The Endothelial Transcription Factor ERG Promotes Vascular Stability and Growth through Wnt/β-Catenin Signaling

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SUMMARY

Blood vessel stability is essential for embryonic development; in the adult, many diseases are associated with loss of vascular integrity. The ETS transcription factor ERG drives expression of VE-cadherin and controls junctional integrity. We show that constitutive endothelial deletion of ERG (ErgEC-KO) in mice causes embryonic lethality with vascular defects. Inducible endothelial deletion of ERG (ErgEC-KO) results in defective physiological and pathological angiogenesis in the postnatal retina and tumors, with decreased vascular stability. ERG controls the Wnt/β-catenin pathway by promoting β-catenin stability, through signals mediated by VE-cadherin and the Wnt receptor Frizzled-4. Wnt signaling is decreased in ERG-deficient endothelial cells; activation of Wnt signaling with lithium chloride, which stabilizes β-catenin levels, corrects vascular defects in ErgEC-KO embryos. Finally, overexpression of ERG in vivo reduces permeability and increases stability of VEGF-induced blood vessels. These data demonstrate that ERG is an essential regulator of angiogenesis and vascular stability through Wnt signaling.

INTRODUCTION

Angiogenesis is essential during embryogenesis and is a critical component of many diseases. Coordination of growth and stability signals is required for effective angiogenesis (Jain, 2003). Diseases such as cancer, diabetic retinopathy, and vascular malformations are associated with vascular instability, which causes increased permeability and edema, excessive and/or dysfunctional angiogenesis, and hemorrhage. New strategies that target the maturation of blood vessels and restore vascular integrity could therefore have important therapeutic implications.

Multiple interactions at endothelial cell-cell junctions control vascular integrity. Crucial among these are the adhesion molecule vascular endothelial (VE)-cadherin and its intracellular partner β-catenin, an essential component of the canonical Wnt pathway (reviewed in Dejana, 2010). β-catenin is a multifunctional protein that can act as a scaffold between VE- and N-cadherins and the actin cytoskeleton, and as a coregulator for the T cell factor (TCF)/lymphoid enhancer-binding factor transcription factor complex. β-catenin levels are controlled by phosphorylation through a cytoplasmic degradation complex (reviewed in Reis and Liebner, 2013; Dejana, 2010). In the presence of Wnt ligands, which bind to a receptor complex containing members of the Frizzled (Fzd) family, the degradation complex is inactivated; β-catenin is stabilized and translocates to the nucleus to promote transcription. In the vasculature, the Wnt/β-catenin pathway controls vascular stability through remodeling, junction assembly, and pericyte recruitment (reviewed in Reis and Liebner, 2013; Dejana, 2010; Franco et al., 2009).

The ETS transcription factor family is implicated in vascular development and angiogenesis (reviewed in Randi et al., 2009). The ETS related gene (ERG), expressed throughout the life of the endothelium, regulates multiple pathways involved in vascular homeostasis and angiogenesis, such as monolayer integrity, endothelial permeability, and survival (Birdsey et al., 2008, 2012; Yuan et al., 2012). Previous studies have indicated a role for ERG in vascular development and angiogenesis (Baltzinger et al., 1999; Birdsey et al., 2008; Liu and Patient, 2008), Vijayaraj et al. reported that global deletion of a subset of endothelial ERG isoforms in mice results in defects in vascular and cardiac morphogenesis, causing embryonic lethality (Vijayaraj et al., 1999).
Figure 1. ERG Is Required for Vascular Development, Physiological Postnatal Angiogenesis, and Pathological Tumor Angiogenesis
(A) Representative whole mount images of E10.5 Erg\textsuperscript{flflo} and Erg\textsuperscript{EC-KO} embryo yolk sacs (magnification × 0.7). Bottom panel shows higher magnification of yolk sacs (magnification × 2).

(legend continued on next page)
et al., 2012). The mechanisms through which ERG controls blood vessel formation are still unclear, and its therapeutic potential is unexplored.

In this study, we use genetic lineage-specific mouse models and multiple in vitro models to show that ERG promotes vascular growth and stability, through control of the canonical Wnt/b-catenin pathway. Crucially, we demonstrate that overexpression of ERG in vivo enhances vascular endothelial growth factor (VEGF)-dependent angiogenesis and promotes stability of VEGF-induced new blood vessels.

RESULTS

Endothelial ERG Is Required for Vascular Development, Angiogenesis, and Tumor Growth

To investigate the role of endothelial ERG in vivo, we used a Cre/loxP strategy. The floxed allele of Erg was obtained by inserting two loxP sites flanking exon 6 (Figure S1A available online). Constitutive endothelial-specific deletion of Erg was achieved by breeding floxed Erg mice with mice expressing the Cre transgene under the control of the Tie2 promoter and enhancer (Kisanuki et al., 2001). Homozygous deletion of endothelial Erg (Tie2Cre-Ergfl/fl), henceforth referred to as ErgEC-KO, resulted in embryonic lethality between embryonic day (E)10.5 and E11.5, with no live offspring (Figure S1B). Analysis of the yolk sacs from ErgEC-KO embryos at E10.5 showed a significant reduction in perfused large vessels, consistent with defects in yolk sac vascular remodeling (Figures 1A, 5E, and S5F). Between E10.5 and E11.5, some mutant embryos appeared pale with no evidence of vessel blood flow, whereas others displayed hemorrhages and an enlarged pericardial cavity, suggesting defective heart function (Figures S1C and S1D). ErgEC-KO embryos were reduced in size compared to littermate controls (Figure 1B), with growth retardation clearly visible at E9.5 (Figure S1E). These results are in agreement with the phenotypes caused by global deletion of endothelial ERG isoforms (Vijayaraj et al., 2012). Endomucin staining of blood vessels in ErgEC-KO embryos at E10.5 revealed an immature disorganized vascular plexus, with significant disruption of the large vessels in the cranial vasculature (Figure 1B, panels a and b), altered development of the hyaloid vessels of the eye (Figure S1F), and irregular blind ending vessels in the head microvasculature (Figure S1G). Mutant embryos also exhibited disorganization of the intersomitic vessels in the trunk (Figure 1B, panel c). These data confirm that ERG is required for vascular development in mice.

To investigate the role of ERG in physiological and pathological postnatal angiogenesis, floxed Erg mice were bred with mice carrying tamoxifen-inducible Cre recombinase under the control of the Cdh5 promoter (Cdh5(PAC)-iCreERT2) (Wang et al., 2010). Following tamoxifen administration, efficient Cre-recombinase deletion of Erg was confirmed by PCR in Ergfl/+;Cdh5(PAC)-iCreERT2 mice (henceforth referred to as ErgEC-KO) (Figure S1H). The reduction in Erg protein levels was demonstrated by western blotting and immunofluorescence microscopy (Figures S1I and S1J). In the retinal vasculature of littermate controls (Ergfl/+), ERG was strongly expressed in endothelial cells (EC) from all regions of the vascular plexus, including tip and stalk cells, arteries, veins, and capillaries (Figure S1K), in line with previous studies (Korn et al., 2014). Deletion of endothelial Erg resulted in significant reduction in vascular coverage (Figure 1C) and density (Figure 1D) in the retinal plexus, and reduction in the number of vascular sprouts at the front (Figure 1E).

Next, we investigated whether ERG is involved in pathological angiogenesis, using the B16F0 melanoma tumor model, which depends on angiogenesis for growth (Reynolds et al., 2002). At day 14, tumor size and microvessel density were significantly reduced in adult ErgEC-KO mice compared to controls (Figures 1F–1H). These studies confirm that ERG is required for postnatal angiogenesis and show that endothelial ERG is involved in tumor angiogenesis and tumor growth.

ERG Controls Vascular Stability and Pericyte Coverage

Co-staining for isolectin B4 and the basement matrix component collagen IV showed a greater number of empty collagen IV sleeves in the capillary plexus (Figure 2A) and at the angiogenic front (Figure S2A) in retinas from ErgEC-KO mice compared to controls, indicating increased vessel regression. Pericyte recruitment, measured by staining with neuron-glial antigen 2 (NG2) and desmin, was significantly decreased along all vessels in the vascular plexus, including veins and arteries in ErgEC-KO mice (Figures 2B, S2B, and S2C). Similar signs of decreased vessel stability were observed in tumors grown in ErgEC-KO mice, with a marked increase in the number of empty collagen IV sleeves (Figure 2C) and a reduction in pericyte coverage of blood vessels (Figure 2D). These data suggest that ERG controls both physiological and pathological angiogenesis through pathways that promote vascular stability.

ERG Controls b-Catenin Stability and Signaling through VE-Cadherin- and Wnt-Dependent Mechanisms

In vitro studies have shown that ERG is essential to maintain the integrity of endothelial junctions, by driving expression of VE-cadherin (Gory et al., 1998; Birdsey et al., 2008). A marked reduction in VE-cadherin expression and junctional localization was also observed in the retinal vasculature of ErgEC-KO mice (Figure 3A), demonstrating that loss of endothelial ERG leads to a disruption of cell-cell junctions in vivo. Isolated primary mouse lung EC from heterozygous Tie2Cre-Ergfl/+ mice
Figure 2. ERG Controls Vascular Remodeling

(A) Collagen IV (green) and isolectin B4 (IB4, red) staining of Erg<sup>EC-KO</sup> and Erg<sup>fl/fl</sup> P6 retinal vessels. Arrows show empty collagen IV sleeves, quantification of number of vessels, (n = 4).

