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BRIEF REPORTS

Endothelial *Erg* expression is required for embryogenesis and vascular integrity

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ABSTRACT. Members of the ETS family of transcription factors are involved in several developmental processes including endothelial cell specification and blood vessel formation, but their exact roles remain unclear. The family member Erg is highly expressed in endothelial cells as compared to other developing cell types including chondrocytes, hematopoietic cells and mesodermal cells. To study the specific roles ERG plays in endothelial cell specification and function during early embryogenesis, we conditionally ablated it by mating ErgloxP/loxP and Tie2-Cre mice. We found that mutant embryos died by mid-gestation and that angiogenesis and vascular integrity were highly compromised. Our study reveals that ERG has essential and cell autonomous roles in endothelial cell development and blood vessel maintenance.

KEYWORDS. embryogenesis, endothelial cells, ERG, vascular integrity, vascular remodeling

INTRODUCTION

ETS transcription factor family members regulate a variety of embryological events and processes, including development and homeostasis of the vascular system. All members contain a highly conserved *ets* DNA binding domain that exhibits a winged helix-turn-helix structure and binds to the GGA(A/T) core DNA sequence.[1] Nearly all genes specifically expressed by endothelial cells have *ets* binding sites in their promoters, and indeed many *ETS* genes are expressed in vascular endothelial cells.[2,3] Although no single *ETS* gene is solely

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expressed in the endothelial cell lineage, *Erg* appears to have the most restricted expression pattern in that lineage and is the most highly expressed *ETS* member in resting endothelial cells.[4-6]

During mouse embryogenesis, endothelial cells first originate from hemangioblasts in blood islands present in the yolk sacs at about E7.5 and subsequently derive from the embryo proper.[7] *Erg* expression is found in the endothelium of both intra- and extra-embryonic tissues as early as E8.0.[6] *Erg* expression is high in the endothelial cell lineage in all vascular beds throughout development and continues into adulthood.[5] The exact roles and function of ERG in the vasculature remain unclear, but some progress has been made recently. A number of studies using cultured endothelial cells have shown that in addition to promoting endothelial cell survival, migration and tube formation,[8-10] ERG also represses the expression of many inflammation-related genes suggesting that ERG keeps endothelial cells in a quiescent state.[11-13] In mouse models, inflammatory stimuli such as those elicited by TNFα and lipopolysaccharide (LPS) greatly suppress the expression of *Erg*,[13,14] confirming that ERG plays an important role in inhibiting endothelial cell activation *in vivo*.

The possible roles of ERG during blood vessel development have also been studied using genetically modified mouse models. A chemically induced mutation in *Erg* (termed Erg^{mld2}) caused mouse embryonic lethality at E11.5 due to absence of definitive hematopoiesis.[15] Surprisingly, development of the vasculature was largely unaffected in those mice, except for the presence of mildly dilated blood vessels.[15] Two targeted mutations in *Erg* were generated recently, each deleting one of the 2 alternative translational start sites that are preferentially used to transcribe *Erg* in endothelial cells and chondrocytes, respectively.[16] Embryos lacking endothelial-enriched *Erg* transcripts died between E10.5-E11.5, with defects in cardiac valve development and widespread vascular remodeling defects.[16]

Despite these and other recent advances, however, the exact roles of ERG in vascular development remain unclear. To address this and related questions, we generated conditional mouse mutants in which *Erg* was selectively deleted in developing endothelial cells. We report here that endothelial *Erg* expression is indispensable for maintenance of vascular integrity and embryo survival.

RESULTS

Mice carrying a floxed *Erg* allele (Erg^{loxP}) were generated previously.[17] We crossed the $Erg^{loxP/loxP}$ mice with *Tie2-Cre* mice to delete *Erg* specifically in the endothelial lineage. Heterozygous *Tie2-Cre;*$Erg^{loxP/+}$ mice were healthy and fertile, but further crosses between female $Erg^{loxP/loxP}$ mice and male *Tie2-Cre;*$Erg^{loxP/+}$ mice yielded no viable homozygous *Tie2-Cre;* $Erg^{loxP/loxP}$ pups at birth, suggesting that those embryos had died in utero (**Table 1**). To

Table 1. Number of Embryos or Mice Recovered from Conditional Knockout Mating ($Erg^{loxP/loxP}$ × Tie2-Cre;$Erg^{loxP/+}$)

	Tie2-Cre; $Erg^{loxP/loxP}$ (ECKO)	Tie2-Cre; $Erg^{loxP/+}$	$Erg^{loxP/loxP}$ (WT)	$Erg^{loxP/+}$ (WT)	Reabsorbed embryos[b]
E9.5	5	4	5	7	0
E10.5	20 (2[a])	18	28	25	9
E11.5	14 (6)	13	13	10	5
E12.5	5 (4)	9	5	7	7
E13.5	1	1	3	2	4
E14.5	0	5	7	2	0
postnatal	0	18	27	33	

[a]Numbers in parentheses indicate numbers of dead embryos.

[b]Embryos that were dead and reabsorbed, with no to minimal embryonic tissue recoverable for genotyping.

determine at what developmental stage the embryos died, we performed timed mating and examined embryos on different gestation days. Up until E9.5, homozygous *Tie2-Cre;Erg*$^{loxP/loxP}$ mutant embryos (hereafter referred as *Erg*^{ECKO} embryos) were anatomically indistinguishable from wild-type (WT) embryos (including *Erg*$^{loxP/loxP}$ and *Erg*$^{loxP/+}$ embryos). However, starting from E10.5 some *Erg*^{ECKO} embryos were clearly delayed in development and displayed signs of cardiovascular insufficiency, including hemorrhage and cardiac edema (**Fig. 1A–C**). Genotyping of recovered embryos showed that death of *Erg*^{ECKO} embryos had occurred mostly between E10.5 and E12.5 (**Table 1**). Occasionally, some *Erg*^{ECKO} embryos remained alive and appeared fairly normal by E13.5, probably because *Erg* deletion was not complete in those embryos.

