Activated protein C reverses epigenetically sustained p66\textsuperscript{Shc} expression in plaque-associated macrophages in diabetes

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Impaired activated protein C (aPC) generation is associated with atherosclerosis and diabetes mellitus. Diabetes-associated atherosclerosis is characterized by the hyperglycaemic memory, e.g., failure of disease improvement despite attenuation of hyperglycaemia. Therapies reversing the hyperglycaemic memory are lacking. Here we demonstrate that hyperglycaemia, but not hyperlipidaemia, induces the redox-regulator p66\textsuperscript{Shc} and reactive oxygen species (ROS) in macrophages. p66\textsuperscript{Shc} expression, ROS generation, and a proatherogenic phenotype are sustained despite restoring normoglycemic conditions. Inhibition of p66\textsuperscript{Shc} abolishes this sustained pro-atherogenic phenotype, identifying p66\textsuperscript{Shc}-dependent ROS in macrophages as a key mechanism conveying the hyperglycaemic memory. The p66\textsuperscript{Shc}-associated hyperglycaemic memory can be reversed by aPC via protease-activated receptor-1 signalling. aPC reverses glucose-induced CpG hypomethylation within the p66\textsuperscript{Shc} promoter by induction of the DNA methyltransferase-1 (DNMT1). Thus, epigenetically sustained p66\textsuperscript{Shc} expression in plaque macrophages drives the hyperglycaemic memory, which—however—can be reversed by aPC. This establishes that reversal of the hyperglycaemic memory in diabetic atherosclerosis is feasible.
oss of thrombomodulin expression and impaired activated protein C (aPC) generation have been associated with atherosclerosis and diabetes mellitus1–6. Plaque morphology differs among diabetic and non-diabetic atherosclerotic disease, indicating at least partially disjoint pathophysiology7–9. Indeed, despite intensive lipid lowering the probability of atherosclerosis and myocardial infarction remains increased in diabetic patients, suggesting that diabetes-specific mechanisms contribute to atherosclerosis independent of elevated blood lipids in hyperglycaemic memory10–12. In striking contrast to the improvement of atherosclerotic disease following lipid lowering in humans and mice11,12, diabetes-associated atherosclerosis is perpetuated despite marked improvement of blood glucose control13. The sustained disease process despite improved blood glucose is referred to as the hyperglycaemic memory14,15. The hyperglycaemic memory is linked with glucose-induced post-translational modifications and epigenetically sustained gene expression6,16,17. Approaches to therapeutically reverse the hyperglycaemic memory are lacking.

The protein p66Shc, which can translocate to the outer mitochondrial space and interact with the electron-transport chain, resulting in increased mitochondrial ROS generation18, has been linked with glucose-induced excess ROS generation and diabetes-associated vascular complications16,19. Glucose-induced p66Shc expression has been observed in podocytes (renal epithelial cells), endothelial cells, and smooth muscle cells16,19,20. Expression of p66Shc is epigenetically controlled, suggesting that sustained p66Shc expression may provide a mechanistic link between the hyperglycaemic memory, sustained vascular ROS generation and inflammation, and progressive atherosclerotic disease despite improved blood glucose control16,19. A recent study demonstrated sustained p66Shc expression in peripheral blood monocytes of diabetic patients despite improved blood glucose control21. However, whether sustained p66Shc expression likewise occurs in atherosclerotic plaque-associated macrophages remains unknown. Likewise, it remains unknown whether p66Shc expression in macrophages is causatively linked with diabetes-associated atherosclerosis and whether targeting p66Shc expression may provide a therapeutic benefit. We previously reported that aPC normalizes glucose-induced p66Shc expression in podocytes16, suggesting that impaired aPC generation in the context of diabetes mellitus may contribute to sustained p66Shc expression and thus to the hyperglycaemic memory in diabetic patients. Here we demonstrate that aPC reverses the glucose-induced, epigenetically sustained p66Shc expression in atherosclerotic plaque-associated macrophages, thus promoting the reversal of hyperglycaemia-induced atherosclerotic plaques.

Results

Hyperglycaemia promotes plaque instability in ApoE−/− mice. To gain insights into specific pathomechanisms of hyperglycaemia versus hyperlipidaemia-induced atherosclerotic plaque development we directly compared hyperglycaemic (induced by low-dose streptozotocin, STZ injection for 5 days, a model of type 1 DM) and hyperlipidaemic (induced by a high-fat diet, HFD) ApoE−/− mice with control ApoE−/− mice. These were followed up for 22 weeks. As expected, body weight, blood lipids, or blood glucose levels differed among treatment groups (Supplementary Fig. 1).

Analyses of Oil Red O-stained aortae en face revealed an increase of lipid deposits in ApoE−/− HFD mice compared to ApoE−/− DM mice (Fig. 1a). Likewise, plaques within the brachiocephalic artery (Fig. 1b, c) and the aortic roots (Fig. 1b, d) were larger in ApoE−/− HFD than in ApoE−/− DM mice. To evaluate whether the reduced plaque size in hyperglycaemic mice simply reflects the lower total plasma cholesterol levels, we compared a subgroup of ApoE−/− DM and ApoE−/− HFD mice matched for total plasma cholesterol levels (922 ± 52 mg dl−1 versus 912 ± 46 mg dl−1 for HFD and DM mice, respectively). Despite matching a subgroup of ApoE−/− HFD and ApoE−/− DM mice for total plasma cholesterol levels lesion size was still smaller in hyperglycaemic ApoE−/− DM mice compared to ApoE−/− HFD mice (Supplementary Fig. 2). The smaller plaque size in ApoE−/− DM mice suggests that the aggravated disease course of hyperglycaemia-related atherosclerosis is not primarily related to an increased plaque size. Hence, we next evaluated cellular composition and other parameters reflecting plaque stability.

Indeed, signs of plaque instability were more frequent in ApoE−/− DM mice than in ApoE−/− HFD mice. Thus, plaques in ApoE−/− DM mice had an increased necrotic core area (Fig. 1e), thinner fibrous caps (Fig. 1f), and an increased frequency of ruptured plaque shoulders (Fig. 1g). Plaque morphology and stability depend in part on cellular composition. In plaques of ApoE−/− DM mice macrophages area (immunohistochemically positive for MOMA-2) were increased, while smooth muscle cells (SMC α-actin-positive area) were reduced in comparison to plaques of ApoE−/− HFD mice (Fig. 1h). The observed shift to more macrophages and less SMC corroborates reduced plaque stability in ApoE−/− DM mice.

