The transcription factor ERG regulates a low shear stress-induced anti-thrombotic pathway in the microvasculature

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Endothelial cells actively maintain an anti-thrombotic environment; loss of this protective function may lead to thrombosis and systemic coagulopathy. The transcription factor ERG is essential to maintain endothelial homeostasis. Here, we show that inducible endothelial ERG deletion (ErgEC-KO) in mice is associated with spontaneous thrombosis, hemorrhages and systemic coagulopathy. We find that ERG drives transcription of the anticoagulant thrombomodulin (TM), as shown by reporter assays and chromatin immunoprecipitation. TM expression is regulated by shear stress (SS) via Krüppel-like factor 2 (KLF2). In vitro, ERG regulates TM expression under low SS conditions, by facilitating KLF2 binding to the TM promoter. However, ERG is dispensable for TM expression in high SS conditions. In ErgEC-KO mice, TM expression is decreased in liver and lung microvasculature exposed to low SS but not in blood vessels exposed to high SS. Our study identifies an endogenous, vascular bed-specific anticoagulant pathway in microvasculature exposed to low SS.
Hemostasis is a physiological mechanism, which aims to maintain vessel integrity through the formation of a blood clot after injury. A crucial function of the healthy endothelium is to maintain a constitutive anti-thrombotic state via the fine regulation of many procoagulant and anti-coagulant pathways balance local hemostasis and thrombosis, with differences between arterial, venous and microvascular beds, suggesting possible advantages in vascular-bed specific therapeutic approaches. However, the molecular basis for such specificity is poorly understood.

Amongst the multiple procoagulant and anti-coagulant pathways controlled by endothelial cells (EC), thrombomodulin (TM) is a key player in the regulation of coagulation and thrombosis. TM is widely expressed by EC of intact vessels and exerts anti-coagulant properties by potentiating activation of protein C; it also has anti-fibrinolytic and anti-inflammatory functions. In vivo, 60% of the mice with genetic constitutive deletion of TM in EC survive beyond birth, but show impairment of protein C activation and succumb to spontaneous thrombosis, resulting in consumption of coagulation factors, coagulopathy and generalized bleeding. In line with its anti-coagulant and anti-inflammatory roles, TM has been implicated in multiple inflammatory and ischemic pathologies. Polymorphisms in the TM gene have been reported to be associated with increased risk of venous thromboembolism, bleeding disorder, coronary artery disease, atrial hemolytic-uremic syndrome and atherosclerotic disease. TM expression was shown to be downregulated on EC overlying the atherosclerotic plaque. Finally, recombinant soluble TM therapy has been shown to be beneficial in treating sepsis and suspected disseminated intravascular coagulation.

Previous studies have demonstrated that different external stimuli are able to transcriptionally regulate TM. Laminar shear stress (SS) has been shown to upregulate TM expression, whereas inflammatory cytokines such as TNF-α repress TM expression via the transcription factor (TF) NF-κB. Multiple TF have been implicated in the regulation of TM expression, including Krüppel-like factor 2 (KLF2). KLF2 has been shown to regulate TM levels in EC in static, low and high SS conditions. Upregulation of TM expression by high SS, mediated by KLF2 (refs20–22), is an important protective anti-thrombotic and anti-atherosclerotic mechanism in large arteries. However, unlike loss of endothelial TM, in vivo deletion of KLF2 in mice leads to a modest decrease in platelet counts in 78% (7/9) and 100% (4/4) of ErgiEC-KO mice, as at 30 and 45 days post-tamoxifen injection, respectively. No clots were identified in lung, brain or kidney tissues. Immunofluorescence (IF) microscopy confirmed spontaneous intravascular thrombi in liver blood vessels, with high fibrinogen/fibrin content (Fig. 1d) and platelet aggregates (Fig. 1e), demonstrating that endothelial ERG protects from thrombosis in the liver.

To determine whether endothelial ERG impacts the coagulation cascade, a thrombin generation assay was performed on mouse plasma from ErgiEC-KO mice and littermate control mice (Supplementary Fig. 1d). The data showed no significant change in the endogenous thrombin potential (ETP) (Supplementary Fig. 1e), but a significant increase in thrombin maximum peak height (Supplementary Fig. 1f). Interestingly, mice generating the highest concentration of thrombin in plasma showed the most pronounced thrombotic phenotype in the liver.

Flow cytometry performed 30 and 45 days post-tamoxifen (Fig. 1f and Supplementary Fig. 1g, respectively), revealed a significant decrease in platelet counts in ErgiEC-KO mice; expression of the platelet marker GPIbβ was unchanged (Supplementary Fig. 1h). Crucially, analysis of mouse plasma 30 days post-tamoxifen showed decreased fibrinogen concentration (Fig. 1g) associated with elevated D-dimer (Fig. 1h) and thrombin-antithrombin (TAT) complex (Fig. 1i) levels in ErgiEC-KO mice; these findings are indicative of coagulopathy. Notably, the penetrance and severity of the phenotype in ErgiEC-KO mice appeared to be heterogeneous and variable (Supplementary Fig. 2a, b).

These data show that the endothelial TF ERG is essential to protect from spontaneous thrombosis in selected vascular beds. Deletion of ERG disrupts regulation of coagulation, leading to a coagulopathy associated with thrombosis and/or bleeding in liver and lung.

**Results**

**ERG deletion in mice results in thrombosis and coagulopathy.** Previous studies have shown that deletion of endothelial ERG in adult mice causes defective angiogenesis, loss of endothelial homeostasis and enhanced tissue inflammation in multiple organs. To investigate whether ERG protects EC from thrombosis in vivo, we used adult mice with inducible endothelial deletion of ERG (ErgiEC-KO). ErgiEC-KO mice were injected with tamoxifen at 6–8 weeks old and analysed 30 and 45 days after injection. Effective downregulation of ERG expression post-tamoxifen injection was confirmed by qPCR on liver and lung lysates (Supplementary Fig. 1a, b). Histological analysis of multiple tissues revealed the presence of hemorrhages in liver and lung in ErgiEC-KO mice, as shown by H&E staining (Fig. 1a, b) and Masson’s Trichrome (Supplementary Fig. 1c). Histological analysis also showed the presence of spontaneous clots in the liver (Fig. 1c), in 78% (7/9) and 100% (4/4) of ErgiEC-KO mice, at 30 and 45 days post-tamoxifen injection, respectively. No clots were identified in lung, brain or kidney tissues. Immunofluorescence (IF) microscopy confirmed spontaneous intravascular thrombi in liver blood vessels, with high fibrinogen/fibrin content (Fig. 1d) and platelet aggregates (Fig. 1e), demonstrating that endothelial ERG protects from thrombosis in the liver.