(B) NG2-positive pericytes (green) associated with isolectin B4 labeled retinal vessels (red) from Erg<sup>EC-KO</sup> and Erg<sup>fl/fl</sup> mice; quantification of pixel intensity, (n = 4).

(C) Sections from B16F0 tumors grown on adult Erg<sup>EC-KO</sup> and Erg<sup>fl/fl</sup> mice were stained for collagen IV (green) and endomucin (red); quantification of pixel intensity, (n = 3). Arrows show empty collagen IV sleeves.

(D) Tumor sections from Erg<sup>EC-KO</sup> and Erg<sup>fl/fl</sup> mice were stained for NG2 (green) and endomucin (red); quantification of pixel intensity, (n = 3). Arrows show NG2-negative, endomucin-positive vessels. Scale bars, 100 μm (A), scale bars, 50 μm (B–D). All graphical data are ± SEM, *p < 0.05, and ***p < 0.001. See also Figure S2.
Figure 3. Endothelial Canonical Wnt Signaling and β-Catenin Stability Are Regulated by ERG

(A) Staining for VE-cadherin (green), ERG (red), and isolectin B4 (IB4, blue) in ErgEC-KO and Ergfl/fl P6 retinas. Scale bar, 50 µm; zoom, 20 µm.

(B) Relative mRNA expression of Erg and VE-cadherin in primary ErgcEC-het mouse lung EC compared to control (n = 6).

(C, D, and E) (C) β-catenin (β-cat; green) and VE-cadherin (VEC; red) staining of FITC-conjugated siCtrl and siERG (FITC; purple) treated HUVEC (n = 3). Scale bar, 20 µm.

(D and E) Western blot and (E) qPCR analysis of β-catenin expression in control (siCtrl) and ERG-deficient (siERG) HUVEC (n = 4).

(F) TCF reporter activity (TOP) in control and ERG-deficient cells treated with control (Ctrl), Wnt3a, or Wnt5a conditioned medium (CM); (n = 3).
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debate a decrease in canonical Wnt signaling: Wnt3a stimulation of

The decrease in Cyclin D1, Axin-2, and TCF-1 (Shtutman et al., 1999; Roose et al., 1999; Jho et al., 2002) were decreased in ERG-deficient

fore speculated that ERG might regulate of the shared genes identified by GSEA was carried out using DAVID analysis (right). The functional categories shown displayed significant enrichment scores (p < 0.01). All graphical data are ± SEM, *p < 0.05, **p < 0.01, and ***p < 0.001. See also Figure S3.

HPAEC (green curve) (Alastalo et al., 2011) and the ranked list of genes downregulated by ERG inhibition in HUVEC (Birdsey et al., 2012). Functional classification (J) GSEA shows enrichment and significant correlation (normalized enrichment score, 2.46; p < 0.001) between genes downregulated in ERG-deficient human umbilical vein EC (HUVEC) identified several Wnt-related genes as candidate ERG targets (Birdsey et al., 2012) (Figure S3B). We therefore speculated that ERG might regulate β-catenin and Wnt signaling in EC.

Inhibition of ERG expression in HUVEC (Figures S3C and S3D) significantly reduced β-catenin junctional staining and protein expression (Figures 3C and 3D). ERG regulates β-catenin protein levels in confluent (Figure 3C) and subconfluent (Figure S3E) EC, suggesting that this pathway functions both in quiescent and angiogenic endothelium. However, β-catenin mRNA levels were unaffected by ERG inhibition in HUVEC (Figures 3E and S3F) or in primary mouse EC from Ergec-het mice (Figure 3H). The decrease in β-catenin protein expression correlated with a decrease in canonical Wnt signaling: Wnt3a stimulation of β-catenin transcriptional activity was lost in ERG-deficient EC (Figure 3F). Moreover, expression levels of the Wnt target genes Cyclin D1, Axin-2, and TCF-1 (Shutman et al., 1999; Roose et al., 1999; Jho et al., 2002) were decreased in ERG-deficient HUVEC (Figure 3G) and primary mouse EC from Ergec-het mice (Figure 3H). Endothelial β-catenin signaling regulates blood brain barrier maintenance through concomitant activation of the tight junction molecule Claudin-3 and repression of plasma-lemma vesicle-associated protein (PLVAP) (Liebner et al., 2008). In line with these findings, Claudin-3 expression was significantly downregulated, while PLVAP was strongly upregulated in Ergec-KO mouse brains (Figure 3G). Together, these results indicate that ERG regulates canonical Wnt/β-catenin signaling in both human and mouse EC.

To confirm the relationship between ERG and β-catenin pathways, we used gene set enrichment analysis (GSEA) to compare the data set from transcriptome profiling of ERG-deficient HUVEC (Birdsey et al., 2012) with the data set from transcriptome analysis of human pulmonary artery EC (HPAEC) following β-catenin inhibition (Alastalo et al., 2011). GSEA showed a significant positive correlation between the genes regulated by ERG and β-catenin (Figure 3J, left). Interestingly, gene ontology analysis of shared genes identified a significant number of regulators of angiogenesis, cell adhesion, migration, and apoptosis (Figure 3J, right). These results suggest a strong relationship between these two pathways.

Since ERG inhibition decreases β-catenin protein, but not mRNA levels, we tested whether ERG regulates β-catenin degradation. β-catenin protein expression was restored in ERG-deficient EC in the presence of the proteosomal degradation inhibitor MG132 (Figure 4A). We investigated whether ERG controls β-catenin stability through VE-cadherin. In ERG-deficient HUVEC, GFP-tagged VE-cadherin overexpression (Figure S4A) partially restored junctional β-catenin protein levels (Figures 4B and 4C, lane 6). However, cellular fractionation studies showed that ERG also controls the nuclear pool of β-catenin (Figure 4C, lane 3), which was not corrected by VE-cadherin overexpression (Figure 4C, lane 5). This suggests that ERG controls β-catenin also through a Wnt signaling-dependent, VE-cadherin-indepen-dent pathway. Activation of Wnt signaling by lithium chloride (LiCl) inhibits GSK3β, and thus degradation of cytoplasmic β-catenin, allowing its nuclear translocation (Stambolic et al., 1996). LiCl was able to partially normalize β-catenin nuclear levels in ERG-deficient EC (Figure 4C, lane 7). Finally, combined Wnt signaling activation (through LiCl) and VE-cadherin overexpression were able to rescue both nuclear and junctional β-catenin pools in ERG-deficient EC (Figure 4C, lanes 9 and 10). These results demonstrate that the balance between VE-cadherin-dependent and Wnt signaling-dependent pathways, which modulates canonical Wnt/β-catenin signals in EC, is controlled by the transcription factor ERG.

Expression of the Wnt Receptor Frizzled-4 Is Regulated by ERG

Wnt ligands bind to receptors of the Fzd family to inhibit the β-catenin degradation complex and activate Wnt signaling (Goodwin and D’Amore, 2002). LiCl treatment was able to partially stabilize β-catenin expression in ERG-deficient EC (Figure 4C, lanes 7 and 8). However, the upstream ligand Wnt3a was unable to do so (Figure S4B), suggesting a receptor-mediated defect upstream of the degradation complex. Wnt3a interacts with the Frizzled-4 (Fzd4) receptor (Reis and Liebner, 2013), which is highly expressed in cultured EC (Goodwin et al., 2006). Fzd4 was identified as a putative ERG target by transcriptome analysis in HUVEC (Birdsey et al., 2012). We confirmed that Fzd4 mRNA (Figure 4D) and protein levels (Figure 4E) were significantly decreased in ERG-deficient HUVEC. Consistently, Fzd4 expression was decreased in mouse EC isolated from Ergec-het mice (Figure S4C). Comparative genomic analysis of the Fzd4 promoter revealed the presence of three highly conserved ERG DNA binding motifs in the 800 base pair (bp) region upstream of the Fzd4 transcription start site (Figures 4F and S4D). Analysis of the Encyclopedia of DNA Elements (ENCODE) chromatin immunoprecipitation sequencing (ChIP-seq) data (Birney et al., 2007) for histone marks H3K4me1 and H3K27Ac and RNA polymerase II occupancy, markers of active promoters, show that the location of these marks correlates with the position of the ERG binding motifs (Figure 4F). ChIP-quantitative (q)PCR demonstrated that ERG interacts directly with the human Fzd4 promoter (Figure 4G); specificity of the interaction was confirmed in ERG-deficient EC (G) qPCR of downstream β-catenin target gene expression in control and ERG-deficient HUVEC: Cyclin D1, Axin-2, and TCF-1 (n = 4).

(H) mRNA expression of Erg, β-catenin, and its target genes Cyclin D1, Axin-2, and TCF-1 in primary Ergec-het mouse lung EC compared to control (n = 6).