Hyperglycaemia induces p66Shc and CD36 in macrophages. Plaque-associated macrophages impair plaque stability in part by generating reactive oxygen species (ROS). We therefore analysed expression of the redox-regulator p66Shc in plaque-associated macrophages of ApoE−/− DM and ApoE−/− HFD mice. Expression of p66Shc was markedly enhanced in CD68-positive laser-dissected plaque macrophages of ApoE−/− DM mice (Fig. 2a and Supplementary Fig. 3). Immunohistochemical analyses confirmed increased p66Shc expression and co-localization of p66Shc with MOMA-2, another macrophage marker, in plaques of ApoE−/− DM mice (Fig. 2b).

As ROS induces expression of the scavenger receptor CD36, we speculated that enhanced p66Shc expression increases CD36 expression in hyperglycaemic mice. First, we ascertained whether p66Shc converses glucose-induced CD36 expression. ApoE−/− or p66Shc−/−/−<sub>DM</sub> mouse bone marrow-derived macrophages (BMDMs) were cultured under normoglycaemic (5 mM glucose plus 20 mM mannitol, NG) or hyperglycaemic (25 mM glucose, HG) conditions for 48 h. HG conditions-induced CD36 expression in ApoE−/−, but not in ApoE−/−/p66Shc−/−/−<sub>DM</sub> mice (Fig. 2c), demonstrating that p66Shc mediates the glucose-dependent induction of CD36 in macrophages. Consistently, CD36 expression was markedly increased in laser-dissected macrophages obtained from plaques of ApoE−/− DM mice (Fig. 2a). Immunohistochemical analyses verified an increased expression and co-localization of CD36 in plaque-associated macrophages of ApoE−/− DM versus ApoE−/− HFD mice (Fig. 2d).

To investigate the potential translational relevance, we analysed p66Shc and CD36 expression in atherosclerotic plaques of non-diabetic and diabetic patients. As in mice, both p66Shc and CD36 expressions were more abundant in atherosclerotic plaques of diabetic patients compared to those of non-diabetic patients (Fig. 3). Again, p66Shc and CD36 expression was predominately observed in macrophages (co-localization of p66Shc and MOMA-2, Fig. 3). Thus, in diabetic mice and humans, macrophages within atherosclerotic plaques express more p66Shc and CD36 compared to non-diabetic mice or humans.

p66Shc is crucial for hyperglycaemia-induced atherosclerosis. To evaluate the pathogenic relevance of p66Shc in myeloid-derived
cells for hyperglycaemia-associated atherosclerosis we conducted bone marrow transplantation experiments. Bone marrow isolated from ApoE−/− or p66Shc−/−ApoE−/− mice was transplanted into lethally irradiated ApoE−/− mice (age 8 weeks, Fig. 4a). Control mice were left untreated (control), while in experimental mice hyperglycaemia or hyperlipidaemia were induced at age 10 weeks. As expected, body weight, total plasma cholesterol, or blood glucose levels differed among groups, but transplantation of ApoE−/− or p66Shc−/−ApoE−/− derived bone marrow had no impact on these parameters (Supplementary Fig. 4).

Following transplantation of p66Shc−/−ApoE−/− derived bone marrow plaque size was reduced, (Fig. 4b). However, the reduction in plaque size was less prominent and only of borderline significance ($P = 0.052$) in the HFD group, while a pronounced reduction of plaque size was apparent in the DM group (Fig. 4b). Indeed, plaque size in p66Shc−/−ApoE−/−
transplanted ApoE−/− DM mice was comparable to that in the controls (Fig. 4b). The markedly reduced plaque size in p66Shc−/−Apoe−/− DM mice was associated with a decreased necrotic core area, increased fibrous cap thickness, decreased frequency of rupture shoulder, and less plaque-associated macrophages. (Fig. 4c, d), reflecting increased plaque stability. Thus, hyperglycaemia-associated impaired plaque stability primarily depends on p66Shc expression in myeloid-derived cells.

To determine whether p66Shc may be a therapeutic target in diabetes-associated atherosclerosis we treated mice with p66Shc morpholinos (VM) to repress p66Shc expression (p66Shc-MO). After 16 weeks of persistent hyperglycaemia ApoE−/− DM mice were injected with p66Shc-MO (DM-p66Shc-MO) or control-MO (DM-Cont-MO; Fig. 5a). Efficient suppression of p66Shc in the aorta was confirmed by semi-quantitative reverse-transcriptase PCR (Supplementary Fig. 5a) and immunofluorescence staining (Supplementary Fig. 5b). Treatment with VM had no impact on body weight, blood glucose, or blood lipid levels (Supplementary Fig. 6). Following suppression of p66Shc plaques within aortic roots (Fig. 5b) and brachiocephalic arteries (Supplementary Fig. 7) were smaller in p66Shc-MO as compared to Cont-MO-treated ApoE−/− DM mice. This was associated with increased signs of plaque stability as reflected by decreased necrotic core area, increased fibrous cap thickness, decreased frequency of rupture shoulder and less plaque-associated macrophages in p66Shc-MO as compared to Cont-MO-treated ApoE−/− DM mice (Fig. 5c, d). These results demonstrate that p66Shc expression in
Sustained p66Shc and CD36 expression in macrophages. To evaluate whether glucose induces persistent p66Shc expression in macrophages we exposed mouse BMDMs to high LDL (50 µg/ml, HL) or high glucose (25 mM, HG) for 48 h in vitro. High LDL (but not low LDL, Fig. 6a) induced p66Shc and CD36 expression. Similarly, high glucose-induced (but not mannitol, Fig. 6b) p66Shc and CD36 expression. We then ascertained whether normalization of LDL for additional 24 h (HL-NL) p66Shc and CD36 expression returned to baseline (Fig. 6a). In contrast, despite normalization of glucose concentration for the last 24 h (HG-NG) p66Shc and CD36 expression remained elevated in BMDMs (Fig. 6b). Thus, high glucose causes a sustained induction of p66Shc and CD36 expression in macrophages in vitro.

aPC reverses sustained p66Shc expression via PAR1. We next determined whether aPC reverses p66Shc expression in glucose-stressed macrophages in vitro. BMDMs were left untreated or exposed for 48 h to high glucose concentrations, followed by normal glucose concentrations for 24 h (HG-NG). Concomitant aPC treatment (20 nM, HG-NG-aPC) during the 24 h period of normalized glucose concentrations markedly reduced p66Shc expression as compared to PBS-treated controls (HG-NG, Fig. 6b). Parallel changes were observed for CD36 expression, indicating that glucose-induced persistent CD36 expression depends on p66Shc, but can be normalized by aPC (Fig. 6b).