**ERG controls expression of multiple regulators of coagulation.** To investigate the molecular mechanisms through which ERG exerts its anti-thrombotic function, we analysed the global transcriptome profile of ERG-deficient HUVEC and identified...
multiple genes directly or indirectly involved in the regulation of coagulation as putative ERG transcriptional targets. These genes were validated by qPCR in HUVEC (Fig. 2a). Interestingly, in vivo analysis showed a tissue-specific pattern in the ERG-dependent regulation of hemostatic genes (including FVIII, PAI1 and eNOS) by ERG (Fig. 2b, c). The most consistently regulated ERG target gene, both in vivo and in vitro, is TM. TM mRNA was significantly downregulated in both liver and lung lysates from ERG-deficient mice (Fig. 2d, e). This was not due to the loss of vasculature, since expression of endothelial markers (CD31, VWF) in lung and liver tissues was not significantly different in ErgEC-KO mice compared to control (Fig. 2b, c). Immunoblotting of lung lysates from ErgEC-KO mice confirmed that TM protein was significantly downregulated compared to control mice (Fig. 2f). These findings suggest that ERG may regulate multiple procoagulant and anticoagulant genes in a tissue-specific manner.
Fig. 1 Endothelial-specific deletion of ERG in adult mice results in thrombosis and coagulopathy. a, b Representative images of H&E staining in a liver and lung sections from Erg<sup>Erg-KO</sup> and Erg<sup>fl/fl</sup> mice showing the presence of extravasated red blood cells (black arrows) in Erg<sup>Erg-KO</sup> mice, 30 days after tamoxifen injection. Scale bar: 100 μm for top panels and 50 μm for bottom panels. c Liver sections from Erg<sup>Erg-KO</sup> and Erg<sup>fl/fl</sup> mice imaged following H&E staining, 30 and 45 days after tamoxifen injection, revealed the presence of spontaneous thrombi in liver from Erg<sup>Erg-KO</sup> mice, as shown by clots (black arrows) partially occluding veins. Scale bar 50 μm. d, e Liver sections from Erg<sup>Erg-KO</sup> mice, 30 days after tamoxifen injection, stained with d an anti-fibrinogen (green) antibody and e a platelet marker (CD41 antibody, green) confirmed the presence of clots (yellow arrowheads) with high fibrinogen content and platelet aggregates in veins (V) and in sinusoids. Tissues are co-stained for CD31 to visualise blood vessels (magenta); nuclei are identified by DAPI (blue). Scale bar 50 μm. f Platelet counts (Platelets x 10<sup>9</sup> per μl expressed as % of control mice) were determined on plasma from Erg<sup>Erg-KO</sup> and Erg<sup>fl/fl</sup> mice, 30 days post tamoxifen (n = 8 per genotype). g Fibrinogen concentration (μg per mL) was measured on plasma from Erg<sup>Erg-KO</sup> and Erg<sup>fl/fl</sup> mice, 30 days post tamoxifen, using mouse Fibrinogen ELISA kit (n = 8 per genotype). h D-dimer concentration (ng per mL) was measured on plasma from Erg<sup>Erg-KO</sup> and Erg<sup>fl/fl</sup> mice, 30 days post tamoxifen, using mouse D-dimer ELISA kit (n = 8 per genotype). i Thrombin-antithrombin (TAT) complex concentration (ng per μL) was measured on plasma from Erg<sup>Erg-KO</sup> and Erg<sup>fl/fl</sup> mice, 30 days post tamoxifen, using mouse TAT ELISA kit (n = 8 per genotype). All graphical data are mean ± s.e.m., *P < 0.05, **P < 0.01, ***P < 0.001, Student’s t-test. Source data are provided as a Source Data file.

and points to TM as a key candidate for the anti-thrombotic effects of ERG.

**Thrombomodulin treatment rescues Erg<sup>Erg-KO</sup> mice phenotype.** To test whether the thrombotic phenotype observed in Erg<sup>Erg-KO</sup> mice was due to the decrease in TM expression, we used a mouse recombinant TM fusion protein targeting red blood cells (RBC-TM) to restore TM protein activity in vivo. RBC-TM has been shown to have a longer half-life and to confer more durable and potent protection from thrombosis in vivo compared to soluble non-targeted thrombomodulin (sTM). The ability of RBC-TM to generate activated protein C (APC) was confirmed in vitro (Supplementary Fig. 3a). Plasma was collected from adult control and Erg<sup>Erg-KO</sup> mice (25 days post-tamoxifen injection) before and after (6 h) injection of RBC-TM (4 mg per kg, intravenous route). Confirmation of ERG deletion and decrease in TM levels were assessed on liver (Fig. 3a and Supplementary Fig. 3b, d) and lung (Fig. 3b and Supplementary Fig. 3c, e) tissues. Crucially, acute treatment with RBC-TM in Erg<sup>Erg-KO</sup> mice was able to rescue TAT levels (Fig. 3c), an early systemic plasma marker for coagulopathy/thrombosis with short half-life (45 mins). As expected, treatment with RBC-TM for 6 h was not sufficient to correct levels of D-dimer (Fig. 3d) and fibrinogen (Fig. 3e), which are circulating biomarkers of coagulopathy that emerge later and have longer half-lives. These data show that an acute treatment with RBC-TM is able to significantly rescue the coagulopathy observed in Erg<sup>Erg-KO</sup> mice.

