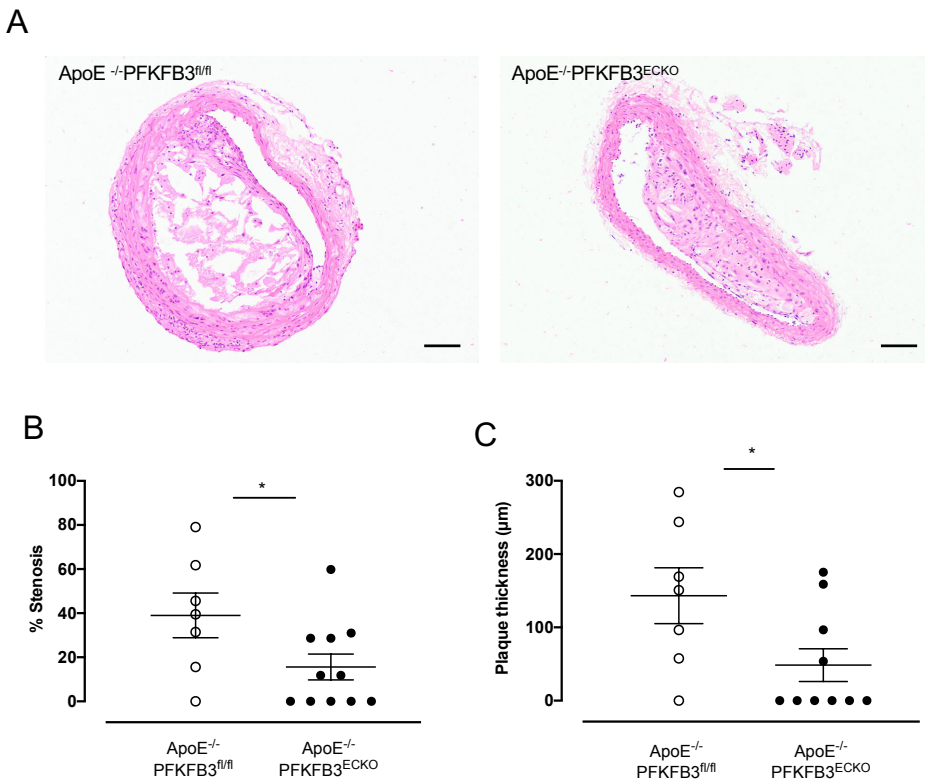ApoE$^{-/-}$PFKFB3^{ECKO}

MAC-3

B

Figure 6. PFKFB3 deficiency in endothelial cells reduces macrophage infiltration in vein graft lesions. (A) Immunohistochemical detection of macrophages in representative vein graft lesions of ApoE$^{-/-}$PFKFB3$^{fl/fl}$ and ApoE$^{-/-}$PFKFB3^{ECKO} mice using MAC3 antibody. Scale bar = 200 μm. (B) Quantification of macrophages in vein graft lesions of ApoE$^{-/-}$PFKFB3$^{fl/fl}$ and ApoE$^{-/-}$PFKFB3^{ECKO} mice. *P<0.05 versus ApoE$^{-/-}$PFKFB3$^{fl/fl}$ mice (Independent samples t-test; n=8-10).