To evaluate the in vivo relevance of these findings we analysed freshly isolated BMDMs, which were in vivo conditioned using different treatment schemes: (A) control mice (Cont., no hyperglycaemia), (B) diabetic mice (DM, 22 weeks of persistent hyperglycaemia), and (C) diabetic mice, in which blood glucose levels were normalized for 6 weeks after 16 weeks of persistent hyperglycaemia using the SGLT2-inhibitor dapagliflozin (DM-NG). The DM-NG group was randomly assigned to receive either PBS (DM-NG-PBS) or aPC (DM-NG-aPC) parallel to the treatment with the SGLT2-inhibitor (Supplementary Fig. 8a). aPC treatment had no impact on blood glucose levels (Supplementary Fig. 8b). Expression of p66Shc was high in BMDMs derived from DM and DM-NG-PBS mice, but markedly reduced in DM-NG-aPC mice (Fig. 6c), demonstrating that aPC normalizes glucose-induced sustained p66Shc expression not only in vitro, but also in vivo.

To identify the receptors through which aPC reduces p66Shc expression in glucose stressed BMDMs we first determined the expression of protease-activated receptors (PARs) and endothelial protein C receptor (EPCR) in BMDMs. Expression of PAR1, PAR2, PAR3, PAR4, and EPCR was readily detectable in BMDMs (Supplementary Fig. 9). To determine the functional relevance of these receptors we isolated BMDMs from PAR1−/−, PAR2−/−, or PAR3−/− mice, while PAR4 or EPCR function was blocked in...
wild-type BMDMs using inhibitory antibodies. PAR1 deficiency efficiently abolished p66Shc inhibition by aPC, whereas PAR2, PAR3 deficiency or PAR4 and EPCR inhibition had no effect (Fig. 6d). Hence, PAR1 is required for the aPC-dependent inhibition of glucose-induced sustained p66Shc expression in BMDMs.

aPC reverses the sustained atherogenic macrophage phenotype. To determine whether aPC modulates p66Shc dependent functional consequences in macrophages we analysed ROS and inflammatory markers. In HG BMDMs 8-Oxo-dG (8-Oxo-2'-deoxyguanosine) and nitrotyrosine, both reflecting ROS generation, were induced and remained high despite normalization of glucose levels (HG-NG, Fig. 7a, b). The sustained expression of these ROS-markers was reversed by concomitant aPC treatment (HG-NG-aPC, Fig. 7a, b). In parallel, IL-6, TNF-α, and NF-κB p65 expression were induced in BMDMs and remained high despite normalization of glucose levels, but were reversed by concomitant aPC treatment (HG-NG-aPC, Fig. 7c, d).

Fig. 6. a. Experimental design of bone marrow transplantation experiments. b. Transplantation of p66Shc−/− deficient bone marrow markedly reduces hyperglycaemia-, but only slightly hyperlipidaemia-induced atherosclerosis. Representative images showing Oil Red O staining of aortic roots (left panel) and dot plot summarizing plaques size (right panel). c. Dot plots summarizing results of necrotic core area (left panel), fibrous cap thickness (middle panel), and frequency of ruptured shoulders (right panel). d. Representative images showing immunofluorescence staining of macrophages (MOMA-2, green; DAPI nuclear counterstain, blue) within lesions (left panel) and dot plot summarizing data (right panel). Cont: normoglycaemic ApoE−/− mice with normal chow diet; DM: hyperglycaemic ApoE−/− mice; HFD: ApoE−/− mice with high-fat diet (HFD). Data shown as dot plots represent mean ± SEM of 6–10 mice per group (b–d); size bars: b, d, 20 μm; **P < 0.01; b–d: two-way ANOVA with Bonferroni-adjusted post hoc comparison of ApoE−/− (Cont, HFD, DM) versus p66Shc−/− ApoE−/− (Cont, HFD, DM respectively) recipient mice.
and Supplementary Fig. 10). In agreement with the observed CD36 expression pattern lipid uptake by BMDMs was increased by HG and remained high despite normalization of glucose levels (HG-NG, Fig. 7e), but was normalized by aPC (HG-NG-aPC, Fig. 7e). Thus, glucose induces sustained pro-inflammatory and pro-atherogenic effects in macrophages in vitro, which can be efficiently reversed by aPC.

**aPC epigenetically inhibits sustained p66Shc expression.** We next analysed p66Shc promoter methylation in macrophages. Compared to control BMDMs (normal glucose, NG) CpG dinucleotides within the p66Shc promoter were hypomethylated in BMDMs maintained under hyperglycaemic conditions (HG, Fig. 8a). The p66Shc promoter remained hypomethylated despite normalization of glucose levels for 24 h (HG-NG, Fig. 8a). However, concomitant aPC treatment restored p66Shc promoter methylation (Fig. 5a). Corresponding changes were observed for p66Shc mRNA levels (Fig. 8b).

To gain further mechanistic insights we determined DNMT expression parallel to p66Shc expression in BMDMs. The expression pattern of DNMT1 was opposite to that of p66Shc (Fig. 8c), while

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**Fig. 5** In vivo silencing of p66Shc by vivo morpholino reduces hyperglycaemia-induced atherosclerosis. **a** Experimental design of vivo morpholino p66Shc silencing experiments. **b** Representative images of Oil Red-O staining of the aortic root lesions (left panel) and dot plot summarizing plaque size (right panel). **c** Dot plots summarizing result of necrotic core area (left panel), fibrous cap thickness (middle panel), and frequency of ruptured shoulders (right panel). **d** Representative images showing immunofluorescence staining of macrophages (MOMA-2, green; DAPI nuclear counterstain, blue) within lesions (left panel) and dot plot summarizing data (right panel). Cont: normoglycaemic control mice; DM: hyperglycaemic ApoE−/− mice treated with PBS; Cont-MO: hyperglycaemic ApoE−/− mice treated with control vivo morpholino; p66Shc-MO: hyperglycaemic ApoE−/− mice treated with p66Shc-specific vivo morpholino. Data shown as dot plots represent mean ± SEM of 6–10 mice per group (b–d); size bars: **b, d** 20 μm; **P < 0.01; one-way ANOVA with Bonferroni-adjusted post hoc comparison of DM and DM-Cont-MO versus Cont and DM-p66Shc-MO versus DM-Cont-MO (b, d) or DM-p66Shc-MO versus DM-Cont-MO and DM (c).
DNMT3a and DNMT3b expression remained unchanged (Supplementary Fig. 11). We observed concomitant changes in DNMT activity (Fig. 8d). Of note, the DNMTs-inhibitor 5-azacytidine (Aza) abolished the inhibitory effect of aPC on glucose sustained p66Shc expression (HG-NG-aPC-Aza, Fig. 8c). This suggests that the induction of DNMT1 by aPC is required for aPC-mediated repression of sustained p66Shc expression.