**ERG regulates thrombomodulin expression in endothelial cells.** We used in vitro models to investigate the mechanisms through which ERG controls TM expression. Inhibition of ERG expression in HUVEC by siRNA treatment, for 12, 24 and 48 h, caused a decrease in TM mRNA (Fig. 4a and Supplementary Fig. 4a) and protein expression, assessed by immunoblotting (Fig. 4b, c and Supplementary Fig. 4b) and IF microscopy (Fig. 4d, e), compared to control siRNA. Consistently, ERG inhibition via a second siRNA sequence confirmed downregulation of TM mRNA expression levels (Supplementary Fig. 4c). ERG also regulates TM expression at the mRNA and protein levels in microvascular EC, in both human dermal microvascular EC (HDMEC) (Supplementary Fig. 4d and Fig. 4d) and human dermal blood EC (HDBEC) (Fig. 4e). Conversely, ERG overexpression (Supplementary Fig. 4e, f) significantly increased TM expression (Fig. 4f) in HUVEC.

TM is a key regulator of thrombin-mediated activation of the natural anticoagulant APC<sub>39</sub>. To investigate whether the regulation of TM expression by ERG results in regulation of its function, we conducted a protein C activation assay on HUVEC treated with control or ERG siRNA and measured the production of APC over time. In line with decreased TM expression, ERG-deficient cells (Supplementary Fig. 4g, h) showed reduced generation of APC after 30 min of incubation with protein C, with a maximum reduction at the 60 min time point compared to siCtrl cells (Fig. 4g).

These results demonstrate that ERG regulates TM levels and thus the TM-protein C system in EC.

**ERG transcriptionally regulates thrombomodulin expression.** We investigated the molecular mechanisms by which ERG induces TM expression in EC. Bioinformatic analysis of the human TM gene locus revealed the presence of highly conserved ERG DNA binding motifs in the promoter region (Fig. 5a and Supplementary Fig. 5a–c). Comparative analysis of chromatin immunoprecipitation sequencing (ChIP-seq) data for markers of active promoters (Histone marks H3K4Me3/H3K27Ac and RNA pol II) in HUVEC (available from ENCODE Consortium) showed that the location of these marks correlates with the position of the putative ERG binding sites (Fig. 5a). Data from ERG ChIP-sequencing<sup>41</sup> (Fig. 5a and Supplementary Fig. 5a; ERG binding peak) and ChIP-qPCR (Fig. 5b, TM promoter regions R1 and R2; sequence of ERG binding peak shown in Supplementary Fig. 5b) confirmed ERG binding to the TM promoter but not in a negative control region distal to the ERG binding peak (Fig. 5b, TM negative control). Specificity of the ERG antibody was confirmed in ERG-deficient HUVEC (Fig. 5b). Functionality of the ERG binding sites (EBS; A/CGGAA) (Supplementary Fig. 5b) present on the proximal TM promoter was shown by luciferase reporter assays (sequence of the TM promoter construct shown in Supplementary Fig. 6). ERG overexpression resulted in transactivation of TM wild-type (TM WT) promoter in HUVEC (Fig. 5c); mutation of the two EBS located upstream the transcription start site (TSS) of the TM gene were sufficient to abrogate this signal, showing that these EBS are functional and are responsible for ERG’s ability to drive TM (Fig. 5c and Supplementary Fig. 6).

The acetyltransferase p300 has been shown to bind to the TM promoter and activate its expression<sup>18</sup>. Pharmacological inhibition of p300 in HUVEC, resulting in decreased global levels of histone 3 lysine (K) 27 acetylation (H3K27Ac) (Supplementary Fig. 7a), led to a decrease in H3K27Ac occupancy on the TM promoter (Fig. 5d) and a decrease in TM mRNA levels (Supplementary Fig. 7b). We have recently shown that ERG is involved in p300 recruitment and acetylation of H3K27 on super-enhancers associated with endothelial cell-specific genes<sup>41</sup>. In line with this concept, we showed that ERG deficient-HUVEC have reduced p300 binding to the TM promoter region (Fig. 5e, regions R1 and R2). Deletion of ERG also led to a significant decrease in H3K27Ac occupancy, in agreement with a reduction in open and active chromatin (Fig. 5f, regions R1 and R2),
suggesting that ERG directly affects chromatin accessibility at the TM promoter. These data demonstrate that ERG binds to the TM promoter and directly drives TM expression in EC, through p300 recruitment, acetylation of H3K27 and opening of the chromatin at the TM promoter region.

**ERG cooperates with KLF2 to drive thrombomodulin expression.** The TF KLF2 has been identified as a key regulator of TM expression in EC. Analysis of the ERG bound sequence on the TM promoter revealed the presence of adjacent ERG and KLF2 binding sites close to the TSS (Supplementary Fig. 5c). Thus, we

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**Fig. 2 ERG controls the expression of multiple regulators of coagulation in a tissue-specific manner.** a qPCR screen analysis of genes involved in the regulation of coagulation in HUVEC was performed at 48 h after ERG inhibition by siRNA (n = 4–6 independent experiments). Data were normalised to GAPDH. b, c qPCR analysis of selected targets genes directly or indirectly involved in coagulation and endothelial markers in whole liver (b) and lung (c) lysates from adult control (Ergfl/fl) and ERG-deficient (ErgiEC-KO) mice (n = 4–5 per genotype). Data were normalised to 18S. d–e qPCR analysis of thrombomodulin (TM) mRNA expression in whole liver (d) and lung (e) lysates from adult Ergfl/fl and ErgiEC-KO mice (n = 5 per genotype). Data were normalised to 18S. f Immunoblot and quantification of WB for TM in protein extracts of lung samples from adult Ergfl/fl and ErgiEC-KO mice 30 days after tamoxifen injection (n = 3 mice per group). Data were normalized to GAPDH. All graphical data are mean ± s.e.m, *P < 0.05, Student’s t-test. Source data are provided as a Source Data file.
investigated whether ERG and KLF2 cooperate in EC to promote TM expression.