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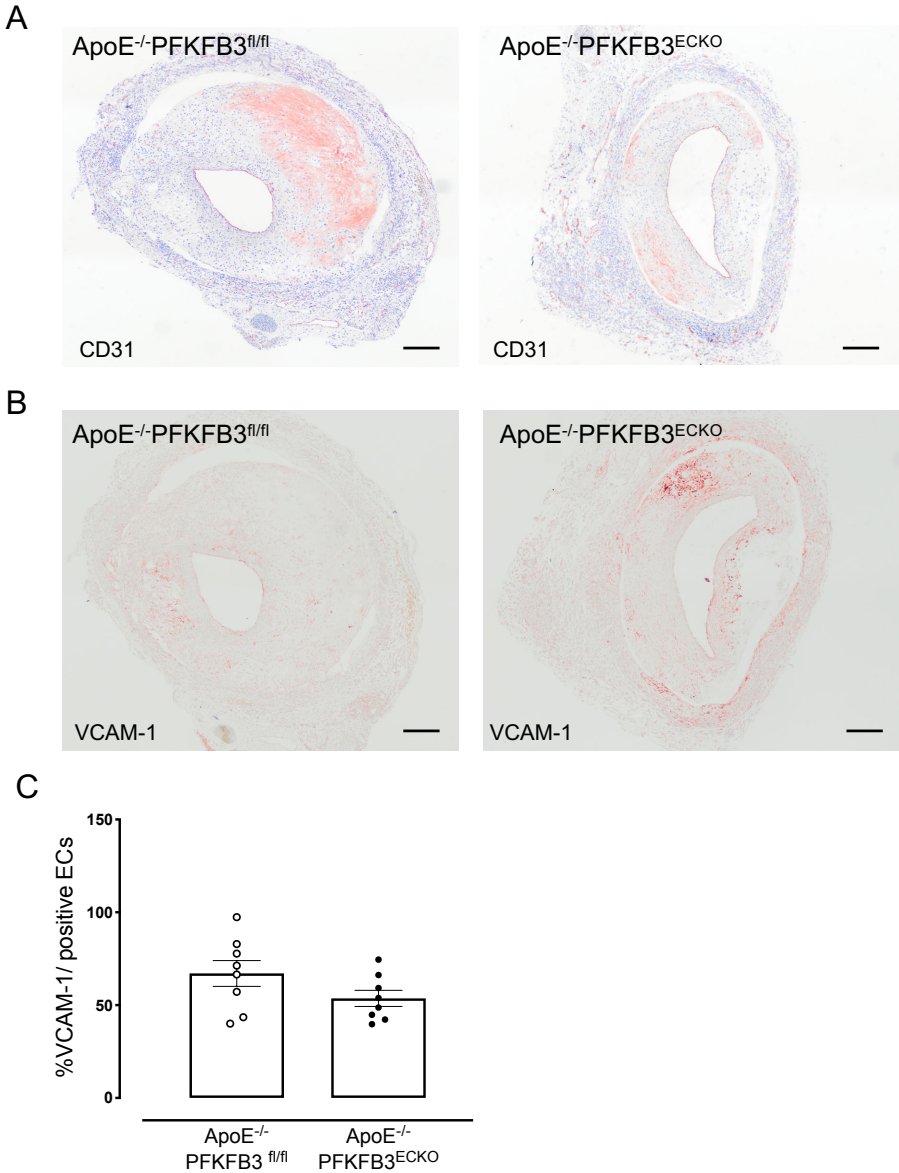
Figure 7. PFKFB3 deficiency in endothelial cells does not influence vascular cell adhesion molecule-1 (VCAM-1) expression in vein graft lesions. (A-B) Immunohistochemical detection of endothelial cells in representative vein graft lesions of ApoE$^{-/-}$PFKFB3$^{fl/fl}$ and ApoE$^{-/-}$PFKFB3^{ECKO} mice using CD31 and VCAM-1 antibody. Scale bar = 200 μm. (C) Quantification of VCAM-1 positive endothelial cells in vein graft lesions of ApoE$^{-/-}$PFKFB3$^{fl/fl}$ and ApoE$^{-/-}$PFKFB3^{ECKO} mice (Independent samples t test, n=8).

PFKFB3 deficiency in ECs has a positive impact on plaque formation in native atherosclerosis

An analysis of total cholesterol did not reveal significant differences between ApoE$^{-/-}$PFKFB3$^{fl/fl}$ and ApoE$^{-/-}$PFKFB3^{ECKO} mice after 12 weeks WD (ApoE$^{-/-}$PFKFB3$^{fl/fl}$ = 574±26 mmol/l vs ApoE$^{-/-}$PFKFB3^{ECKO} = 520±51 mmol/l; P=0.36). Nonetheless, plaque thickness (Figure 6A-B) and percentage stenosis was reduced in ApoE$^{-/-}$PFKFB3^{ECKO} mice as compared to ApoE$^{-/-}$PFKFB3$^{fl/fl}$ mice (Figure 8).