(I) qPCR analysis of total brain mRNA from control and Ergec-KO mice for Erg, Claudin-3, and PLVAP.

(J) GSEA shows enrichment and significant correlation (normalized enrichment score, 2.46; p < 0.001) between genes downregulated in β-catenin siRNA-treated HPAEC (green curve) (Alastalo et al., 2011) and the ranked list of genes downregulated by ERG inhibition in HUVEC (Birdsey et al., 2012). Functional classification of the shared genes identified by GSEA was carried out using DAVID analysis (right). The functional categories shown displayed significant enrichment scores (p < 0.01). All graphical data are ± SEM, *p < 0.05, **p < 0.01, and ***p < 0.001. See also Figure S3.
ERG overexpression resulted in a 6-fold transactivation of a Fzd4 promoter luciferase construct in HUVEC (Figure 4H). Finally, Fzd4 overexpression in ERG-deficient EC was able to partially rescue Wnt3a activation of β-catenin transcriptional activity (Figure 4I). These data demonstrate that ERG controls transcription of the Fzd4 receptor in EC and point to a molecular mechanism for the VE-cadherin-independent control of Wnt signaling by ERG.

**ERG Controls Angiogenesis through Wnt Signaling**

Wnt/β-catenin signaling can promote EC proliferation (Masckauchan et al., 2005) and induce cell cycle progression through transcriptional activation of Cyclin D1 (Shtutman et al., 1999). Therefore, we tested whether ERG may also control EC proliferation through Wnt signaling. As shown in Figure 5A, inhibition of ERG expression by siRNA decreased HUVEC proliferation; LiCl, which prevents β-catenin degradation, rescued proliferation of ERG-deficient HUVEC. These results indicate that ERG controls endothelial proliferation through the Wnt/β-catenin pathway. We have previously shown that ERG deficiency causes increased EC apoptosis (Birdsey et al., 2008). Combination of VE-cadherin overexpression and LiCl treatment could completely prevent cell death in ERG-deficient cells, indicating that ERG controls EC survival through Wnt/β-catenin signaling (Figure S5A).

To test the functional relevance of Wnt signaling in ERG-dependent angiogenesis, we used an in vitro sprouting assay (Nakatsu et al., 2007). ERG-deficient HUVEC formed markedly decreased numbers of significantly shorter sprouts (Figures 5B, panel b, 5C, and 5D). However, pretreatment of ERG-deficient cells with LiCl to inhibit β-catenin degradation was able to partially restore normal sprouting behavior of HUVEC (Figure 5B, panel d), by rescuing the number (Figure 5C) and length (Figure 5D) of the sprouts. These results suggest that Wnt/β-catenin signaling is required for ERG to control sprout formation during angiogenesis.

To confirm that ERG controls angiogenesis and vascular development in a Wnt/β-catenin-dependent manner in vivo, we carried out a rescue experiment by pharmacological stabilization of Wnt/β-catenin signaling (Griffin et al., 2011). Light microscopy examination of the yolk sacs from NaCl (control) and LiCl treated mice revealed a dramatic increase in perfused vessels in the yolk sacs of Erg<sup>EC-KO</sup> mutants following LiCl treatment (Figure 5E). Endomucin staining revealed disrupted vessel morphology in the yolk sacs from NaCl-treated Erg<sup>EC-KO</sup> embryos, with reduced microvasculature branching and decreased diameter of the larger vitelline vessels (Figures 5E and 5F). LiCl treatment of Erg<sup>EC-KO</sup> mutants resulted in significant increase in vitelline vessel diameter and in the remodeling of the microvascular plexus (Figures 5E and 5F), in line with the increase in perfusion.

Wnt targets CyclinD1 and Axin2, previously shown to be decreased in ERG-deficient endothelium (see Figures 3G and 3H), were significantly decreased in NaCl-treated Erg<sup>EC-KO</sup> yolk sacs compared to controls (Figure S5B). LiCl-treatment of Erg<sup>EC-KO</sup> yolk sacs normalized Cyclin D1 and Axin2 levels to those observed in LiCl-treated control embryos (Figure 5G). Interestingly, ERG targets VE-cadherin and Fzd4 were not normalized, in line with the direct transcriptional role of ERG in their regulation.

These results demonstrate that endothelial ERG controls embryonic vascular development and angiogenesis through the Wnt/β-catenin signaling pathway.

**ERG Overexpression Stabilizes VEGF-Induced Blood Vessels and Promotes Angiogenesis In Vivo**

The data presented so far show that the transcription factor ERG controls angiogenesis through pathways mediating vascular stability and growth. The importance of the coordinated regulation of these pathways is highlighted by the variable and disappointing results of clinical trials for therapeutic angiogenesis in ischemic diseases, using the proangiogenic growth factor VEGF. VEGF has been shown to induce the formation of unstable and highly permeable vessels in vivo (Reginato et al., 2011), giving rise to local edema and inefficient tissue perfusion. Therefore, we investigated the ability of ERG to stabilize new vessels induced by VEGF in vivo. C57BL/6 mice received a subcutaneous injection of Matrigel supplemented with VEGF-A<sub>165</sub> and ERG (Ad.ERG) or LacZ (Ad.LacZ) adenovirus; basic fibroblast growth factor (bFGF), which can induce stable new vessels in this model (Bussolati et al., 2004), was used as control. Immuno-fluorescence staining for the adenovirus hexon coat protein showed localization of the adenovirus to endomucin-positive neovessels in the Matrigel plugs (Figure S6A). In addition, qPCR analysis confirmed significant expression of V5-tagged

Figure 4. ERG Controls β-Catenin Stability through VE-Cadherin- and Wnt-Dependent Mechanisms

(A) Western blot of β-catenin expression in control and ERG-deficient cells treated in presence or absence of MG132 (n = 4).
(B) ERG (magenta), VEC (red), β-catenin (green), and DAPI (blue) staining of control and ERG-deficient HUVEC transduced with GFP-tagged control (Ctrl-GFP) or VE-cadherin (VEC-GFP) adenovirus. Scale bar, 20 μm.
(C) Western blot (left) and quantification (right) of β-catenin expression in nuclear/cytoplasmic fractions of ERG-deficient HUVEC transduced with GFP or VEC-GFP adenovirus in presence or absence of LiCl. For normalization, tubulin was used as a cytoplasmic control and HDAC1 as a nuclear marker (n = 3).
(D) and (E) (D) qPCR and (E) western blot analysis of Fzd4 expression in control and ERG-deficient cells (n = 3).
(F) There are three putative ERG binding sites (black bars) located within the Fzd4 locus upstream of the transcription start site (arrow); sequence conservation between 100 vertebrates is shown across this region. ENCODE ChIP-seq data profiles for H3K4me1, H3K27Ac, and RNA polymerase II indicate open chromatin and active transcription. Location of qPCR amplicon covering region R1 is indicated.
(G) ChIP-qPCR using primers to region R1 on ERG-bound chromatin from HUVEC treated with siCTRL or siERG. Primers for a downstream region within the Fzd4 gene were used as a negative control. Data are shown as fold change over IgG (n = 3).
(H) Luciferase reporter assay, an ERG cDNA expression plasmid (pcDNA-ERG), or an empty expression plasmid (pcDNA) were cotransfected with a Fzd4 promoter-luciferase construct (pG34-Fzd4) in HUVEC and luciferase activity was measured. Values are represented as the fold change in relative luciferase activity over the empty pGL4 vector alone.
(i) TCF reporter (TOP) activity in control and ERG-deficient HUVEC treated with Wnt3a. Cells were transfected with control pCMV6 or pCMV6-Fzd4 plasmids and transduced with VEC-GFP adenovirus (n = 3). All graphical data are ± SEM, *p < 0.05, **p < 0.01, and ***p < 0.001. See also Figure S4.
ERG in Matrigel samples treated with Ad.ERG compared to Ad.Lacz control (Figure S6B). To evaluate the stability of the new vessels, vascular permeability was measured using two different sized dextran tracers. In the presence of bFGF (Figure 6A, top panel), the lower molecular weight tetramethylrhodamine (TRITC)-dextran was fully contained within the vascular structures and colocalized with the larger molecular weight Fluorescein isothiocyanate (FITC)-tracer, confirming that bFGF induces the formation of stable, nonleaky vessels. Plugs containing VEGF-A165 and Ad.Lacz revealed a less organized vascular network with the smaller molecular weight dextran dispersed both inside and outside of the vessels (Figure 6A, middle panel, and Movie S1A). Interestingly, overexpression of ERG in the presence of VEGF-A165 resulted in reduced diffusion of the smaller molecular weight dextran (Figure 6A, bottom panel, and Movie S1B), indicating a more stable, less permeable vasculature. Quantification of the net amount of extravasated TRITC-dextran tracer shows that Ad.ERG caused an approximate 4-fold reduction in tracer extravasation in VEGF-A165-induced new vessels compared to Ad.Lacz control (Figure 6B). Consistently, quantification of FITC-dextran area showed that ERG overexpression in the presence of VEGF-A165 resulted in an increase in perfused vessels within the Matrigel plug after 7 and 10 days, compared to control (Figures 6C and S6C). ERG overexpression also resulted in a significant increase in the number of new vessels within the Matrigel plug; however, this difference was observed only at the later time point (Figure 6D), suggesting that the increase in blood vessel number is secondary mainly to stabilization of VEGF-induced angiogenesis. These results confirm that ERG promotes stabilization of VEGF-induced angiogenesis in vivo.