Single ERG or KLF2 siRNA treatment or a combination of the two led to a comparable decrease in TM mRNA expression (Supplementary Fig. 8a) or protein levels (Supplementary Fig. 8b, c), showing no additive effect. ERG siRNA did not affect KLF2 mRNA (Supplementary Fig. 8d) and protein levels (Supplementary Fig. 8e); similarly, KLF2 siRNA did not affect ERG levels (Supplementary Fig. 8a–c). However, inhibition of KLF2 expression caused a significant decrease in ERG’s ability to drive TM promoter activity (Fig. 6a) and to promote TM expression (Supplementary Fig. 8f). Moreover, inhibition of ERG expression caused a significant decrease in the KLF2-dependent transactivation of the TM promoter and of TM expression (Fig. 6b and Supplementary Fig. 8g, h). Finally, co-expression of ERG and KLF2 significantly increased transactivation of the TM promoter compared to ERG or KLF2 alone (Fig. 6c). These findings indicate that ERG and KLF2 co-operate in driving TM expression. Interestingly, mutations of the two ERG binding sites (EBS) upstream of the TSS, which abolished ERG-dependent transactivation (see Fig. 5c), also led to a complete loss of KLF2 transactivation ability (Fig. 6d) suggesting that ERG binding to DNA is required for KLF2 transcriptional activity. ChiP-qPCR analysis showed binding of ERG and KLF2 to the same promoter regions (Fig. 6e, f, regions R1 and R2). Inhibition of KLF2 expression in HUVEC had no effect on ERG enrichment on the TM promoter and of TM expression (Fig. 6b and Supplementary Fig. 8g, h). Moreover, inhibition of ERG expression caused a significant decrease in the KLF2-dependent transactivation of the TM promoter and of TM expression (Fig. 6b and Supplementary Fig. 8g, h). Finally, co-expression of ERG and KLF2 significantly increased transactivation of the TM promoter compared to ERG or KLF2 alone (Fig. 6c). These findings indicate that ERG and KLF2 co-operate in driving TM expression. Interestingly, mutations of the two ERG binding sites (EBS) upstream of the TSS, which abolished ERG-dependent transactivation (see Fig. 5c), also led to a complete loss of KLF2 transactivation ability (Fig. 6d) suggesting that ERG binding to DNA is required for KLF2 transcriptional activity. ChiP-qPCR analysis showed binding of ERG and KLF2 to the same promoter regions (Fig. 6e, f, regions R1 and R2). Inhibition of KLF2 expression in HUVEC had no effect on ERG enrichment on the TM promoter and of TM expression (Fig. 6b and Supplementary Fig. 8g, h). Moreover, inhibition of ERG expression caused a significant decrease in the KLF2-dependent transactivation of the TM promoter and of TM expression (Fig. 6b and Supplementary Fig. 8g, h). Finally, co-expression of ERG and KLF2 significantly increased transactivation of the TM promoter compared to ERG or KLF2 alone (Fig. 6c). These findings indicate that ERG and KLF2 co-operate in driving TM expression. Interestingly, mutations of the two ERG binding sites (EBS) upstream of the TSS, which abolished ERG-dependent transactivation (see Fig. 5c), also led to a complete loss of KLF2 transactivation ability (Fig. 6d) suggesting that ERG binding to DNA is required for KLF2 transcriptional activity. ChiP-qPCR analysis showed binding of ERG and KLF2 to the same promoter regions (Fig. 6e, f, regions R1 and R2). Inhibition of KLF2 expression in HUVEC had no effect on ERG enrichment on the TM promoter and of TM expression (Fig. 6b and Supplementary Fig. 8g, h). Moreover, inhibition of ERG expression caused a significant decrease in the KLF2-dependent transactivation of the TM promoter and of TM expression (Fig. 6b and Supplementary Fig. 8g, h). Finally, co-expression of ERG and KLF2 significantly increased transactivation of the TM promoter compared to ERG or KLF2 alone (Fig. 6c). These findings indicate that ERG and KLF2 co-operate in driving TM expression.

Fig. 3 Treatment with RBC-thrombomodulin rescues the thrombotic phenotype of ErgEC-KO mice. a, b qPCR analysis of ERG, CD31 and TM gene expression in whole a liver and b lung lysates from adult control (Ergfl/fl) and ERG-deficient (ErgEC-KO) mice (n = 3); 25 days after tamoxifen injection and 6 h after RBC-TM injection. Data were normalized to 18S. Graphical data are mean ± s.e.m., *P < 0.05, Student’s t-test. c–e Adult ErgEC-KO and control mice were bled before and after (6 h) injection of red blood cells-targeted thrombomodulin (RBC-TM, 4 mg per kg) to analyse plasma markers for coagulopathy. a Thrombin-antithrombin (TAT) complex concentration (ng per µL) was measured on plasma from ErgEC-KO and Ergfl/fl mice, using mouse TAT ELISA kit (n = 3–4 per genotype). b D-dimer concentration (ng per mL) was measured on plasma from ErgEC-KO and Ergfl/fl mice, using mouse D-dimer ELISA kit (n = 3–4 per genotype). c Fibrinogen concentration (µg per mL) was measured on plasma from ErgEC-KO and Ergfl/fl mice, using mouse Fibrinogen ELISA kit (n = 3–4 per genotype). All graphical data are mean ± s.e.m., NS: Not significant, *P < 0.05, **P < 0.01, Student’s t-test. Data for each ErgEC-KO mouse before and after RBC-TM injection are also presented in separated graphs (bottom graphs) to see individual response to the treatment. Source data are provided as a Source Data file.
Fig. 9a–c). These data suggest a model of cooperativity where, in homeostatic conditions, ERG promotes opening of the chromatin at the TM promoter via p300 recruitment and supports KLF2 recruitment to DNA via direct interaction (Fig. 6h).

**ERG regulates thrombomodulin in low shear stress conditions.** Although expressed in EC under static conditions in vitro, KLF2 and TM are important flow-responsive genes: laminar shear stress (SS) upregulates expression of KLF2, which then promotes increased TM expression.\(^{15,21}\) Whether ERG is a flow-responsive TF is unknown. We first interrogated the effect of low (5 dynes per cm\(^2\)) and high (20 dynes per cm\(^2\)) laminar SS on ERG and KLF2 protein levels in HUVEC. As expected, KLF2 protein levels were upregulated in HUVEC exposed to low and high SS compared to static conditions; in contrast, ERG protein levels were unaffected by SS (Fig. 7a). ERG-KLF2 complex formation was increased under low SS compared to
static conditions; high SS did not further increase this interaction (Fig. 7b).