Figure 8. PFKFB3 deficiency in endothelial cells decreases plaque thickness and percentage of stenosis in ApoE$^{-/-}$ mice. Scale bar= 100 µm. (A) Representative plaque lesions of ApoE$^{-/-}$PFKFB3$^{fl/fl}$ and ApoE$^{-/-}$PFKFB3^{ECKO} mice fed a western diet for 12 weeks. (B) Quantification of stenosis and plaque thickness. *P<0.05 versus ApoE$^{-/-}$PFKFB3$^{fl/fl}$ (Independent samples t-test; n=7-11).

Discussion

IP angiogenesis is frequently observed inside human vein graft lesions and is recognized as a contributing factor of plaque vulnerability.[3, 6, 9, 10, 21]

In the present study, we crossed EC-specific PFKFB3 knockout mice with ApoE^{-/-} mice to investigate the role of EC glycolysis modulation in vein graft IP angiogenesis. To our knowledge, this is the first study using a conditional EC-specific PFKFB3 knockout mouse in the context of advanced atherosclerosis.

First of all, we did not observe adverse effects or changes in general metabolism after PFKFB3 deletion in ECs. Circulating liver enzymes, blood glucose, insulin and total cholesterol were not affected. Also glucose and insulin tolerance tests were similar in ApoE^{-/-}PFKFB3^{fl/fl} versus ApoE^{-/-}PFKFB3[ECKO] mice. Ketone-body β-hydroxybutyrate was not changed in both groups. These findings suggest that PFKFB3 deletion in ECs does not lead to severe side effects or to a major metabolic switch in ApoE^{-/-} mice.

Next, and in line with previous studies, we found that PFKFB3 deletion impaired vessel sprouting from aortic rings. Along these lines ApoE^{-/-}PFKFB3[ECKO] mice showed a significantly reduced number of microvessels in vein graft lesions.

Moreover, IP microvessels in ApoE^{-/-}PFKFB3[ECKO] showed less leakage of erythrocytes inside the graft lesion. These findings are consistent with recent in vitro and in vivo observations showing that PFKFB3 inhibition reduces VE-cadherin endocytosis and promotes normalization of the endothelial barrier.[15]

We also observed a reduction in plaque size in vein grafts of ApoE^{-/-}PFKFB3[ECKO] mice, which suggests that PFKFB3 may play a direct role in plaque progression. Interestingly, EC-specific deletion of PFKFB3 also promoted an atheroprotective effect in ApoE^{-/-} mice, which normally develop plaques without IP neovessels. These results suggest that PFKFB3 in ECs may affect early onset of atherosclerosis. Although this study was not designed to investigate the impact of EC-specific PFKFB3 deletion on atherogenesis, our findings are in agreement with the general concept that glycolysis inhibition prevents endothelial activation, recruitment of monocytes and vascular inflammation. Such compelling possibility

corresponds with data from in vitro studies showing that PFKFB3 is linked to pro-inflammatory signalling of ECs in response to blood flow shear stress. Indeed, turbulent blood flow in atheroprone regions leads to inhibition of Krüppel-like Factor 2 activity, which correlates with PFKFB3 upregulation, increased EC glycolysis and inflammatory activation.[22] The importance of PFKFB3 in plaque progression has also been suggested by a recent study showing a positive correlation between PFKFB3 expression and an unstable plaque phenotype in both carotid and coronary plaques in humans. [23] Furthermore, administration of the PFKFB3 inhibitor PFK158 in mice led to a reduction in advanced plaques with a vulnerable phenotype and an increase in plaque stability.[23]

Most interestingly, we detected a reduction in the percentage of macrophage infiltration in vein graft lesions of ApoE$^{-/-}$PFKFB3^{ECKO} mice. This finding is in agreement with the presence of crosstalk between EC metabolism and macrophages in pathological conditions as previously reported.[24, 25] For example, in tumor settings a metabolic competition for glucose between EC and macrophages reduces EC hyperactivation and prevent abnormal vessel leakage. [26] PFKFB3 inhibition also abolishes the inflammatory response caused by lipoprotein(a) with concomitant attenuation of transendothelial monocyte migration in atherosclerotic plaques.[27] It is therefore possible that the observed reduction in macrophage infiltration in vivo is in part due to an improved restoration of EC junctions after PFKFB3 deletion.