**Sustained p66Shc impairs atherosclerosis regression.** We next evaluated whether glucose-induced epigenetic p66Shc expression contributes to the hyperglycaemic memory in macrovascular disease and whether this can be reversed by aPC. First, we established a “memory effect” in glucose, but not in lipid exposed ApoE−/− mice (see Experimental scheme, Supplementary Fig. 12a). Aortic root plaque size was prominently reduced following lowering of blood lipid levels in HFD-adeno hApoE versus HFD-adeno LacZ mice (Supplementary Fig. 12b–e). Conversely, the size of atherosclerotic plaques remained unchanged following restoration of normoglycaemia (Supplementary Fig. 12b–e), reflecting sustained plaques size secondary to the hyperglycaemic memory.

Using this model, we were able to determine whether aPC reverses the hyperglycaemic memory effect in the context of atherosclerosis in vivo. Indeed, concomitant aPC treatment in...
addition to blood glucose reduction markedly reduced the size of aortic root plaques when compared to mice in which only blood glucose levels were normalized (DM-NG-aPC versus DM-NG-PBS, Fig. 9a). Furthermore, concomitant aPC treatment (DM-NG-aPC) decreased necrotic core area, the frequency of rupture shoulders, plaque-associated macrophages, while increasing fibrous cap thickness (Supplementary Fig. 13a,b). Importantly, concomitant aPC treatment reduced p66Shc expression, ROS generation (8-Oxo-dG), and IL-6 expression, while normalization of blood glucose levels alone had no impact on these parameters (Fig. 9b,c and Supplementary Fig. 13c,d).

Treatment of mice with 5-azacytidine in addition to aPC treatment and blood glucose normalization (DM-NG-aPC-Aza) abolished aPC’s effects (Fig. 9 and Supplementary Fig. 13). Treatment with aPC or 5-azacytidine had no impact on body weight, blood glucose levels, or total plasma cholesterol levels (Supplementary Fig. 14).

Taken together, these data demonstrate that aPC in addition to normalization of blood glucose levels reverses the high-glucose-induced sustained p66Shc expression and aPC-mediated reversion of plaque phenotype in ApoE-/- mice.

**Discussion**

Reduced thrombomodulin expression and aPC plasma levels have been clinically associated with diabetes and its vascular complications. While impaired thrombomodulin-dependent protein C activation has been mechanistically linked with diabetic microangiopathy, the role of aPC in diabetic macroangiopathy remained unknown. Here we demonstrate that aPC reverses the hyperglycaemic memory by correcting glucose-induced p66Shc expression in macrophages, but also identifies a potential therapeutic approach to the atherosclerosis-associated hyperglycaemic memory. The current data suggest that targeting p66Shc expression in macrophages either directly (as demonstrated by the VM approach) or indirectly by employing aPC-based therapies is a feasible approach to combat the hyperglycaemic memory in atherosclerosis.

By directly comparing hyperglycaemic ApoE-/- (ApoE-/- DM) mice to non-diabetic ApoE-/- mice on a high-fat diet (ApoE-/- HFD) we first show that the expression of the redox-regulator p66Shc in bone marrow-derived cells is a major determinant of plaque development and instability in hyperglycaemic conditions. While normalization of blood glucose levels alone fails to reduce glucose-induced and epigenetically sustained p66Shc expression in macrophages, aPC in addition to blood glucose normalization reverses glucose-induced p66Shc promoter hypomethylation and p66Shc expression in macrophages and associated ROS generation. These aPC-mediated consequences are congruent with aPC’s anti-oxidant effects and its effect on
p65\textsuperscript{Nuc} expression in glucose stressed podocytes (renal epithelial cells)\textsuperscript{16,26}. We specifically addressed the consequences of persistently elevated glucose level and associated changes for the hyperglycaemic memory in the current manuscript. The results show that hyperglycaemia per se drives plaque instability in murine models of hyperglycaemia-associated atherosclerosis. These findings add to previous important work, demonstrating that insulin resistance promotes unstable plaques through chronic inflammation\textsuperscript{27–29}. Hence, we propose that insulin resistance and hyperglycaemia both promote plaque instability, and that the concurrence of insulin resistance and hyperglycaemia in insulin resistance type 2 diabetic patients may synergistically promote plaque instability. Accordingly, we cannot exclude that other metabolic changes,
such as obesity, increased insulin levels, or impaired insulin signaling, contribute to a “memory” effect in type 2 diabetic patients. Indeed, epigenetic control of p66Shc in association with obesity has been recently reported. Here the methyltransferase SUV39H1 was identified as a key regulator modulating p66Shc expression by orchestrating recruitment of JMJD2C (a demethylase) and SRC-1 (an acetyltransferase) to the p66Shc promoter. Intriguingly, SUV39H1 forms a complex with DNMT1, suggesting that SUV39H1 and DNMT1 co-ordinately modulate the epigenetic landscape controlling p66Shc expression. Further characterization of the complex modulating the p66Shc-associated epigenetic landscape and thus p66Shc expression may...
Fig. 9 aPC reverses hyperglycaemic induced and epigenetically sustained p66Shc expression, promoting atherosclerotic plaque regression in mice. 

a Experimental design (left top) and representative Oil Red O-stained images of aortic roots (left bottom) and dot plot summarizing data (right).