We next investigated the role of ERG in the regulation of TM expression in static versus low and high SS conditions in HUVEC treated with either ERG or control siRNA. As expected, KLF2 and TM mRNA levels were upregulated in HUVEC exposed to SS compared to static conditions, whilst ERG mRNA levels were unaffected (Fig. 7c). Crucially, whilst high SS induced upregulation of TM expression in ERG-deficient cells, low SS was not able to induce TM upregulation in ERG-deficient cells (Fig. 7c), indicating that ERG controls TM expression selectively in conditions of low SS and is dispensable for the high SS-dependent regulation of TM. These results show that the cooperation between ERG and KLF2 is essential for TM expression specifically in low SS conditions and suggest that other partners/pathways support KLF2-dependent transcription in high SS conditions.

HUVEC are the most widely used EC model in vitro but do not recapitulate all the properties of EC from different vascular beds. Therefore, to investigate the role of ERG in the regulation of TM in EC from microvascular or arterial origins, we conducted flow experiments on human microvascular EC (HDHBC) (Fig. 7d) and human aortic endothelial cells (HAEC) (Supplementary Fig. 10). The experiments on HDHBC showed similar results compared to HUVEC, demonstrating that ERG regulates TM expression specifically in low SS conditions also in microvascular EC (Fig. 7d). However, loss of ERG in HAEC did not affect low and high SS-induced upregulation of TM expression (Supplementary Fig. 10), indicating that ERG is not involved in the regulation of TM expression in these cells. These data indicate the presence of distinct molecular mechanisms of gene regulation in EC from different vascular beds. Interestingly, these differences are retained ex vivo, possibly via epigenetic memory of the cells being exposed to high SS in vivo.

High laminar SS has been shown to mediate chromatin remodelling by inducing acetylation of histone marks, thus promoting flow-dependent regulation of gene expression. We have recently shown that ERG is required for acetylation of H3K27 in HUVEC in static conditions; this was confirmed by decreased levels of H3K27Ac, assessed by IF microscopy, in ERG-deficient HUVEC (Fig. 7e). Here, we asked whether ERG promotes chromatin remodelling and accessibility under different SS conditions using H3K27Ac global levels as a readout. As expected, high SS increased levels of H3K27Ac compared to low SS (Fig. 7e). Interestingly, H3K27Ac levels were significantly decreased in ERG-siRNA treated cells exposed to low SS compared to control HUVEC, but were not affected by the loss of ERG in high SS conditions (Fig. 7e). These data show that ERG is required for chromatin accessibility in low SS conditions but is not required under high SS.

**DISCUSSION**

In the present study, we identify a pathway that controls the anti-thrombotic properties of the endothelium, via the transcription factor ERG.

Previous studies have shown that ERG is required for maintenance of the endothelial lineage and to promote vascular homeostasis, by controlling multiple functions including survival, permeability and inflammation (reviewed previously). Here, we show that ERG also drives pathways that prevent spontaneous clotting and thus exerts anti-thrombotic properties in EC. Other TF have been reported to control pro- and anti-thrombotic pathways, and their deletion or overexpression has been shown to affect thrombus formation in a variety of in vivo injury models. However, the finding that loss of a single endothelial TF is
Fig. 5 ERG binds to and transactivates the thrombomodulin promoter in vitro. **a** Putative ERG binding sites (black bars) are located within the TM promoter upstream and downstream of the transcription start site (black arrow); ERG ChIP-sequencing data show a significant ERG peak on TM proximal promoter. ENCODE sequence conservation between 100 vertebrates is shown across this gene. ENCODE ChIP-seq data profiles for H3K4Me3 (tri-methylation of lysine (K) 4 on histone 3), H3K27Ac (acetylation of lysine (K) 27 on histone 3) and RNA polymerase II (RNA pol II) in HUVEC indicate open chromatin and active transcription. Location of qPCR primers covering regions R1 and R2 (black bar) are indicated. **b** ERG ChIP-qPCR using primers to region R1 and R2 on ERG-bound chromatin from siCtrl or siERG-treated HUVEC. Primers for a region within 5′UTR region of TM gene (TM neg con) were used as negative control. Data are shown as fold change over IgG (n = 3 independent experiments). Graphical data are mean ± s.e.m., *P < 0.05, **P < 0.01, ***P < 0.001, Student’s t-test. **c** TM promoter luciferase reporter assay. ERG cDNA expression plasmid (ERG) or empty expression plasmid (pcDNA) were co-transfected with TM promoter-luciferase constructs (TM wild type (WT), TM mutant 1 or TM mutant 2) or a pGL4 empty vector in HUVEC, and luciferase activity was measured. Values represent the fold change in relative luciferase activity over the empty pGL4 vector alone (n = 4 independent experiments). Graphical data are mean ± s.e.m., *P < 0.05, **P < 0.01, ***P < 0.001, One-way ANOVA; ##P < 0.01 compared to pcDNA-TM WT condition, Student’s t-test. **d** H3K27Ac ChIP-qPCR using primers to region R1 and R2 on H3K27Ac-bound chromatin from HUVEC treated with DMSO or p300 inhibitor (10 µM) for 1 h. Data are shown as fold change over IgG (n = 3 independent experiments). **e** ChIP-qPCR using primers to region R1 and R2 on p300-bound chromatin from siCtrl or siERG-treated HUVEC. Data are shown as fold change over IgG. All graphical data for ChIP experiments are mean ± s.e.m., *P < 0.05, **P < 0.01, ***P < 0.001, Student’s t-test. Source data are provided as a Source Data file.
sufficient to cause spontaneous thrombosis is to our knowledge unique, and in line with the essential role of ERG in maintaining vascular homeostasis.