The reduction of IP angiogenesis in ApoE$^{-/-}$PFKFB3^{ECKO} mice, as described in this study, is in line with previous findings in our group showing decreased IP angiogenesis following administration of the glycolysis inhibitor 3PO in a mouse model of advanced atherosclerosis.[18] Moreover, 3PO significantly reduced initiation of plaque formation in a preventive study design.

Altogether, our findings indicate that endothelial PFKFB3 plays a critical role in IP angiogenesis and lesion progression, and that PFKFB3 inhibition is a promising approach to prevent plaque development and to reduce the complications of vein bypass grafting.
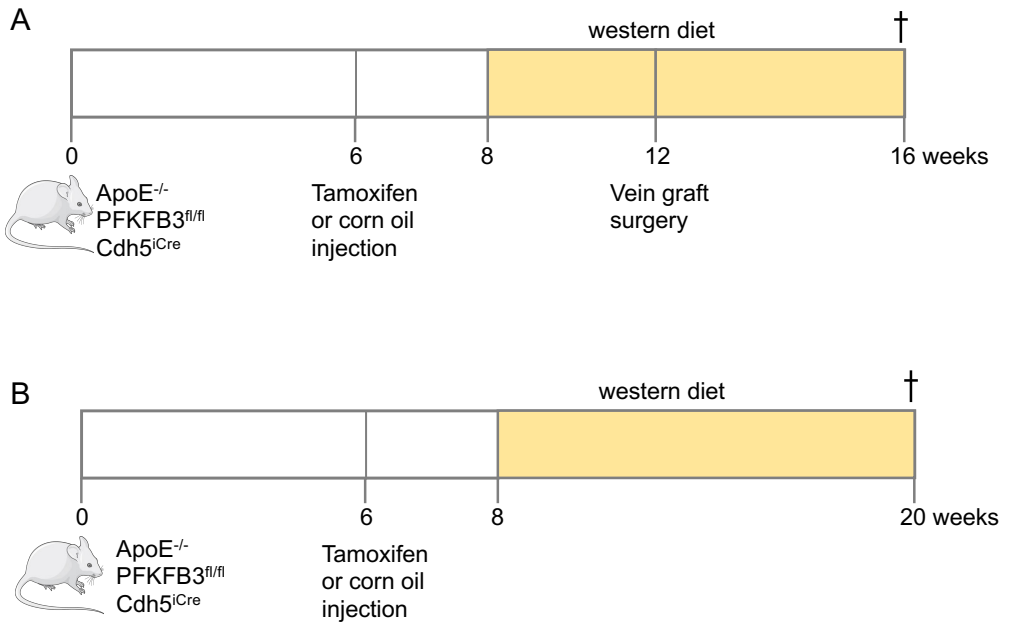
Acknowledgments

Data availability

All primary data that support the findings of this study are available from the corresponding author upon reasonable request.

Supplemental material

A

B

Figure I. Schematic overview of the experimental design. ApoE$^{-/-}$PFKFB3$^{fl/fl}$Cdh5^{iCre} mice (6 weeks old) were injected with tamoxifen (0.1 g/kg body weight) for 5 consecutive days to induce PFKFB3 deletion in ECs. Control mice were injected with corn-oil using the same protocol. After 2 weeks, mice were fed a western-type diet. Four weeks later, vein graft surgeries were performed. Mice were sacrificed 4 weeks after surgery. (B) atherosclerotic plaque study: ApoE$^{-/-}$PFKFB3$^{fl/fl}$Cdh5^{iCre} (6 weeks old) were injected with tamoxifen (0.1 g/kg body weight) for 5 consecutive days to induce PFKFB3 deletion in ECs . Control mice were injected with corn-oil using the same protocol. After 2 weeks, mice were fed a western-type diet. Twelve weeks later, mice were sacrificed.