b, c Representative co-immunofluorescence images for p66Shc (b, red), 8-Oxo-dG (c, red), macrophages (MOMA-2, b, c, green), and DAPI nuclear counterstain (b, c, blue; boxed areas shown at higher magnification in the lower panel). Dot plot summarize data for p66Shc-MOMA-2 (b) and 8-Oxo-dG-MOMA-2 co-localization (c, both Icorr, correlation index). Cont: normoglycaemic ApoE−/− mice with normal chow diet; DM: hyperglycaemic ApoE−/− mice; DM-NG-PBS: ApoE−/− DM mice receiving the SGLT2 inhibitor and PBS from week 16 to week 22; DM-NG-aPC: ApoE−/− DM mice with concomitant SGLT2 inhibitor and aPC treatment from week 16 to week 22; DM-NG-aPC-Aza: DM-NG mice with concomitant SGLT2 inhibitor, aPC, and 5-azacytidine. Data shown as dot plots represent mean ± SEM of 8–10 mice per group (a, c); size bars: a–c: 20 µm; **P < 0.01 (one-way ANOVA with Bonferroni-adjusted post hoc comparison of DM and DM-NG-aPC versus control and DM-NG-PBS and DM-NG-aPC-Aza versus DM-NG-aPC).
**Fig. 10** aPC-mediated reversal of hyperglycaemia-induced persistent p66Shc expression and plaque instability depends on DNMT1. 

**a** Experimental design.

**b** Representative images of Oil Red O-stained aortic root lesions (left panel) and dot plot summarizing data (right panel). 

**c** Dot plots summarizing morphometric analyses of necrotic core area (left panel), fibrous cap thickness (middle panel), and frequency of ruptured shoulders (right panel).

**d** Representative images showing immunofluorescence staining of macrophages within lesions (MOMA-2, green; DAPI nuclear counterstain, blue; left panel) and dot plot summarizing data (right panel).

**e** Representative co-immunofluorescence images for 8-Oxo-dG (red), macrophages (MOMA-2, green), and DAPI nuclear counterstain (blue) within aortic root lesions (left, ×10 magnified images shown in lower panel) and dot plot summarizing data (Icorr, correlation index, right). 

Cont: normoglycaemic ApoE−/− mice with normal chow diet; DM-NG: ApoE−/− DM mice receiving the SGLT2 inhibitor and PBS from week 16 to week 22; DM-NG-aPC-DNMT1-MO: DM-NG mice with concomitant SGLT2 inhibitor, aPC, and DNMT1-MO treatment; DM-NG-aPC-Cont-MO: DM-NG mice with concomitant SGLT2 inhibitor, aPC, and control morpholino treatment. Data shown as dot plots represent mean ± SEM of 8–10 mice per group; size bars: b, d, e 20 μm; **P < 0.01; b–e one-way ANOVA with Bonferroni-adjusted post hoc comparison of DM-NG and DM-NG-aPC-DNMT1-MO versus DM-NG-aPC-Cont-MO.)
identify additional therapeutic targets. This may lay ground for individualized treatment strategies in the context of diabetes-associated atherosclerosis, as recently proposed32.33.

The translational relevance of the current finding is supported by a recent clinical study which demonstrated that p66Shc expression is epigenetically increased in peripheral blood monocytes of type 2 diabetic patients34,35. In agreement with these clinical results we observed increased p66Shc expression in plaque-associated macrophages of diabetic, but not of non-diabetic patients and mice. The concordant observation in human peripheral blood monocytes31, human plaque-associated macrophages (Fig. 3), and the murine data presented within this manuscript argue against sustained p66Shc expression in ApoE−/−/DM mice as a consequence of toxic streptozotocin effects36. Congruently, we were not able to detect increased cell death, markers of the DNA damage response, or liver toxicity in STZ-injected ApoE−/− mice. Taken together, these studies identify p66Shc as an epigenetically controlled gene modulating the phenotype of monocytes and macrophages in diabetes. We speculate that the p66Shc-dependent modulation of the macrophage phenotype constitutes a diabetes-specific facet of trained innate immunity34,35. Alternative approaches may allow modulation of trained innate immunity in the context of diabetes.

The pivotal role of p66Shc in bone marrow-derived cells, the co-localization of p66Shc with macrophages, and the expression of pro-atherogenic (CD36) and pro-inflammatory (NF-κB p65, IL-6, and TNFα) genes in hyperglycaemia-associated macrophages are entirely congruent with the previously proposed pathogenetic function of macrophages in diabetes-associated atherosclerosis in animal36–38 and clinical studies39. In extension of previous work we thus identify p66Shc as a specific regulator of diabetes-associated atherosclerosis40. Different pathways through which increased and sustained p66Shc expression may impair plaque stability, such as increased macrophages cell death, enhanced ROS-induced inflammasome activation, or impaired effecotylicosis may contribute to impaired plaque stability in hyperglycaemic mice. Similar to p66Shc, ACSL-1 (long-chain acyl-CoA synthetase 1, an enzyme that catalyzes the thioesterification of fatty acids) promotes atherosclerotic plaque development specifically in a diabetic milieu41. Collectively, these studies support the concept of a specific pathophysiology underlying diabetes-associated atherosclerosis, thus providing a rationale for “individualized” therapeutic strategies in patients with diabetes-associated atherosclerosis.

Hyperglycaemia is thought to cause mitochondrial dysfunction, thus promoting diabetes-associated vascular complications42. The role of p66Shc for diabetic vascular complications, as shown here and previously16,19,43 supports a pivotal role of mitochondrial ROS generation. Excess ROS generation activates the redox-sensitive transcription factor NF-κB, promoting a pro-inflammatory micromilieu characterized by increased IL-6, TNFα, CCL2, or VCAM-1 expression44. Intriguingly, inhibition of pro-inflammatory NF-κB signalling by aPC has been demonstrated before45,46, but the underlying mechanism remained unknown. Given the current insights we propose that aPC restricts p66Shc-mediated ROS generation and thus NF-κB signalling. Additionally, increased ROS generation promotes NO-uncoupling and inflammasome activation, which are closely linked with diabetic vascular complications47. The observed induction of IL-6 and TNFα in glucose stressed macrophages is entirely congruent with these observations and with the perception of diabetic vascular complications as inflammation driven diseases48,49. The current data indicate that endothelial dysfunction with impaired thrombomodulin protein C activation accelerates and perpetuates the hyperglycaemia-induced vascular inflammation by unrestained p66Shc-mediated ROS generation in macrophages.

Other mechanisms of increased ROS generation have been linked with diabetes-associated accelerated atherosclerosis50. Intriguingly, enhanced p66Shc expression induces NADPH oxidase activity, while repressing manganese superoxide dismutase (MnSOD) expression, resulting in increased ROS generation in peripheral blood monocytes51. Accordingly, enhanced p66Shc expression may be a common pathomechanism inducing mitochondrial and other mechanisms of increased ROS generation, thus generating a pro-inflammatory and pro-atherogenic micromilieu.