Pericyte recruitment is a critical step in vascular stability and maturation, and lack of pericytes has been shown to cause increased permeability (Hillström et al., 2001). Since pericyte recruitment was decreased in two models of angiogenesis in the ErgΔEC-KO mice (see Figure 2), we investigated whether ERG overexpression could increase the recruitment of vascular pericytes in the in vivo Matrigel plug model. Indeed, pericyte recruitment was increased by desmin staining compared with the Ad.ERG-treated plugs compared to controls (Figures 6E and 6F). These results suggest that ERG may promote stabilization of angiogenesis also through control of pericyte recruitment.

**DISCUSSION**

Over the last decade, major progress has been made in understanding the molecular mechanisms that regulate angiogenesis. However, the pathways that control vessel stability are less well characterized. In this study, we identify a transcriptional program regulated by ERG that controls vascular stability and growth through the Wnt/β-catenin pathway, in both a physiological and pathological context.

We show that constitutive deletion of endothelial ERG in the mouse embryo causes embryonic lethality with severe vascular disruption. These observations are in line with a previous report where global deletion of a subset of endothelial ERG isoforms resulted in vascular defects and lethality between E10.5 and E11.5 (Vijayaraj et al., 2012). Instead of a strategy based on a posteriori knowledge of ERG isoform expression, the Cre/LoxP system allowed us to delete all endothelial isoforms of Erg, by targeting exon 6, which encodes a region of the protein present in all isoforms. A previous transgenic model, where ERG’s function was disrupted by a mutation in the DNA binding ETS domain (ErgMed2/Med2), caused embryonic lethality at a later stage (E13.5) (Loughran et al., 2008) and did not appear to display early vascular defects, suggesting that ERG’s functions in the vasculature are not exclusively mediated by its DNA binding activity.

Using the inducible endothelial specific Cdh5(PAC)-iCreERT2 line, we show that ERG is required for angiogenesis in the developing retina of newborn mice and for tumor blood vessel growth in adult mice. ERG deficiency results in vessel regression and reduced pericyte recruitment, demonstrating that ERG controls vascular stability. Interestingly, ERG overexpression in the in vivo Matrigel plug model resulted in increased pericyte recruitment to vessels. This suggests that ERG may promote stabilization of angiogenesis in part through control of pericyte recruitment. A recent paper has described a role for ETS factors (including ERG) in arterial specification and reported increased ERG expression in arterial-derived EC in vitro (Wythe et al., 2013). However, in the mouse retinal vasculature, ERG was strongly expressed in all EC with no detectable difference between arteries and veins.

In the in vivo developmental vascular defects in the ErgΔEC-KO embryos are reminiscent of those associated with deletion of endothelial β-catenin. Endothelial deletion of ERG causes embryonic lethality earlier than the E12.5 reported for endothelial deletion of β-catenin (Cattelino et al., 2003). This study proposed that EC might not require β-catenin for early vascular development, but rather for maintenance of vascular integrity and vascular patterning at later stages. ERG’s regulation of other genes involved in earlier stages of vascular development, such as VE-cadherin, may be partly accountable for this difference in phenotypes. Constitutive endothelial deletion of ERG also causes diffuse hemorrhages and defects in vascular remodeling, similar to those observed in the β-catenin deficient embryos. In both lines, vitelline vessels of the yolk sac are significantly smaller in diameter; however, unlike ErgΔEC-KO embryos, endothelial-specific loss of β-catenin does not affect vessel formation.
Figure 6. ERG Stabilizes Angiogenesis In Vivo

Matrigel containing bFGF or VEGF with adenovirus expressing either Lacz (Ad.Lacz) or ERG (Ad.ERG) was injected into C57BL6 mice. There were two labeled dextran molecules of different molecular weights, 2×10^6 MW (FITC, green) and 4.4×10^4 MW (TRITC, red), that were injected intravenously 15 min prior to harvesting plugs.

(legend continued on next page)
in the head, but causes hyperbranching of intersomitic vessels (Corada et al., 2010). Thus, embryonic mouse phenotypes of ERG versus β-catenin endothelial deletion show similarities, but not complete overlap, as expected, given the complex role of ERG as a transcriptional regulator of multiple vascular pathways. Crucially, the yolk sac vascular defects in the ErgCdc-KO and expression of Wnt targets were rescued by in vivo treatment with LiCl. Although we cannot completely rule out non-EC effects of LiCl, these experiments clearly demonstrate that ERG controls vascular development through Wnt signaling.

Interestingly, similar angiogenic defects are observed in the retinas from ErgCdc-KO and from the reported endothelial-specific β-catenin and Fzd4 knockout mice (Xu et al., 2004; Ye et al., 2009; Phng et al., 2009; Corada et al., 2010). Whether ERG is implicated in human ocular diseases, including Norrie disease and familial exudative vitreoretinopathy, which are associated with Fzd4 and its ligand Norrin (Xu et al., 2004), remains to be established. In line with our data, a link between Fzd4 and ERG has been previously observed in prostate cancer (Gupta et al., 2010). Interestingly, our results show that ERG deficiency results in about 50% reduction in Fzd4 protein, but completely abrogates Wnt luciferase reporter activity in response to Wnt3a. This suggests that ERG’s control of other nodes in this pathway, including repression of the Wnt inhibitor DACT1 (Zhang et al., 2006) and activation of the transcription factor TCF4 (Wang et al., 2002), may be important.

Canonical Wnt signaling promotes EC survival, junction stabilization, proliferation, and pericyte recruitment and is essential for vessel stability (Cattelino et al., 2003; Phng et al., 2009; reviewed in Franco et al., 2009; Dejana, 2010). In this study, we establish ERG as a regulator of canonical Wnt/β-catenin signaling, and therefore identify a connection between two key transcriptional regulators essential for EC function. We show that ERG controls cell survival, proliferation, angiogenesis, and vessel stability through β-catenin. Whether ERG controls pericyte recruitment via Wnt signaling remains to be elucidated; preliminary evidence suggests that ERG regulates expression of the junction molecule and β-catenin transcriptional target N-cadherin (data not shown), which plays a crucial role in pericyte attachment during vessel formation (Giampietro et al., 2012; Gerhardt et al., 2000). Our data show that ERG controls Wnt/β-catenin levels and signaling through VE-cadherin-dependent and independent pathways both in confluent, quiescent monolayers and in subconfluent, proliferating cells. The balance between VE-cadherin and Wnt-dependent signals controls β-catenin cellular localization and activity. It has been suggested that β-catenin function to increase cell plasticity and sensitivity to extracellular signals (Franco et al., 2009). Transcriptional activity of ERG itself has been shown to be modulated by extracellular signals (Wythe et al., 2013). Thus, we propose that in EC, ERG is required to maintain homeostatic levels of β-catenin protein, the output of which can be modulated according to the growth and survival signals it encounters, providing the balance between proliferation and stability required in a nascent blood vessel.

Dysregulation of the Wnt/β-catenin signaling pathway is frequently observed in many types of cancer. Constitutive Wnt signaling activation caused by mutations in β-catenin or genes that control β-catenin stability has been associated with aberrant cell proliferation and subsequent cancer progression (reviewed in Giles et al., 2003). Wnt signaling has been shown to be a critical mediator of ERG-induced oncogenesis in several types of cancer, where aberrant ERG overexpression is a marker of aggressive malignancy and associated with increased proliferation (Gupta et al., 2010; Wu et al., 2013; Li et al., 2011; Mochmann et al., 2011). This is in contrast with its role in healthy endothelium, where ERG promotes homeostasis and stability. The reasons for this discrepancy are unknown and may be linked to the lack of balance between growth and survival signals, due to disrupted cell-cell signaling in malignant cells, thus driving the cells to a proliferative fate. Thus, strategies to control ERG’s activity in malignant cells through cell-cell adhesion signals might be worthy of investigation.

Finally, in this study, we explore the potential for ERG in promoting vascular stability during VEGF-induced angiogenesis. Numerous studies have shown that the new vasculature induced by VEGF in vivo, to promote revascularization in ischemic diseases (Zachary and Morgan, 2011), can be dysfunctional due to vascular instability and increased permeability (Reginato et al., 2011). Here, we show that overexpression of ERG can reduce permeability and promote VEGF-induced angiogenesis in vivo. Combined with the homeostatic and anti-inflammatory role of ERG (Sperone et al., 2011; Yuan et al., 2009), these results establish the ERG pathway as a potential target to promote vascular quiescence and stability.

EXPERIMENTAL PROCEDURES

Detailed methods are available in the Supplemental Experimental Procedures.