A
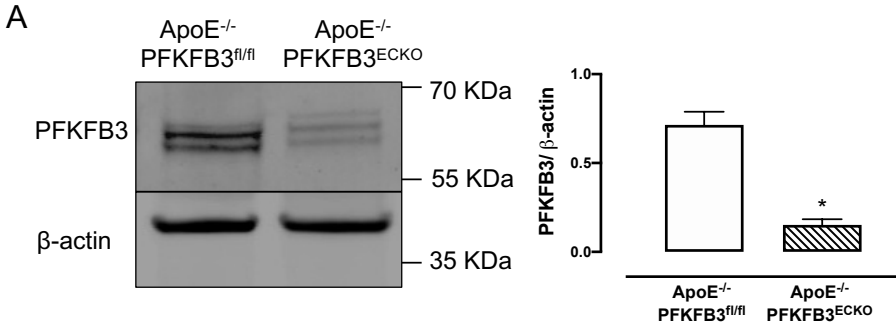
B

Thoracic aorta segments
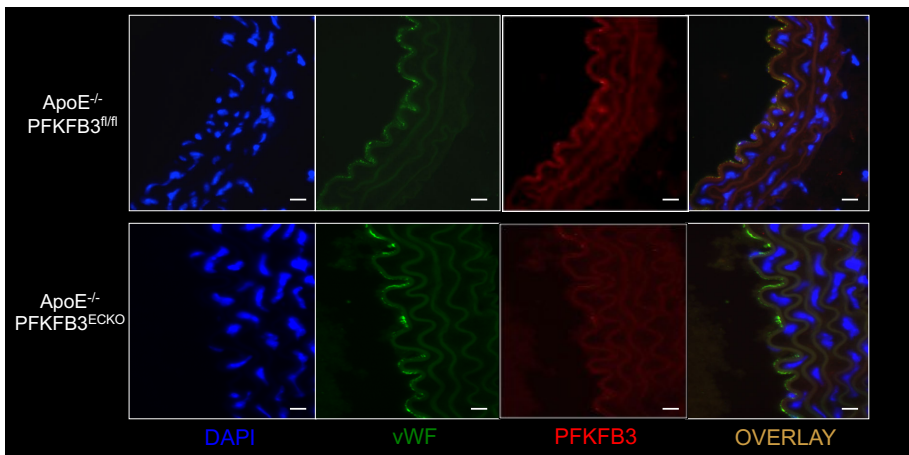
Figure II. Validation of PFKFB3 gene deletion in endothelial cells. (A) Representative western blot showing protein levels of PFKFB3 and β-actin. Bars represent relative protein quantification of PFKFB3 normalized to the reference protein β-actin. *P<0.05 versus ApoE$^{-/-}$PFKFB3$^{fl/fl}$ (Independent samples t-test; n=3). (B) Representative thoracic aorta segments of ApoE$^{-/-}$PFKFB3$^{fl/fl}$ and ApoE$^{-/-}$PFKFB3^{ECKO} stained with antibodies against PFKFB3 or von Willebrand Factor (endothelial cell marker). Nuclei were stained with DAPI.