Mitochondrial dysfunction in bone marrow-derived cells promotes unstable plaques42. Here we show that p66Shc (a mitochondrial redox regulator) promotes CD36 expression and lipid uptake in glucose-stimulated macrophages. As excess lipid uptake promotes cell death and thus the evolution of an unstable acellular—or necrotic—core, increased and perpetuated p66Shc expression may contribute to the impaired plaque stability observed in diabetic patients10.

While providing insights, the current study has potential limitations. We aimed to directly compare mice with hyperlipidaemia and hyperglycaemia-associated atherosclerosis. However, due to the study design total plasma cholesterol levels and plasma HDL levels were not comparable between groups. While a subgroup analyses of ApoE−/−/DM and ApoE−/−/HFD mice gave similar results (Supplementary Fig. 2), we cannot exclude an impact of different plasma levels of lipid species such as HDL cholesterol. Yet, we are confident that the observed changes largely reflect hyperglycaemia-dependent effects as the endpoints analysed (e.g., p66Shc expression expression and its sequelae: ROS generation, CD36, IL-6, TNFα-expression) were increased in the hyperglycaemic milieu compared to the hyperlipidaemic milieu despite lower total cholesterol levels in the hyperglycaemic milieu. Of note, the mixed phenotype in diabetic ApoE−/− mice, characterized by hyperglycaemia and hyperlipidaemia, reflects the situation in diabetic patients, in which likewise both risk factors are increased. Accordingly, some consider the ApoE−/− streptozotocin model to be the most appropriate mouse model to study accelerated diabetes-associated atherosclerosis52. The recent clinical observation showing epigenetically sustained p66Shc expression in diabetic patients53 and the increased p66Shc expression in atherosclerotic lesions from diabetic as compared to non-diabetic patients in the current study supports the translational relevance of the model used.

Finally, the question arises how the current finding may be taken further. Approaches to directly target p66Shc may be appropriate in addition to exploiting aPC-based signalling. In the current study morpholinos targeting p66Shc efficiently restricted hyperglycaemia-induced lesion development. Targeting morpholinos to relevant cell types, e.g., macrophages, through a receptor-dependent mechanism, e.g., via CD36, may constitute an approach to enable a targeted therapy, thus avoiding unwanted side effects. Alternatively, biased PAR1 agonists may be suitable to restrict p66Shc expression in plaque macrophages. These questions need to be addressed in future studies.

Methods
Reagents. The following antibodies were used in the current study: rabbit anti-p66Shc (Merck, Millipore, United States); rabbit anti-CD36, mouse anti-nitrotyrosine, rabbit anti-PAR4 (Santa Cruz, Germany); rabbit anti-DNMT3a, rabbit anti-DNMT3b, rabbit anti-DNMT3h, rabbit anti-β-actin, goat anti-rabbit IgG HRP (Cell Signaling Technology, Germany); mouse anti-8-hydroxy-2′-deoxygenosine (Trevisen, United States); rat anti-MOMA-2, rabbit anti-mouse IgG HRP (abcam, Germany); rat anti-endothelial cell protein C receptor (EPCR), rabbit anti-GAPDH (Sigma-Aldrich, Germany). The following secondary antibodies for immunofluorescence were used: FITC goat anti-rabbit IgG, FITC rabbit anti-goat IgG, Texas red rabbit anti-mouse IgG, and FITC donkey anti-goat IgG (Vector Laboratories, United States).
Other reagents were as follows: DMEM, trypsin-EDTA, penicillin, streptomycin, FCS, FBS, and HEPES (PAAS Laboratories, Austria); BCA reagent (Perbio Science, Germany); vectashield mounting medium with DAPI (Vector Laboratories, United States); PVDF membrane and immobilon™ western chemiluminescent HRP substrate (Merck, Millipore, United States); streptozotocin (Enzo Life Sciences, Germany); saffron, Oil Red-O, L929 cell line (Sigma-Aldrich, Germany); Accu-Chek test strips, Accu-Chek glucosemeter, protease inhibitor cocktail (Roche Diagnostics, Germany); albumin fraction V, hematoxylin Gill II, acrylamide, agarose (Carl Roth, Germany); aqueous-mounting medium (ZYTOMED, Germany); human LDL (Alfa Aesar, Germany); ‘high-fat diet’ (HFD) experimental food (Western Type Diet, 43% carbohydrates, 15% proteins and 42% fat; Nuvanc® Off, Trizool Research and Life Technologies (Germany); RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas, Germany); rompun 2% (Bayer, Germany); ketamine 10% (beta-pharm, Germany).

**Analysis of mice.** After 22 weeks of age the mice’s body weight was measured and mice were killed5,56. Blood samples were obtained from the inferior vena cava of anaesthetized mice (500 μl) and centrifuged at 2000 × g for 10 min and the heart, aortic arches, including brachiocephalic arteries, were embedded in O.C.T. compound and snap frozen. Brachiocephalic arteries were sectioned from distal to proximal at 6 μm thick slices. The thoracic aorta was fixed in 4% buffered formalin for 20 min, washed twice in PBS for 10 min and stored for not more than 1 day at 4 °C before analysis.

**Analysis of blood lipids.** Blood lipids were measured with the Cobas 6000 Chemistry System from Roche (Basel, Switzerland).