Mice and Breeding

Generation of Erg foxed mice was carried out by genOway. LoxP sequences were inserted around exon 6. Deletion of this exon leads to a frameshift mutation resulting in a premature stop codon in exon 7. Erglox mice were crossed with the following Cre transgenic deleter lines: Cdh5(PAC)-CreERT2 (Wang et al., 2010) and Tie2-Cre (Kisanuki et al., 2001). All experiments were conducted in accordance with the Animals (Scientific Procedures) Act of 1986.

Postnatal Retinal Angiogenesis

Mice were administered Tamoxifen (50 μg per mouse; Sigma) by intraperitoneal injection (IP) at postnatal (P) day 1, P2 and P3. Retinas were collected at P6 and processed as described (Pitulescu et al., 2010).

(A) 3D rendering of confocal microscopy images of whole-mount Matrigel plugs perfused with the dextran tracers. Cross sectioning through neovessels (right) shows localization of the tracers.

(B) Vessel permeability was quantified by measuring the amount of dextran-TRITC present outside of the dextran-FITC positive vessels, arbitrary units (n = 3).

(C) Perfused vessels were quantified by measuring the area of dextran-FITC within the Matrigel plug after 3, 7, and 10 days (n = 4).

(D) Vessel density was quantified by measuring the area of isolectin B4 within the Matrigel plug after 3, 7, and 10 days (n = 4).

(E) Endomucin (red), desmin-positive pericytes (green), and Draq5 (blue) staining of cryosections from Matrigel plugs implanted for 7 days; scale bar, 20 μm.

(F) Quantification of pericyte coverage, pixel intensity (n = 8). All graphical data are ± SEM, *p < 0.05, and **p < 0.01. See also Figure S6.
In Vivo Matrigel Angiogenesis Assay
C57BL/6 mice received a subcutaneous injection of Matrigel (BD Biosciences), as described (Birdsey et al., 2008). Matrigel was supplemented with 80 nanogram (ng)/ml bFGF (R&D Systems), or with 100 ng/ml murine VEGF-A 165 (Pe-protech) containing 10² plaque-forming unit adenovirus expressing either Lacz or ERG. After 3, 7, or 10 days, 100 µl of a 1:1 mixture of 10 mg/ml Dextran:FITC (2 x 10⁴ MW) and Dextran:TRITC (4.4 x 10⁴ MW) was injected intravenously 15 min prior to harvesting plugs. Images were acquired whole-mount using confocal microscopy. Velocity software (Perkin Elmer) was used to reconstruct 3D images of the vessels from serial Z-sections. The extent of TRITC-dextran tracer extravasation was quantified by subtracting the signal corresponding to the FITC-dextran tracer (intravascular) from the signal corresponding to the TRITC-dextran tracer (intravascular + extravascular).

Isolation of Mouse Lung Endothelial Cells
Primary mouse lung EC were isolated from control Erg⁰/² and ErgcEC-het mice as described (Reynolds et al., 2002). Rat APC-CD31, anti-ICAM-2, and anti-rat PE antibodies (all BD Biosciences) were used to assess the EC purity by flow cytometric analysis using a Cya flow cytometer (Beckman Coulman).

ChIP-qPCR
ChIP was performed using ChIP-IT express (Active Motif) as previously described (Birdsey et al., 2012).

Plasmid Transfections and Reporter Assays
For Fzd4 transactivation assays, a 1,010-bp region of the Fzd4 promoter (SwitchGear, Active Motif) was cloned into the pGL4 Luciferase Reporter Vector (Promega). TCF reporter constructs TOPFLASH and FOPFLASH were used to measure the transcriptional activity of β-catenin/TCF (Korinek et al., 1997). In some experiments, cells were cotransfected with a pCMV6-Fzd4 expression construct (Origene). Reporter assays were performed using the Dual-Luciferase Reporter Assay System (Promega).

BrdU In Vitro Proliferation Assay
Cell proliferation was determined in vitro using a BrdU proliferation ELISA kit (Roche) according to the manufacturer’s instructions.

Fibrin Gel Bead Assay
The 3D in vitro model of angiogenesis was performed as described previously ( Nakatsu et al., 2007 ).

Statistical Analysis
Values are presented as means ± SEM. Statistical significance was determined by using unpaired two-tailed Student’s t test. Differences were considered significant with a p value < 0.05.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, six figures, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.11.016.

AUTHOR CONTRIBUTIONS
G.M.B. designed, carried out, and supervised in vivo and in vitro experiments, analyzed and interpreted results, and wrote the manuscript. A.V.S. designed and carried out in vitro experiments, analyzed, interpreted, and conceptualized results, and wrote the manuscript. N.D. designed and performed in vivo experiments, analyzed, and interpreted results. B.G. provided advice on bioinformatic analysis and interpretation and contributed to scientific discussion; Y.Y. performed bioinformatic analysis and interpretation; and L.R. and K.H.D. performed and supervised the tumor angiogenesis experiments, analyzed results, and contributed to scientific discussion. L.O.A. and S.T.K. performed experiments and analyzed results. I.M.A. provided advice on retina isolation and optimized ERG retinal staining; E.D. provided reagents and contributed to scientific discussion; J.C.M. contributed to scientific discussion; H.G. provided advice and contributed to scientific discussion; R.H.A. assisted in the design of the transgenic mice, provided reagents, advice, and contributed to scientific discussion; and A.M.R. provided funding, conceived, designed, and supervised the study, interpreted results, and wrote the manuscript.

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The Endothelial Transcription Factor ERG Promotes Vascular Stability and Growth through Wnt/β-Catenin Signaling

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Figure S1, Related to Figure 1

Generation of Erg floxed mouse; characterization of vascular phenotypes in Tie2Cre-EndoEC-KO (ErgEC-KO) and ErgEC-KO/Cdh5(PAC)-iCreERT2 (ErgiEC-KO) mice

(A) Schematic structure of the murine floxed Erg gene. The two translation start sites within the alternatively spliced exons 3 and 4 are shown (ATG). The location of the loxP sites flanking exon 6 are indicated (black arrowheads). The predicted ERG protein domains are indicated, showing the approximate location of the pointed domain (PNT), alternative domain (AD) and ETS DNA-binding domain (ETS). (B) Genotype frequencies of live births from Erg floxed mice, obtained from 8 breeding females. (C) Whole mount images of ErgEC-KO embryos. Arrows indicate perfused vessels in the head. Scale bar, 1 mm. (D) Morphology of ErgEC-KO embryos (left) compared to ErgEC-KO (middle and right) embryos, which display hemorrhaging in the head (arrows), and fluid accumulation around the pericardial cavity (arrowhead). Scale bar, 1 mm. (E) Confocal microscopy showing endomucin-staining of blood vessels in control ErgEC-KO embryos at gestation ages E9.5, E10.5 and E11.5. Scale bar, 500 µm. (F) Confocal microscopy of E10.5 ErgEC-KO embryos labelled with antibodies to endomucin (green) and ERG (red) in blood vessels surrounding the developing eye. Scale bar, 50 µm. (G) Capillary vessel detail from head regions of ErgEC-KO embryos shows the presence of multiple blunt-ended capillaries (arrowheads). Scale bar, 50 µm. (H) PCR amplification was carried out on genomic DNA isolated from tamoxifen-treated ErgiEC-KO and ErgEC-KO mice to detect the presence of the loxP sequences around Erg exon 6 (Flox PCR), the Cre transgene (Cre PCR), or the intronic sequences that span Erg exon 6 (Cre excision PCR). Lane 1, ErgiEC-het; lane 2, ErgiEC-KO; lane 3, ErgiEC+/+; lane 4, ErgEC-KO. (I) Representative western blot analysis (top) and relative ERG expression (bottom) from heart protein lysates. (J) Whole mount retinas isolated at postnatal (P) day 6 from tamoxifen-treated mice were labelled for ERG (green) and isolectin B4 (IB4, red). Arteries (A) and veins (V) are indicated. (K) Whole mount retinas from P6 control mice (ErgEC-KO) were stained with antibodies to ERG (green) and the vasculature was labelled with isolectin B4 (red); arrows indicate tip cells containing ERG-positive nuclei.
Figure S2, Related to Figure 2

Collagen IV, NG2 and desmin staining in the retinal vasculature of Erg\textsuperscript{fl/fl} and Erg\textsuperscript{EC-KO} mice

(A) Staining for collagen IV (green) and isolectin B4 (IB4, red) at the angiogenic front of P6 retinas from Erg\textsuperscript{EC-KO} and Erg\textsuperscript{fl/fl} mice. Arrows show empty collagen IV sleeves. (B) NG2-positive pericytes (green) associated with isolectin B4 labeled arteries (red) in P6 retinas, n=4. Graphical data are ± SEM, *P < 0.05. (C) Desmin-positive pericytes (green) and isolectin B4 (red) staining of retinal vasculature from P6 Erg\textsuperscript{EC-KO} and Erg\textsuperscript{fl/fl} mice. Scale bars, 50 µm.
Figure S3

A. Isotype control (3%) unselected (37%) ICAM-2 selection (76%)

B. Relative gene expression fold change

C. siCtrl

D. siERG

E. siCtrl

F. siERG

Relative ERG gene expression fold change

Relative ERG protein expression fold change

Mean fluorescence intensity

Total ERG

Total β-catenin

Nuclear β-catenin

Cytoplasmic β-catenin
Figure S3, Related to Figure 3
Characterisation of primary mouse lung endothelial cells; validation of siCtrl and siERG treatment in HUVEC