Multiple ERG targets are likely to be directly or indirectly implicated in the control of coagulation. However, the gene target consistently regulated by ERG across different tissues and in vitro was the anticoagulant TM. Studies on the transcriptional regulation of TM have shown that KLF2 drives TM expression in EC, particularly under high laminar SS conditions. In this study, we show that ERG increases KLF2 binding to the TM promoter by facilitating chromatin access via p300 recruitment and H3K27 acetylation. We find that the ERG binding sites on the proximal TM promoter are essential for ERG and KLF2-dependent transactivation. These data suggest that both direct cooperation between ERG and KLF2 and epigenetic regulation controlled by ERG contribute to the fine regulation of TM expression.
Fig. 6 ERG cooperates with KLF2 to drive thrombomodulin expression. **a–d** TM promoter luciferase reporter assays. Values represent the fold change in relative luciferase activity over the empty pGL4 vector alone. **b** pcDNA or ERG expression plasmid (ERG) were co-transfected with a TM wild type (WT) promoter-luciferase construct (TM WT) in siCtrl or siKLF2-treated HUVEC, and luciferase activity was measured (n = 3 independent experiments). **c** pcDNA or KLF2 cDNA expression plasmid (KLF2) were co-transfected with TM WT promoter construct in siCtrl or siERG-treated HUVEC (n = 3 independent experiments). Graphical data presented in **a and b** are mean ± s.e.m., *P < 0.05, **P < 0.01, Student’s t-test. **c** pcDNA, KLF2 or both KLF2 and ERG plasmids were co-transfected with TM WT promoter construct (n = 4 independent experiments). Graphical data are mean ± s.e.m. ***P < 0.001, ****P < 0.0001, One-way ANOVA, compared to pcDNA-TM WT condition. **d** pcDNA or KLF2 plasmid were co-transfected with TM promoter constructs (TM WT, TM mutant 1 or TM mutant 2) (n = 4 independent experiments). Graphical data are mean ± s.e.m., **P < 0.01, Student’s t-test. 

In this study, we report a mechanism of differential transcriptional control of TM expression depending on SS. We show that ERG is essential for chromatin remodelling and for KLF2-dependent regulation of TM selectively in low SS conditions in vitro. Our findings suggest that ERG, together with low levels of KLF2, provides the basal essential anticoagulant properties required throughout the endothelium of the microcirculation; however, extra protection from unwanted thrombus formation in a larger vessel, particularly arteries, is provided by pathways induced by high laminar SS. Indeed, high SS has been shown in vitro to promote endothelial epigenetic changes leading to an increase in open and accessible chromatin. This could allow chromatin access for KLF2 and other TF to bind DNA and drive gene expression, without requiring ERG. In line with the concept of the additional KLF2-dependent protection, Boon et al. have demonstrated that increased KLF2 expression in arterial high SS conditions results in activation of an endothelial athero-protective phenotype, by inhibiting the TGFβ-SMAD pathway. Interestingly, we have recently shown that ERG represses the TGFβ-SMAD pathway in EC to prevent endothelial–mesenchymal transition and fibrinogenesis; whether ERG and KLF2 also cooperate in the regulation of the TGFβ pathway remains to be investigated. These studies suggest a model of cooperation between ERG and KLF2 in the transcriptional control of endothelial homeostasis, where ERG, partly by promoting KLF2 binding to DNA, is essential to maintain a basal, healthy and anti-thrombotic endothelium in the microvasculature of multiple organs. This pathway appears dispensable in blood vessels exposed to high SS, where the role of KLF2 becomes dominant, and possibly other ETS factors (such as ETS1) or other epigenetic mechanisms may step in to provide cooperation. Future studies will investigate the TF networks involved in shear-dependent, cooperative transcriptional and epigenetic networks.

In vivo, endothelial deletion of KLF2 alone in adult mice does not cause a spontaneous thrombotic phenotype; double deletion of KLF2 and KLF4 is required to cause a profound compromise in vascular integrity and dysregulation of the coagulation system. Crucially, deletion of endothelial ERG is sufficient to cause thrombosis and coagulopathy, with decreased platelet counts, decreased circulating levels of fibrinogen and increased D-dimer and TAT plasma concentrations. The penetrance and severity of the phenotype in ErgEC-KO mice appears to be variable and vascular bed-specific. In fact, the thrombotic and hemorrhagic phenotypes observed in ErgEC-KO mice occur selectively in the liver and the lung, showing intriguing tissue-specificity of this pathway. Our data also revealed an increased rate of thrombin generation in ErgEC-KO mice and an ERG-dependent regulation of a set of genes potentially involved in the control of coagulation (such as PAI1 and eNOS). Interestingly, acute treatment with a red blood cells-targeted TM was able to restore the levels of TAT, an early biomarker of coagulopathy and thrombosis, showing that loss of TM expression is in part responsible for the prothrombotic phenotype observed in ERG-deficient mice. Future experiments will investigate the contribution of other pathways in the prothrombotic phenotype of ErgEC-KO mice.

Although regulation of TM expression is clearly a pivotal mechanism through which ERG exerts its anti-thrombotic function, it is likely that the thrombotic phenotype caused by inducible deletion of ERG results from a combination of pathways, which may have different relevance depending on tissue-specific mechanisms, still to be characterised. EC from different sites of the vascular tree appear indeed to use a site-specific combination of hemostatic proteins to prevent excessive clot formation. Specifically, the liver synthesizes and releases most of the proteins involved in the clotting cascade (such as fibrinogen, protein C and protein S). This high local concentration of coagulation factors and the unique architecture of liver sinusoids, presenting fenestrated and discontinuous EC, might explain why the most pronounced phenotype in ErgEC-KO mice is observed in this tissue. Moreover, one of ERG’s key functions is to promote vascular stability though the transcriptional control of genes, such as VE-cadherin and claudin-5. Thus, it is possible that the hemorrhagic phenotype, observed in the lung of adult ErgEC-KO mice, may be due to the combination of disrupted blood vessels with increased permeability and impairment of coagulation, leading to bleeding.