**Bone marrow transplantation.** ApoE−/− or p66−/− or ApoE−/− mice were sacrificed and bones were isolated from the hind limb (tibia, femur) and flushed with medium (RPMI 1640 with 2% FBS, 10 units/ml heparin, penicillin, and streptomycin) using a 25 G needle55. To remove osseous particles the solution was filtered through a sterile 40 μm filter. Cells were then washed onto a 50 μl tube. Cells were centrifuged at 900 × g, 10 min, 4 °C. Supernatant was discarded and the cell pellet was washed twice with 50 ml of serum-free RPMI (RPMI 1640 with 20 mM HEPES, penicillin, and streptomycin). Following centrifugation at 900 × g (5 min, 4 °C) cells were re-suspended in 25 ml of serum-free RPMI. Cell number was determined using a cell counter (TC20™ automated cell counter, Biorad, Germany). Cells were suspended in serum-free RPMI to a final concentration of 1 × 10^6 cells/ml. Recipient mice (ApoE−/− or 8 weeks old) were lethally irradiated (11 Gy, once) and injected with 5 × 10^6 bone marrow cells (volume 0.2 ml) via the tail vein 4–6 h after irradiation. The mice were kept on antibiotic solution (1 mg ml−1 penicillin, 1 g l−1 streptomycin) in the drinking water for 2 weeks after irradiation and then switched to water without antibiotics. Four weeks after bone marrow transplantation, efficient replacement of bone marrow was ascertained by FACS analysis. Recipient ApoE−/− mice were then divided into three groups: mice received either a high-fat diet (HFD group) or a normal chow diet without (control group) or with persistent hyperglycaemia (DM-NG) or with concomitant aPC (1 mg kg−1 intraperitoneally once daily, DM-NG-aPC). Atherogenic mouse models. ApoE−/− mice (age 6 to 8 weeks) were either fed high-fat diet (HFD) or normal chow diet or were made diabetic (DM) by injecting streptozotocin (STZ, 60 mg kg−1 intraperitoneally, once daily for 5 consecutive days, freshly dissolved in 0.05 M sterile sodium citrate, pH 4.5)16,24. Control mice were injected with equal volume of 0.05 M sodium citrate, pH 4.5 for 5 days and maintained on a normal chow diet47,49. Blood glucose and body weight were measured once weekly16,53. On average 85–90% of mice became diabetic (blood glucose > 300 mg dl−1) within the first 4 weeks and these were included as diabetic mice in the experiments. Mice not developing persistently elevated blood glucose levels and maintaining blood glucose levels < 200 mg dl−1 despite STZ injections were included in the control group1. The endpoints analysed did not differ between STZ-injected, but normoglycaemic mice and sodium–citrated injected mice (controls), HFD or hyperglycaemia (minimum 300 mg/dl) was maintained for up to 22 weeks. In some experiments diabetic mice were treated with the SGLT2 inhibitor dapagliflozin (25 mg kg−1 in the drinking water)50,51 16 weeks after the last STZ injection to reduce blood glucose levels without (DM-NG) or with concomitant aPC (1 mg kg−1 intraperitoneally once daily, DM-NG-aPC) or concomitant aPC plus 5-azacytidine (additionally 0.25 mg kg−1 intra-peritoneally on alternative days, DM-NG-aPC-Aza) treatment for further 6 weeks. Mice were killed and plaque morphology was analysed as previously described1. A group of HFD mice was randomly assigned to (a) persistent hyperlipidaemia (HFD-adeno LaCz) or (b) normalization of blood lipids for 6 weeks after 16 weeks of hyperlipidaemia re-expressing human ApoE in the liver (HFD-adeno hApoE)54. Mice were randomly allocated to different groups. All animal experiments were performed using 6–12 mice per group and data from all mice was included in the study to ensure biological reproducibility of in vivo data.

Atherosclerotic plaque morphology was analysed using the Image Pro Plus software from Media Cybernetics as previously described5. Briefly, plaque characteristics were determined as described in the following section (please see also Supplementary Fig. 18). Total plaque size (in mm2) is defined as the size of the plaque comprising all the atheroscleroma (fibrous cap, necrotic tissue, fibrous tissue etc.) within the vessel lumen. Necrotic core (in percent) is the area staining blue upon MOVATs stain and is reported as the percentage of the total plaque size. Fibrous cap thickness (in mm) is the minimal thickness of the fibrous tissue overlaying a necrotic core. If multiple necrotic cores are present within one plaque, the fibrous cap of the thickest fibrous cap is defined as used for further analyses. Frequency of rupture shoulder (in percent) is defined by the detection of a broken fibrous cap upon MOVATs staining and/or intraplaque haemorrhage, as indicated by intraplaque erythrocytes, and then calculating the number of ruptured shoulders compared to the total shoulder number.

**Vivo Morpholinos oligomer treatment.** Morpholinos (MO) were obtained from Gene Tools, LLC, United States55. The following oligonucleotides were used: 5’-ATCCAGGACCGTTTACGCTG-3’ against DNA methyltransferase, DNMT1, transcript variant 1; 5’-ATCCAGACGCGGCTTGAATGTTG-3’ as the p66−/− mismatch control; 5’-CAGGTTGCTGACAGCAAGACGGCTC-3’ against DNA methyltransferase, DNMT1, transcript variant 1, blocking the translation of the DNMT1 transcript variant 1; 5’-CAGGTTGCTGACAGCAAGACGGCTC-3’ as the DNMT1 mismatch control. MOs were dissolved in PBS (100 μl 6 μg MO per kg body weight; intravenously) and were injected every other day for 6 weeks into a subset of diabetic ApoE−/− mice.
100% ethanol for 1 min and stained in alcohol saffron for 8 min. Brachiocephalic arteries were washed in 100% ethanol for 1 min, moved to Xyloid for 10 min and covered with ice-cold aceton medium. The Glycol-solubilized brachiocephalic arteries and aortic roots were analysed for plasma background image. For histological analysis images were captured with an Olympus BX43-Microscope (Olympus, Hamburg, Germany). The Image Pro Plus software (version 6.0) was used for image analysis.

For immunofluorescence frozen sections of brachiocephalic arteries or aortic valves with maximum plaque size were fixed in ice-cold aceton for 8 min. washed twice with ice-cold PBS and incubated in 2% BSA in PBS for 1 h. Sections were then incubated for overnight at 4°C with one or two primary antibodies against pS6, DNMT1, CD68, MOMA-2, or 8-Oxo-2′-deoxyguanosine. The sections were incubated without primary antibodies were used as negative controls for background correction. After overnight incubation the sections were washed three times with 1 × PBS 5 min each time followed by incubation with fluorescently labelled corresponding secondary antibodies. After washing nuclear counterstaining was conducted using mounting medium with DAPI. Images were visualized, captured, and analysed using a fluorescence microscope. All histological analyses were performed by two independent blinded investigators.

Immunohistochemistry and immunofluorescence images were captured with an Olympus BX43-Microscope (Olympus, Hamburg, Germany). The Image J software was used for image analysis. To detect immuno- fluorescence of fluorescently detected proteins we determined a correlation index (correl) that reflects the automatically computed fraction (plug in available: https://sites.google.com/site/colocalizationcolormap/home) of positively correlated pixels in an image and is computed fraction (plug in available: https://sites.google.com/site/colocalizationcolormap/home) of positively correlated pixels in an image and is measured using ImageJ. Equal loading was confirmed in immunoblotted samples by GAPDH antibody.