(A) Flow cytometric analysis for the endothelial marker CD31 in freshly isolated lung cells (unselected) and ICAM-2 positively selected lung cells isolated from Erg^fl/fl^ mice (n=4). (B) Microarray analysis of differential gene expression in HUVEC was performed at 24 and 48 hours after Erg inhibition (Birdsey et al., 2012), with fold change of selected genes represented as high (red) and low (green) expression compared to the median (grey). qPCR analysis of mRNA levels of DACT1 and TCF-4 in 48 hour siCtrl and siERG-treated HUVEC, normalized to GAPDH and expressed relative to siCtrl (n = 4). (C) Immunofluorescence microscopy of FITC-conjugated siCtrl (green) and siERG-treated HUVEC. Cells were co-stained for ERG (red), scale bar, 20 µm. (D) Quantification of western blot analysis of ERG expression in siCtrl and siERG HUVEC (n=4). (E) ERG (magenta), VE-cadherin (VEC; red), β-catenin (β-cat; green) and DAPI (blue) staining of sparse control and ERG-deficient HUVEC. Scale bar, 20 µm. Volocity® software quantification of mean ERG and β-catenin fluorescence intensity. Quantification of mean β-catenin fluorescence intensity in the cytoplasm required the exclusion of nuclear areas (objects that touch DAPI), whereas quantification of mean β-catenin intensity in the nucleus required the exclusion of cytoplasmic areas (objects that do not touch or overlap with DAPI) (n=8). (F) ERG mRNA levels from siCtrl and siERG-treated HUVEC were quantified using qPCR, normalized to GAPDH (n=3). All graphical data are ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001.
Figure S4

A) + GFP + VEC-GFP

siCtrl

siERG

B) Relative β-catenin protein expression fold change

Wnt3a

β-catenin

siCtrl siERG

**

ERG

GAPDH

C) Relative Fzd4 gene expression fold change

Erg fl/fl

Erg cEC-het

0.0

0.2

0.4

0.6

0.8

1.0

*

D) ERG A

human TCCCCTCCGGCCGCTCCTACCCCTCAAAAACCTTTATTATGAAACCACCTGTCGCCAGAGGA

mouse TTCCCCCGGGGCTTCCCTCAAAAACCTTTATTATGAAACCACCTGTCGCCAGAGGA

**

ERG B

human GCCGCGGCGCGCAAGTTGACGAGGCCCTCCTCCGACCGGACGGGAGTCTCCGCTCGGGCTGGTGAAG

mouse GAATTTCATTTCATACGGTGAGGAAATCAGAGCGCGAGACCGTCGGCGGGTCTGCAGCTC

ERG C

human TGGCTGCTGGGCCTCCTCCGACCGGACGGGAGTCTCCGCTCGGGCTGGTGAAG

mouse TGGCTGCTGGGCCTCCTCCGACCGGACGGGAGTCTCCGCTCGGGCTGGTGAAG

**

ERG D

human GCCGCGGCGCGCAAGTTGACGAGGCCCTCCTCCGACCGGACGGGAGTCTCCGCTCGGGCTGGTGAAG

mouse GAATTTCATTTCATACGGTGAGGAAATCAGAGCGCGAGACCGTCGGCGGGTCTGCAGCTC

ERG E

human GCCGCGGCGCGCAAGTTGACGAGGCCCTCCTCCGACCGGACGGGAGTCTCCGCTCGGGCTGGTGAAG

mouse GAATTTCATTTCATACGGTGAGGAAATCAGAGCGCGAGACCGTCGGCGGGTCTGCAGCTC

ERG F

human GCCGCGGCGCGCAAGTTGACGAGGCCCTCCTCCGACCGGACGGGAGTCTCCGCTCGGGCTGGTGAAG

mouse GAATTTCATTTCATACGGTGAGGAAATCAGAGCGCGAGACCGTCGGCGGGTCTGCAGCTC

*
**Figure S4, Related to Figure 4**

ERG regulation of β-catenin signaling in endothelial cells through VE-cadherin and Frizzled-4

(A) GFP autofluorescence was visualized using confocal microscopy of control and ERG-deficient HUVEC transduced with GFP-tagged control or VE-cadherin adenovirus (VEC-GFP). Scale bar, 20 µm (n=3). (B) Western blot analysis of β-catenin expression in extracts of control and ERG-deficient cells treated with β-catenin stabilizer Wnt ligand 3a (Wnt3a) (n=3). (C) Fzd4 mRNA expression in primary ErgEC-het mouse lung EC compared to control (n=6). (D) Sequence comparison of genomic region upstream of the Fzd4 transcription start site in human and mouse. ERG consensus sequences, (A/C)GGAA(G/A) or AGGA(A/T)(G/A), are shown (conserved: green boxes, ERG A-C; non-conserved: empty boxes, ERG D-F). Asterisks denote conserved nucleotides across both species. Nucleotide numbers relative to the Fzd4 transcription start site. All graphical data are ± SEM, *P < 0.05, **P < 0.01.
Figure S5

ERG regulates cell survival through Wnt signaling; decreased ERG target expression in the yolk sacs from Erg<sup>cEC-KO</sup> embryos

(A) Analysis of cell apoptosis by measuring caspase -3 or -7 activity in control or ERG-deficient cells. Cells were treated with control GFP or VE-cadherin-GFP (VEC) adenovirus in the presence and absence of LiCl (n=3). (B) qPCR analysis of NaCl-treated Erg<sup>fl/fl</sup> and Erg<sup>cEC-KO</sup> embryo yolk sacs. Data are expressed as fold change versus NaCl-treated Erg<sup>fl/fl</sup> and are ± SEM from at least three mice per group. All graphical data are ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001.
Figure S6

A

|                | Endomucin | Adeno Protein | Merged | % of adeno-positive vessels |
|----------------|-----------|---------------|--------|-----------------------------|
| bFGF           | ![Image](bFGF.png) | ![Image](bFGF.png) | ![Image](bFGF.png) | 3 %                         |
| VEGF + Ad.Lacz | ![Image](VEGF.png) | ![Image](Ad.Lacz.png) | ![Image](Merged.png) | 55 %                        |
| VEGF + Ad.ERG  | ![Image](VEGF.png) | ![Image](Ad.ERG.png) | ![Image](Merged.png) | 67 %                        |

B

![Graph](graph.png)

C

|                | IB4       | FITC Dextran | Merged |
|----------------|-----------|--------------|--------|
| ![Image](IB4.png) | ![Image](FITC.png) | ![Image](Merged.png) |        |

Day 3  Day 7  Day 10

Relative ERG-V5 mRNA expression

Ad.Lacz  Ad.ERG
Figure S6, Related to Figure 6

Validation of Ad.Lacz or Ad.ERG transduction of endothelial cells in Matrigel plugs in vivo
Matrigel mixture containing basic FGF, or VEGF combined with adenovirus expressing either Lacz (Ad.Lacz) or ERG (Ad.ERG), was injected subcutaneously into C57BL6 mice. (A) Endomucin (green), adenovirus hexon protein (red) and Draq5 (blue) staining of Matrigel cryosections. Quantification of percentage of endomucin and hexon protein-positive vessels. (B) Representative V5-ERG mRNA expression in sections of Matrigel plugs harvested after 3, 7 and 10 days, levels expressed relative to an Ad.LacZ sample at day 3. (C) Isolectin B4 (IB4, red) and FITC dextran (green) staining of cryosections from Matrigel plugs containing basic FGF.

Supplemental Videos, Related to Figure 6
3-dimensional reconstruction of neovessels inside Matrigel plugs supplemented with VEGF and adenovirus expressing either Lacz (Video S1A) or ERG (Video S1B). Perfused vessels are labeled with FITC-dextran (green) and vessel leakage is visualized with TRITC-dextran (red).
Supplemental Experimental Procedures

Mice and breeding
The targeting vector to insert loxP sequences around Erg exon 6 was constructed from a proprietary 129Sv/Pas BAC library (genOway) and PCR amplification from 129Sv/Pas ES cell genomic DNA. The linearized targeting construct was transfected by electroporation into 129Sv ES cells. Following positive selection, 334 G418-resistant ES cell clones were screened for homologous recombination of the targeting vector using both PCR and Southern blotting. Two clones were identified as being correctly targeted and these were injected into recipient blastocysts from C57BL/6J female mice. High chimeric male mice were generated and bred with C57BL/6J Flp recombinase-expressing females to remove the neomycin selection cassette and to generate heterozygous mice carrying the Erg floxed allele (Erg^fl/+).