In conclusion, our study shows that ERG is crucial for the maintenance of the endothelial anti-thrombotic environment. We demonstrate that ERG is a direct transcriptional regulator of TM, driving its expression in a context-specific manner by cooperating with KLF2. The identification of vascular bed-specific pathways has potentially important implications for human pathologies, given that thrombotic diseases show selective preferences for specific organs’ vascular beds. This pathway could be involved in the pathogenesis of human disease associated with thrombosis in microvessels and/or with coagulopathy, such as disseminated intravascular coagulation, hemolytic uremic syndrome or sepsis.
Methods

**Mice and breeding.** All animal experiments were conducted according to and in compliance with the guidelines from Directive 2010/63/EU of the European Parliament for animal testing and research, with ethical approval from Imperial College London under UK Home Office Licence number PPL PEDBB/1586 and in compliance with the UK Animals (Scientific Procedures) Act of 1986. The inducible endothelial-specific ERG knockout mouse model (ErgiEC-KO) was generated by breeding Erg^{fl/fl} mice with Cdh5(PAC)-CreERT2 mice; 10–12 weeks old male and female mice were used for this study. Endothelial deletion of ERG was induced in adult mice, between 6–8 weeks, by tamoxifen injection (five injections of 0.5 mg daily). All experiments were conducted using littermate controls; mice were monitored, and tissues were harvested 25, 30 or 45 days post-tamoxifen injection.

**Cell culture and flow experiments.** Pooled human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (C2519A) (Wokingham, United Kingdom) and cultured in Endothelial Cell Growth Medium-2 media (EGM-2) (Lonza) or isolated from umbilical cords and cultured in M199 media. Human aortic endothelial cells (HAEC) (single donor, Promocell, C-12271) were cultured in EGM-2 media (Lonza). Human dermal microvascular endothelial cells (HDMEC)
Fig. 7  ERG drives thrombomodulin expression in vitro selectively in low shear stress conditions.  a Representative image and quantification of ERG (green) and KLF2 (magenta) expression in HUVEC cultured under static conditions or exposed to low shear stress (LSS, 5 dynes per cm²) or high shear stress (HSS, 20 dynes per cm²) for 24 h by immunofluorescence; nuclei are identified by DAPI (blue). Scale bar 40 μm. Quantification represents the mean pixel intensity for ERG or KLF2 signal (arbitrary unit, A.U.) per cell (n = 3–4 images per condition).  b Representative image of proximity ligation assay (PLA) for nuclear ERG-KLF2 interaction was performed on confluent HUVEC cultured under static conditions or exposed to low shear stress (LSS, 5 dynes per cm²) or high shear stress (HSS, 20 dynes per cm²) for 24 h; nuclei are identified by DAPI (blue). Quantification represents the PLA signal (mean number of dots per cell) and is expressed as a percentage compared to static condition (n = 3–4 images per condition). Scale bar, 40 μm.  c qPCR analysis of ERG, KLF2 and TM mRNA expression in siControl or siERG-deficient HUVEC under static conditions or after 24 h under LSS or HSS (n = 4–5).  d qPCR analysis of ERG, KLF2 and TM mRNA expression in siControl or siERG-deficient HDBEC under static conditions or after 24 h under LSS or HSS (n = 3 independent experiments).  e Representative image and quantification of H3K27Ac expression (magenta) in siControl or siERG-depleted HUVEC cultured under static conditions or exposed to LSS or HSS for 24 h by immunofluorescence; nuclei are identified by DAPI (blue). Scale bar 40 μm. Quantification represents the mean pixel intensity for H3K27Ac signal (arbitrary unit, A.U.) per cell (n = 6 images per condition). All graphical data are mean ± s.e.m., *P < 0.05, **P < 0.01, ***P < 0.001, Student’s t-test for top panel and one-way Anova for bottom panel. Source data are provided as a Source Data file.
Hematologic analysis. Mice were anesthetised intraperitoneally with ketamine (75 mg per kg)/medetomidine (1 mg per kg) and bled from the retro-orbital plexus into tubes containing 3.8% citrate anticoagulant. Platelet counts were evaluated by flow cytometry using calibrated beads (Saxon Europe) and rat anti-mouse GPIb-β-DyLight 488 (X488; Emfret) according to the manufacturer’s instructions. Briefly, whole blood was diluted 1/20 with modified Tyrode’s buffer and platelets were stained with rat anti-mouse GPIb-β-DyLight 488 for 15 min at room temperature. Samples were analysed using a FACSCalibur/LSRFortessa™ flow cytometer and Flowing Software (v2.5.1).

Fig. 8 ERG regulates thrombomodulin expression specifically in microvascular beds. a, c Representative image by immunofluorescence and b, d quantification of TM expression (magenta) in a, b liver and c, d lung sections from Ergfl/fl and ErgiEC-KO mice 30 days after tamoxifen injection; tissues are co-stained for CD31 to visualise blood vessels (green); nuclei are identified by DAPI (blue). Scale bar 50 µm. Quantification represents the ratio between the sum of pixel intensity for TM signal (arbitrary unit, A.U.) and CD31 area (µm²) (reflecting vascular coverage) (n = 4 mice per genotype). e Representative immunofluorescence image and f quantification of endothelial TM expression (magenta) in aorta sections from Ergfl/fl and ErgiEC-KO mice 45 days after tamoxifen injection; tissues are co-stained for ERG (green); nuclei are identified by DAPI (blue). Scale bar 50 µm. Quantification represents the ratio between the sum of pixel intensity for TM signal (arbitrary unit, A.U.) and the number of EC per field identified by DAPI (n = 5 mice per genotype). All graphical data are mean ± s.e.m., *P < 0.05, **P < 0.01, ***P < 0.001, Student’s t-test. Source data are provided as a Source Data file.
ELISA. Hemolytic plasma samples were excluded. Plasma levels of D-dimer, Thrombin-antithrombin (TAT) and fibrinogen, were respectively determined using a mouse D-dimer ELISA kit (Clone Corp), a mouse TAT ELISA kit (Abcam) and a mouse fibrinogen ELISA kit (Abcam) according to the manufacturer’s instructions. Samples were run in duplicate with n = 3–8 animals per group.

Thrombin generation assay. Thrombin generation in citrated mouse plasma was assessed by calibrated automated thrombography (CAT)55,56. Briefly, single mouse plasma preparations (40 µl per well) were used with 1 pM tissue factor (Dade Innovin, Dade Behring), 4 µM phospholipid vesicles and 16.6 mM CaCl2. Contact activation coagulation was inhibited by adding corn 2 trypsin inhibitor (65 µg per ml plasma) and thrombin generation quantified by adding 0.42 mM of Z-GlyArgAMC-HCl (Bachem). Samples were run in duplicate with n ≥ 4 animals per group.