Isolation and culture of bone marrow-derived macrophages. Bone marrow-derived macrophages (BMDM) were isolated and cultured as described elsewhere. Briefly, 7 to 10 weeks old C57BL/6 mice were sacrificed by cervical dislocation and bones were isolated from hind limbs (tibia, femur). Bones were kept in and flushed with RPMI-1640 complete medium to isolate bone marrow cells. Bone marrow cells were further washed with 1 × PBS and re-suspended in culture medium RPMI supplemented with 30% L929 cell-conditioned medium and 20% FBS. This procedure was repeated twice to remove dead cells. After the final wash the pelleted cells were re-suspended in above culture medium. Cells were cultured for 7 to 10 days until ~80% confluence. The purity of cells was confirmed by CD11b staining and FACS analyses and was consistently found to be higher than 90%. These cells were used as BMDM for experiments.

Immunoblotting. Proteins were isolated and immunoblotting was performed as described[48,49]. Cell lysates were prepared in RIPA buffer (50 mM Tris at pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, and 1 mM NaF supplemented with protease inhibitor cocktail). Lysates were centrifuged (10,000 × g, 4 °C to separate phases. The aqueous phase containing RNA was transferred to a 1.8% agarose gel. Universal methylated mouse DNA (Zymo research) was used as a positive control. The sequences of the primers used were as follows: methylated forward, 5′-TTTT TTT TTT GGG TGG TAT GTA GTC-3′; methylated reverse, 5′-GAC GGG AAA AAA AAA AAA AA-3′; unmethylated forward, 5′-TTTT TTT TTT TTT TTT TTGA GTT TAT GTA GTC-3′; and unmethylated reverse, 5′-CCA ACA CAA AAA AAA AAT AAA AA-3′.

DNMT activity assay. To determine DNMTs activity nuclear proteins were extracted from whole cell lysates as described[43]. Culture medium was removed from cells and cells were washed with 1 × PBS and afterwards scraped in 400 μl of cytoplasmic extraction buffer A containing 10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 0.6% NP-40, 0.5 mM DTT, protease inhibitor cocktail. Lysates were kept on ice for 10 min. Lysates were briefly vortexed and then centrifuged at 4 °C. Supernatants containing the cytoplasmic protein fraction were discarded. The pellet was re-suspended in 100 μl nuclear extraction buffer B containing 10 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 10 mM EDTA, 0.5 mM DTT, protease inhibitors. Lysates were incubated for 20 min on ice followed by centrifugation at 13,000 × g for 4 min. Supernatants containing the nuclear extracts were collected and DNMT activity was measured by using EpiQuik™ DNMT Activity assay ultra kit (EPIGENETEK) following the manufacturer’s instructions.

Analyses of human samples. Diabetes was diagnosed in patients according to the American Diabetes Association criteria[64,65]. Diabetic (N = 10) and non-diabetic (N = 11) patients with atherosclerotic disease were recruited from the cardiology clinic at the University Hospital Magdeburg. All 10 diabetic patients were type 2 diabetic patients (T2DM). All patients and controls were Caucasian. All patients were newly admitted to the university hospital at the Otto-von-Guericke University, Magdeburg, for treatment of ACS-stenosis, which was henceforth the primary diagnosis in all patients. Detailed information about the patient’s clinical characteristics is given in Supplementary Table 2. Tissue biopsies of atherosclerotic plaques were obtained from internal carotid artery during carotid disobliteration. Samples were immediately embedded in O.C.T. compound and snap frozen. Tissue biopsies were sectioned at 6-μm thickness and used for immunofluorescence staining, as described above. Tissues were incubated with Abs against cell adhesion molecules, followed by incubation with anti-rabbit Alexa Fluor 546. Labeled sections were imaged using a confocal microscope (Zeiss LSM880). Immunofluorescent images were captured with a Zeiss LSM880 microscope and laser scanning confocal microscopy. Sections were examined using Zeiss LSM software. The images were analyzed using Zeiss Zen Software (Carl Zeiss Microscopy, Inc., Thornwood, NY). The images were subsequently processed using Adobe Photoshop CS6 (Adobe Systems, Inc., San Jose, CA). The images were corrected for background fluorescence using the Tiffen Effects tool (Tiffen Filters, Inc., New York, NY). Immunofluorescence and densitometry were performed using ImageJ software (National Institutes of Health, Bethesda, MD). The intensity of staining was measured as the integrated optical density (IOD) of each channel. The IOD was normalized to the corresponding DAPI channel signal. The mean IOD of each channel was used to calculate the relative staining intensity.
software were used for statistical analyses. Values of P < 0.05 were considered statistically significant.

Data availability. The authors declare that the data supporting the findings of this study are available within the article and its Supplementary Information.

Received: 22 January 2018 Accepted: 3 July 2018
Published online: 06 August 2018

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Acknowledgements

We thank Kathrin Deneser, Julia Judin, Juliane Friedrich, René Rudat, and Rumiya Makarova for excellent technical support. This work was supported by grants of the ‘Deutsche Forschungsgemeinschaft’ (IS-67/5-3, IS-67/8-1, IS-67/11-1, CRC854/B26, CRC1118/B07N, RTG2408/P7&PP9 to B.I., RTG2408/P5, SH 849/1-2 to K.S., KO 5736/1-1 to S.K., and SFB 1118 to PPN), of the ‘Stiftung Pathobiochemie und Molekulare Diagnostik’ (SPMD to K.S.), the National Institute of Health (TACTIC; UM1-HL120877 to C.T.E.), and by a DAAD scholarships to M.M.A.

Author contributions

K.S. and J.G. designed, performed, and interpreted in vivo, in vitro, and ex vivo experiments. S.N. performed histological analyses. M.M.A., S.K., F.B., L.K., S.R. assisted in mouse experiments. T.F. assisted with laser dissection microscopy. Z.H. and R.B. provided human samples and assisted in preparing the manuscript. E.C. provided PAR1+/− mice and assisted in preparing the manuscript. P.P., C.T.E., provided reagents and assisted in preparing the manuscript. K.S. and B.I. conceptually designed and interpreted the experimental work and prepared the manuscript.

Additional information

Supplementary information accompanies this paper at https://doi.org/10.1038/s42003-018-0108-5.

Competing interests: The authors declare no competing interests.

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