Immunofluorescence analysis of mouse tissue
Embryos and yolk sacs were fixed in 4 % PFA overnight at 4 °C and then transferred to 1 % BSA, 0.5 % Triton X-100 overnight at 4 °C. Sections were incubated overnight at 4 °C with primary antibodies to rat anti-endomucin (1:100, clone V7C7, Santa Cruz) and in some cases with rabbit anti-ERG (1:200, sc-353, Santa Cruz) diluted in blocking buffer (1% BSA, 0.5 % Tween-20). Washes were carried out with PBST three times for 20 min each and then sections were incubated overnight at 4 °C with secondary antibodies to anti-rat IgG Alexa Fluor (AF) 488 and anti-rabbit IgG AF 546 (Invitrogen) diluted in blocking buffer. Following washes in PBST, embryos and yolk sacs were mounted in Fluoromount G (Southern Biotech). Confocal microscopy was carried out on a Carl Zeiss LSM510 META.

Retinas were incubated in primary antibodies to rabbit anti-ERG (1:200, Santa Cruz), and/or rabbit anti-NG2 (1:200, Millipore), mouse anti-desmin (1:100, DAKO), goat anti-Collagen IV (1:20, Millipore), rat anti-VE-cadherin (BV13, 1:50, eBioscience), biotinylated isolectin B4 (1:250, Vector Laboratories, Peterborough, UK). Secondary antibodies were anti-rabbit IgG AF 546, and/or anti-rat IgG AF 488, anti-rabbit IgG AF 488, anti-goat IgG AF 546, anti-mouse IgG AF 555, streptavidin-AF 633 (all at 1:500, Invitrogen). Confocal microscopy was carried out on a Carl Zeiss LSM510 META. Images were analyzed with ImageJ (NIH) and Volocity (PerkinElmer). For quantification of retinal vascular progression, the distance of vessel growth from the optic nerve to the periphery was measured. For vessel density, the number of vessel branch points per field was counted. For vessel regression analysis, the ratio of the numbers of isolectin B4 or endomucin-positive blood vessels to collagen IV-positive sleeves were calculated.

Matrigel cryosections were processed for immunostaining using primary antibodies to biotinylated anti-FITC (eBioscience), rabbit anti-TRITC (Abcam), mouse anti-hexon (Clontech), FITC-isolectin B4 (Vector Laboratories), rat anti-endomucin (Santa Cruz) or mouse anti-desmin (DAKO). Secondary antibodies were streptavidin AF 555, anti-rat IgG AF 488, and anti-rabbit IgG AF 488 or anti-mouse
IgG AF 555 (all Invitrogen). Images were captured using a Carl Zeiss LSM510 META or LSM 780 confocal microscope and staining intensity was quantified using Volocity® software.

**Syngeneic tumor experiments**
Three month old control Erg^{fl/fl} and Erg^{IEC-KO} mice were injected daily with tamoxifen IP (500 μg) over 5 days prior to tumor cell injection. Mouse melanoma B16F0 (1x10^6) cells were injected subcutaneously into the flank of mice. After allowing the tumors to grow for 14 days, animals were killed and the tumors were excised. The tumor volume was measured using a digital calliper. Tumors were bisected and either fixed in 10 % formalin or snap-frozen in OCT solution for subsequent immunohistochemical analysis. Tumor cryosections were processed for immunostaining using primary antibodies to rat anti-endomucin (clone V7C7, Santa Cruz) and rabbit anti-NG2 (Millipore) or goat anti-collagen IV (Millipore). Secondary antibodies were anti-rat IgG AF 488 and anti-rabbit IgG AF 546 or anti-goat IgG AF 546. Blood vessel density was determined by counting endomucin-positive structures across the whole tumor section and expressed as numbers of blood vessels/mm^2.

**Isolation of mouse lung endothelial cells**
Primary mouse lung endothelial cells were isolated from the lungs of control Erg^{fl/fl} and Erg^{IEC-het} mice. Lungs were minced using GentleMACS C tubes and GentleMACS Dissociator (Miltenyi Biotec, UK), digested with 0.1% collagenase type I (Invitrogen, UK), and sieved through a 70 μm-pore cell strainer (BD Falcon, USA). Endothelial cells were selected by magnetic immunosorting (sheep anti-rat Dynabeads; Invitrogen, UK) with a negative sort for FcγRII/III receptor–positive macrophages and a positive sort for ICAM-2–positive endothelial cells. Cells were cultured in EGM-2 media (Lonza), in flasks precoated with a mixture of 0.1% gelatin (Sigma), PureCol (Invitrogen) and human plasma fibronectin (Chemicon).

**Cell treatments**
HUVEC were seeded onto 1 % gelatin-coated plates and grown in EGM-2 medium (Lonza). The following day, GeneBloc antisense oligonucleotides (100 nM) or siRNA (30 nM) were transfected into HUVEC using AtuFect01 lipid (1 μg/ml, Silence Therapeutics) in EGM-2 media for 24 h or 48 h. In some experiments, HUVEC were treated overnight with lithium chloride (10 mM, Sigma) or for 6 h with MG-132 (10 μM, Calbiochem). For Wnt3a stimulation, HUVEC were transfected with siRNA for 24 h and treated for 6 h with 200 ng/ml rWnt3a (R&D).

**Adenoviral transduction of HUVEC**
HUVEC (5 × 10^4 cells) grown on gelatin-coated 13-mm diameter glass coverslips were transduced with adenovirus (VE-cadherin [VEC]–GFP and GFP; kindly provided by F. W. Luscinskas, Harvard Medical School, Boston, MA) as described previously (Shaw et al., 2001). After 48 h, cells were transfected with ERG or control antisense. Forty-eight hours later, cells were fixed and stained for immunofluorescence or lysates were used for immunoblotting.
**Immunofluorescence analysis of HUVEC**

HUVEC were cultured on gelatin-coated 13-mm diameter glass coverslips and treated with either ERG or control antisense for 48 h. In some instances, FITC-conjugated GeneBlocs were used. For analysis of sparse cells, HUVEC were treated with either siCtrl or siERG for 6 h and then reseeded at 5 x 10^3 cells per well onto gelatin-coated 13-mm diameter glass coverslips in a 24 well plate. Immunofluorescence labelling was carried out using the following primary antibodies: rabbit anti-ERG (Santa Cruz Biotechnology), goat anti-VE-cadherin (Santa Cruz Biotechnology) and mouse anti-active-β-catenin (Upstate-Millipore). Secondary antibodies were anti-mouse AF 488, anti-rabbit AF 555, anti-rabbit AF 488, anti-rabbit AF 546, anti-goat AF 546, streptavidin AF 633 (all from Invitrogen); biotinylated anti-mouse IgG (Vector Laboratories). Nuclei were visualized using either TOPRO-3 (Invitrogen), DAPI (Invitrogen) or DRAQ5 (BioStatus, Shepshed, UK). Images were captured using a Carl Zeiss LSM510 META or LSM 780 confocal microscope. Volocity® software was used to quantify mean ERG and β-catenin fluorescence intensity. Quantification of mean β-catenin fluorescence intensity in the cytoplasm required the exclusion of nuclear areas (objects that do touch DAPI), whereas quantification of mean β-catenin intensity in the nucleus required the exclusion of cytoplasmic areas (objects that do not touch or overlap with DAPI).

**Plasmid transfections and reporter assays**

To measure transcriptional activity of β-catenin/TCF, cells were transfected with siRNA and after 24 h transfected with TOPFLASH or FOPFLASH reporter plasmids (Korinek et al., 1997), along with Renilla luciferase plasmid, using GeneJuice transfection reagent (Merck Chemicals), according to the manufacturer’s conditions. TOPFLASH experiments were carried out on cells treated overnight with control, Wnt5a or Wnt3a conditioned media (CM) derived from L cells and diluted 1:1 with endothelial cell growth medium (Liebner et al., 2008). In some experiments, cells were co-transfected with a pCMV6-FZD4 expression construct (OriGene Technologies, Rockville, MD). For Frizzled-4 transactivation, a Fzd4 promoter sequence (pLightSwitchProm-Fzd4; SwitchGear, Active Motif) was subcloned into a pGL4 luciferase reporter (Promega) and co-transfected into HUVEC with Renilla luciferase and either an ERG-2 cDNA expression plasmid (pcDNA-ERG) or an empty plasmid (pcDNA). Luciferase reporter activity was normalized to the internal Renilla luciferase control and is expressed relative to control treatment using the Dual-Luciferase Reporter Assay System (Promega) and a Synergy HT microplate reader.

**Fibrin gel bead assay**

HUVEC treated with control or ERG siRNA in the presence or absence of LiCl (10 mM), were mixed with Cytodex 3 microcarrier beads (GE Healthcare) at a concentration of 4 x 10^3 cells per bead in 1.5 ml of EGM-2 medium for 4 h. The beads coated with cells were then embedded in a fibrin clot and incubated with EGM-2 complete medium in the presence of human skin fibroblast cells. Medium and treatment were renewed every other day. For quantification of in vitro sprouting, images of beads were captured on an IX70 Olympus microscope with a 10X objective. Images were then analyzed using ImageJ; the number of sprouts per bead was determined and sprout length was measured in arbitrary units.
BrdU in vitro proliferation assay
HUVEC were transfected with control or ERG siRNA for 8 hr in a 6-well plate and then plated in a 96-well plate at a density of $5 \times 10^3$ cells per well in M199 with 10% FBS. Cells were treated overnight with LiCl (10 mM, Sigma). Cell proliferation was determined in vitro using a BrdU proliferation ELISA kit (Roche) according to the manufacturer’s instructions.