Production of mouse red blood cells-targeted thrombomodulin. Fusion protein targeted to mouse glycophorin A was produced as described below39. Briefly, a stable Drosophila S2 cell lines expressing red blood cells-targeted thrombomodulin (RBC-TM) was maintained in Schneider’s medium (Thermo Fisher Scientific) and transitioned to Insect-Xpress (Lonza, Walkersville, MD) for protein production. Supernatants were harvested after induction with CuSO4. Protein also had a triple FLAG and was purified using anti-FLAG (M2) affinity resin (Sigma-Aldrich) and tested for purity via SDS-PAGE and HPLC (Waters, Huntington Valley, PA, USA) using a Yarra™ 3 µm SEC-2000 LC Column 300 x 4.6 mm size exclusion column with a flow rate of 0.35 ml per min (Phenomenex, Torrance, CA, USA). RBC-TM migrated on SDS-PAGE as a single approximately 85 kDa band.

Characterization of RBC-TM in vitro. Characterization of the antithrombotic properties of RBC-TM was assessed by performing APC generation assay in vitro. Free RBC-TM (4 or 40 µg per ml) was incubated with 5 nM bovine thrombin (Sigma-Aldrich, St Louis, MO) and 300 nM human protein C (Sigma-Aldrich, St Louis, MO) for 20 min at room temperature. Thrombin was quenched with 50 U per ml hirudin (Sigma-Aldrich, St Louis, MO), and APC was measured using APC substrate S-2366 (Diapharma, West Chester, OH, USA). All samples were run in quadruplicates. The rate of substrate hydrolysis was measured by monitoring the change in absorbance at 405 nm over time at room temperature using a Spectramax M2 Microplate reader ( Molecular Devices, CA, USA). Data presented as maximum signal reached at 20 min.

Acute treatment with RBC-TM in vivo. Endothelial deletion of ERG was induced in adult mice (6 weeks) by tamoxifen injection. Twenty-four days post-tamoxifen injection, mice were bled via the tail vein for analysis of hemostasis-related plasma biomarkers (TAT, D-dimer and fibrinogen). The following day (25 days post tamoxifen), mice were intravenously (tail vein) injected with RBC-TM (4 mg per kg) and sacrificed 6 hours after this acute treatment. Mice were bled from the retro-orbital plexus for plasma markers analysis and tissues were collected for histology.

Statistical analysis. All results presented in this study are representative of at least three independent experiments. ‘n’ represents the number of biological replicates unless otherwise stated. Data are shown as the mean ± standard error of the mean (s.e.m.). Statistical significance was determined by two-tailed Student’s t test or one-way ANOVA with Tukey post hoc test using Prism ver. 6.0 (GraphPad). Differences were considered statistically significant with a P value < 0.05.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Data supporting the findings of this work are available within the paper and its Supplementary Information file. The source data (including uncropped immunoblots) underlying Figs. 1f–i, 2a–f, 3a–e, 4a–g, 5b–f, 6a–f, 7a–e, 8b, d, f and Supplementary

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**Table 1 Complete list of antibodies used for this study**

| Antibody (host) | Company | Catalogue number | Application and dilution |
|-----------------|---------|------------------|--------------------------|
| CD31 (rabbit)  | Abcam   | ab28364          | IF (mouse tissue, 1/200) |
| CD41 [MWReg30] (rat) | Abcam | ab33661          | IF (mouse tissue, 1/200) |
| Isolectin B4 (Ib4) | Vector | FL-1201          | IF (mouse tissue, 1/200) |
| Wheat Germ Agglutinin (WGA) | Thermo Fisher Scientific | W1261 | IF (mouse tissue, 1/400) |
| ERG (mouse)     | Santa Cruz | sc-376293       | PLA (HUEVC, 1/200)/WB (HUEVC, 1/2000) |
| ERG (rabbit)    | Abcam   | ab133264         | IF (HUEVC, 1/200)/WB (HUEVC, 1/1000) |
| ERG (rabbit)    | Abcam   | ab92513          | IF (mouse tissue, 1/200)/WB (mouse tissue, 1/1000) |
| Fibrinogen (rabbit) | Abcam | ab34269          | IF (mouse tissue, 1/200) |
| GAPDH (mouse)   | Millipore | MBA374          | WB (HUEVC, mouse tissue, 1/10000) |
| H3K27Ac (rabbit) | Active Motif | 39133          | IF (HUEVC, 1/200) |
| KLF2 (rabbit)   | Abcam   | ab203591         | PLA (HUEVC, 1/100)/WB (HUEVC, 1/500) |
| Actin, α-smooth muscle-Cy3 (mouse) | Sigma | C6198           | IF (mouse tissue, 1/400) |
| TM (mouse)      | Dako    | Clone 1009       | IF (HUEVC, 1/100) |
| TM (mouse)      | Santa Cruz | sc-13164        | WB (HUEVC, 1/1000) |
| TM (goat)       | R&D     | AF3894           | IF (mouse tissue, 1/200)/WB (mouse tissue, 1/1000) |

List of antibodies used for immunofluorescence (IF), western blot (WB) and proximity ligation assay (PLA). Dilution of the antibodies used for each specific application is specified in the right column (in brackets).
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Author contributions
C. Peghaire conceived, designed and carried out experiments, analysed and conceptualised results, wrote the paper. N.P.D., M.L., I.I.S.-C. and J.A. designed and performed experiments, analysed and interpreted results, contributed to scientific discussion. V.K., C.R., C.P. performed experiments and analysed results, contributed to scientific discussion. I.I. performed experiments. R.K. and V.R.M. produced, validated and provided the mouse RBC-TM fusion protein. J.C.M. and G.M.B. contributed to scientific discussion. A.M.R. provided funding, conceived, designed and supervised the study, interpreted results and wrote the paper.

Competing interests
The authors declare no competing interests.

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