A

control   + VEGF

ApoE$^{-/-}$PFKFB3$^{fl/fl}$ ApoE$^{-/-}$PFKFB3^{ECKO}

ApoE$^{-/-}$PFKFB3$^{fl/fl}$ ApoE$^{-/-}$PFKFB3^{ECKO}
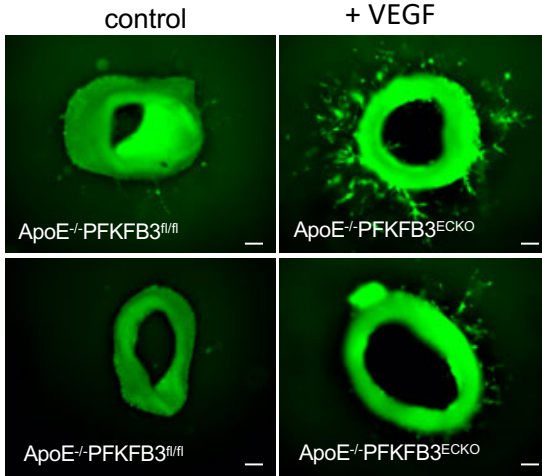
B
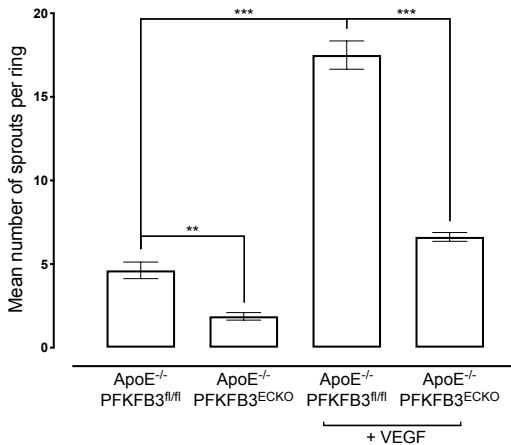
Figure III. PFKFB3 deficiency in endothelial cells inhibits aortic sprouting. (A) Aortic rings from ApoE$^{-/-}$PFKFB3$^{fl/fl}$ and ApoE$^{-/-}$PFKFB3^{ECKO} were embedded in collagen type I and treated with Opti-MEM supplemented with 2.5% FBS (control) in the presence or absence of VEGF (50 ng/mL). At day 6, rings were fixed and stained to delineate ECs. Images of ring sprouting were obtained and quantified. (B) Sprouts were quantified, n=4 mice. ***P<0.0001 One way ANOVA, effect of PFKFB3^{ECKO}: **P<0.005, effect of VEGF: ***P<0.0001, interaction: ***P<0.0001.

A

GTT

ITT
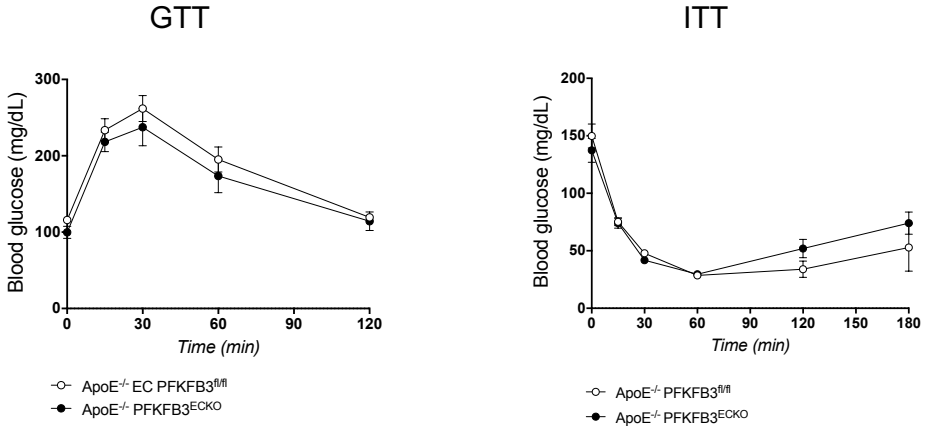
Figure IV. Glucose clearance in ApoE$^{-/-}$PFKFB3$^{fl/fl}$ and ApoE$^{-/-}$PFKFB3^{ECKO} mice after intraperitoneal injection with glucose or insulin. A glucose tolerance test (GTT) and insulin tolerance test (ITT) was performed after 12 weeks of western diet. Blood glucose was measured at different time points after injection of glucose (1 g/kg, i.p.) or insulin (1 U/kg, i.p.).

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