Apoptosis assay
Apoptosis was quantified by measuring caspase 3 and 7 activation, using the Caspase-Glo 3/7 Assay (Promega, Southampton, United Kingdom) on a Bio-Tek Synergy HT multidetection microplate reader.

Western blotting
Whole cell protein lysates were prepared from HUVEC using Cellytic reagent (Sigma). In some experiments, subcellular fractionation of cells into cytoplasmic and nuclear extracts was performed using the Nuclear Extract kit (Active Motif) according to the manufacturer’s instructions. Immunoblotting of cell lysates was performed according to standard conditions. Immunoblots were labelled with the following primary antibodies: anti-active β-catenin (Upstate-Millipore), anti-ERG (Santa Cruz Biotechnology), anti-Fzd4 (Santa Cruz Biotechnology), anti-GAPDH (Millipore), anti-GFP (Santa Cruz Biotechnology), anti-HDAC1 (Abcam), anti-tubulin (Sigma-Aldrich) and anti-VE-cadherin (BD Biosciences). Primary antibodies were detected using fluorescently labelled secondary antibodies: goat anti-rabbit IgG DyLight 680 and goat anti-mouse IgG Dylight 800 (Thermo Scientific). Detection and quantification of fluorescence intensity were performed using an Odyssey® CLx imaging system (LI-COR Biosciences, Lincoln) and Odyssey® 2.1 software. In some instances, HRP-conjugated secondary antibodies were used for chemiluminescence detection and protein levels were quantified by densitometry and normalized against loading controls.

Bioinformatic analyses
ERG transcription factor motif discovery was performed using the JASPAR database (http://jaspar.genereg.net) (Vlieghe et al., 2006). Genome-wide ChIP-seq data for H3K4me1 and H3K27ac histone modifications and RNA polymerase II occupancy in HUVEC and sequence conservation based on Multiz alignment analysis of 100 vertebrate species were obtained from the ‘ENCODER histone modification tracks’ of the UCSC Genome Browser (http://genome.ucsc.edu). Mouse and human sequences were compared using ClustalW (Larkin et al., 2007; Goujon et al., 2010).

Gene set enrichment analysis (GSEA) was carried out using Gene Set Enrichment Analysis Software (GSEA, version 2) (http://www.broad.mit.edu/gsea) (Subramanian et al., 2005). The query dataset were the genes identified as being down-regulated following 24 h ERG inhibition in HUVEC (Birdsey et al., 2012), which were compared against genes identified by transcriptome analysis of 24 h β-catenin inhibition in human pulmonary artery endothelial cells (Alastalo et al., 2011). The Database
for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009) was used to identify over-represented gene ontology (GO) categories. The functional clustering tool within DAVID was used to group together GO annotations that have similar gene members and assign an enrichment score (ES). We used an ES > 1.3 (which corresponds to \( P < 0.05 \)) to identify genes that may be over-represented in particular annotation categories.

**Real-time polymerase chain reaction**
RNA was extracted from tissues and HUVEC using the RNeasy kit (Qiagen). First strand cDNA synthesis was carried out using Supercrypt III Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was performed using PerfeCTa SYBR Green Fastmix (Quanta Biosciences) on a Bio-Rad CFX96 system.

**ChIP-qPCR**
HUVEC previously transfected with ERG or control siRNA were crosslinked for 10 min with formaldehyde (to a final concentration of 1%). Chromatin was sheared using a Bioruptor UCD-200 ultrasound sonicator (Diagenode), resulting in DNA fragments of 500–1000 bp in size. Chromatin was immunoprecipitated with 2 μg antibody to ERG (sc-353, Santa Cruz Biotechnology), or negative control rabbit IgG (PP64, Chemicon, Millipore). Immunoprecipitated DNA was then used as template for quantitative PCR using primers specific for the Fzd4 genomic loci.
| Primers                  | Oligonucleotide Sequences                  |
|-------------------------|-------------------------------------------|
| Axin 2 (human) Forward  | 5’- CATTTCCCGAGAACCCACCGCC -3’            |
| Axin 2 (human) Reverse  | 5’- GTGTTGGGCTCTCCAACCTCA -3’             |
| Axin 2 (mouse) Forward  | 5’- GGTCCTGGCAACTCAGTAACA -3’             |
| Axin 2 (mouse) Reverse  | 5’- CTGATGTGAGCCTCCTCTCTTTTTT -3’         |
| β-catenin (human) Forward | 5’- TGGTGCGGCTCTCCAACTCCA -3’            |
| β-catenin (human) Reverse | 5’- CATGCCGACCACCCTCTCCACA -3’           |
| β-catenin (mouse) Forward | 5’- GGTGGCAAGGAGGCAACCAC -3’             |
| β-catenin (mouse) Reverse | 5’- CAGGTCAGCTGTGATAGCCA -3’             |
| Claudin 3 (mouse) Forward | 5’- GAGTGCTTTTTCTGTTGGCG -3’             |
| Claudin 3 (mouse) Reverse | 5’- CTCCGTGATGCTGTTGGC -3’               |
| Cyclin D1 (human) Forward | 5’- TCAAGTGTAACCCGGACTGCCT -3’           |
| Cyclin D1 (mouse) Reverse | 5’- GCCTGGCCAGGCTTTGACT -3’              |
| Cre Forward             | 5’- GCCTGCATTACCGGCATGCAACGA -3’          |
| Cre Reverse             | 5’- GTCAGTGGAGAGGCGCCAC -3’               |
| Cre excision Forward    | 5’- CTATGGGATGAGACATGGAATTTCACCATGAC -3’  |
| Cre excision Reverse    | 5’- AAGCAGTTTCCCTGGATACACCTATTCC -3’      |
| DACT1 (human) Forward   | 5’- ACATCGTGCTACTCAGGGTT -3’              |
| DACT1 (human) Reverse   | 5’- TGCAGATTGGGGCCACCTACTCA -3’           |
| ERG (human) Forward     | 5’- GGAGTGGGCGGTGAAAGA -3’                |
| ERG (human) Reverse     | 5’- AAGGATGTCGCGTGGATGAC -3’              |
| ERG exon 6 (mouse) Forward | 5’- CCGGATACCTGTTGGAGATG -3’            |
| ERG exon 6 (mouse) Reverse | 5’- CTCTGCGCTTATTGGTGTCA -3’              |
| ERG flp Forward         | 5’- AGATTTTGTTCGTGTTAACAAGGGCTGGT -3’     |
| ERG flp Reverse         | 5’- AATGAGACAGAAAAGGATAGGTCGAGATGGG -3’   |
| ERG-V5 Forward          | 5’- CTCCAGCCCTCCCGTGACA -3’               |
| ERG-V5 Reverse          | 5’- TTGACAACCGCGGCCCCTCTA -3’             |
| Frizzled4 (human) Forward | 5’- GCTGCAGCCAGCTCAGGTTCT -3’            |
| Frizzled4 (human) Reverse | 5’- CGCATGGGCCAATGGGGATGT -3’            |
| Frizzled4 (mouse) Forward | 5’- TGGGAGGAGAGGAGGAGGAGGAG -3’          |
| Frizzled4 (mouse) Reverse | 5’- ACCGAGAAAGGAGAAACTGC -3’              |
| Fzd4 R1 promoter (ChIP) Forward | 5’- TTAGAAGAAACGCTGACCC -3’              |
| Fzd4 R1 promoter (ChIP) Reverse | 5’- GTCTGGCTGCTGATTCTTCAT -3’             |
| Fzd4 Ctrl 3’UTR (ChIP) Forward | 5’- GCCATGCTGGGACTTTCA -3’               |
| Fzd4 Ctrl 3’UTR (ChIP) Reverse | 5’- TTGACAGGCATGGTGCAG -3’                |
| GAPDH (human) Forward   | 5’- CAAGGTGATCATGCAACTCTTTG -3’           |
| GAPDH (human) Reverse   | 5’- GGGGACATACAGCTTCTTGT -3’              |
| HPRT (mouse) Forward    | 5’- GTTAGGCTCAATGCAACAAAT -3’             |
| HPRT (mouse) Reverse    | 5’- TCAAGGGCATATCCAACCAACAC -3’           |
| PLVAP (mouse) Forward   | 5’- CAGAGGGTGTGACTAAGGAG -3’              |
| PLVAP (mouse) Reverse   | 5’- CGAGGGTGTGACTAAGGAG -3’               |
| TCF-1 (human) Forward   | 5’- TTCTTGGCAAGAGTGCCAT -3’               |
| TCF-1 (human) Reverse   | 5’- AGGCAGCGTGCTTCTTTGGA -3’              |
| TCF-1 (mouse) Forward   | 5’- GTAAGGTCCCGAGGTTGCTCAG -3’            |
| TCF-1 (mouse) Reverse   | 5’- TACTTTGGTGAAGGGCGAAG -3’              |
| TCF-4 (human) Forward   | 5’- GGTCAGATCTGAAATATGAGGGA -3’           |
| TCF-4 (human) Reverse   | 5’- CATCCCTGCCCTTCTCTCAG -3’              |
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