Quantitative RT-PCR analysis of *Erg*^{ECKO} embryos exhibiting vascular defects and those lacking obvious vascular defects did show that in fact *Erg* expression was dramatically reduced in the former embryos (**Fig. 1D**) but was only partially reduced by about 50% in the latter embryos as compared to WT embryos (**Fig. 1D**). Furthermore, ERG protein was absent from the vast majority of vascular endothelial cells in *Erg*^{ECKO} embryos exhibiting the vascular phenotype (**Fig. 1E–F**), while vascular endothelium in phenotypically normal *Erg*^{ECKO} embryos still contained substantial amount of ERG protein (data not shown). These data verified that *Erg* was effectively deleted from endothelial cells in *Erg*^{ECKO} embryos with a vascular phenotype, while deletion was only partial and ineffective in embryos lacking those defects.

Next, we focused our analysis on mutant embryos with vascular defects that were alive at the time of harvesting. Since vascular defects in those embryos often arose around E10.5, most subsequent analyses were carried out at that stage. To increase the efficiency of gene deletion, we generated a null allele of *Erg* (*Erg*$^{-}$) by crossing male *Erg*$^{loxP/loxP}$ mice with female *Tie2-Cre* mice, thus resulting in *Erg* deletion in the germline.[18] Timed mating between *Erg*$^{loxP/-}$ female mice and male *Tie2-Cre;Erg*$^{loxP/+}$ mice was then used. Mutant *Tie2-Cre;Erg*$^{loxP/loxP}$ and *Tie2-Cre;Erg*$^{loxP/-}$ embryos resulting from these crosses were indistinguishable by genotyping and phenotyping and were collectively referred to as *Erg*^{ECKO}, while embryos of all other genotypes contained at least one *Erg*$^{+}$ or undeleted *Erg*^{loxP} allele and all developed normally. Data presented below were generated from phenotypically abnormal *Erg*^{ECKO} embryos compared with WT (*Erg*$^{loxP/loxP}$ or *Erg*$^{loxP/+}$) embryos.

Whole-mount PECAM1 staining was performed to visualize blood vessels in embryos and visceral yolk sacs. Yolk sacs of *Erg*^{ECKO} embryos contained fewer branches and lacked a typical highly organized vascular hierarchical organization (**Fig. 2A–B**). The vasculature in the craniofacial region of *Erg*^{ECKO} embryos often lacked large blood vessels (**Fig. 2C–D**), while vessels in the trunk region were much less affected (**Fig. 2E F**). These data suggested a role of ERG in blood vessel branching and remodeling, particularly for vessels in the yolk sac and embryonic head, while other factors might be involved in vessel remodeling in the embryonic trunk region. Interestingly, a vascular remodeling defect was also seen in the yolk sacs of macroscopically normal mutant embryos, albeit less severe, suggesting that ERG is required at different threshold levels for maintenance of proper vascular function in different organs or that Cre activity was higher in the yolk sacs.

Histological analysis and immunohistochemical staining with PECAM1 antibodies showed that circulating cells had leaked out of blood vessels at sites of hemorrhage (**Fig. 3A–D**), suggesting that compromised blood vessel integrity had resulted in hemorrhage. Double immunohistochemical staining of PECAM1 and a mural cell marker, PDGFRB,[19] showed that there was reduced coverage of mural cells on vessels in mutant embryos (**Fig. 3E–F**).

To identify genes that may represent downstream targets of ERG, we performed microarray analyses and compared the gene expression profile of an E10.5 *Erg*^{ECKO} embryo versus a WT *Erg*$^{loxP/loxP}$ embryo. GSEA (Gene Set Enrichment Analysis) and GATHER (Gene Analysis Tool to Help Explain Relationships)

FIGURE 1. Hemorrhagic phenotype in Erg^{ECKO} embryos. Gross anatomical appearance of E10.5 WT (**A**) and Erg^{ECKO} embryos (**B–C**). Mutant embryos show hemorrhages (arrowheads) and cardiac effusion (arrows). (**D**) Erg gene expression was analyzed by qRT-PCR in E10.5 and E11.5 WT embryos (WT), Erg^{ECKO} embryos that presented a vascular phenotype (ECKO), and Erg^{ECKO} embryos that appeared normal (ECKO*). Student's t-test was performed to compare expression levels in Erg^{ECKO} groups to WT. *: $P < 0.05$; **: $P < 0.01$. N =3 ~6 per group. (**E–F**) Sections of WT (**E**) and Erg^{ECKO} embryos (**F**) were stained simultaneously with anti-PECAM1 (blue) and anti-ERG (red). Representative images showing microvessels in the pharyngeal arches region were from a pair of E11.5 embryos. Note the almost complete absence of ERG in the Erg^{ECKO} embryo where just very few endothelial cells still contain ERG protein (arrow in **F**). (**G–H**) Sections of the same embryos as shown in **E and F**, respectively, were subjected to the same staining as in **E-F** except that anti-ERG was replaced with mouse IgG at the same concentration. This confirms the specificity of the ERG staining. Scale bar in A-C: 500 μm; E-H: 50 μm.

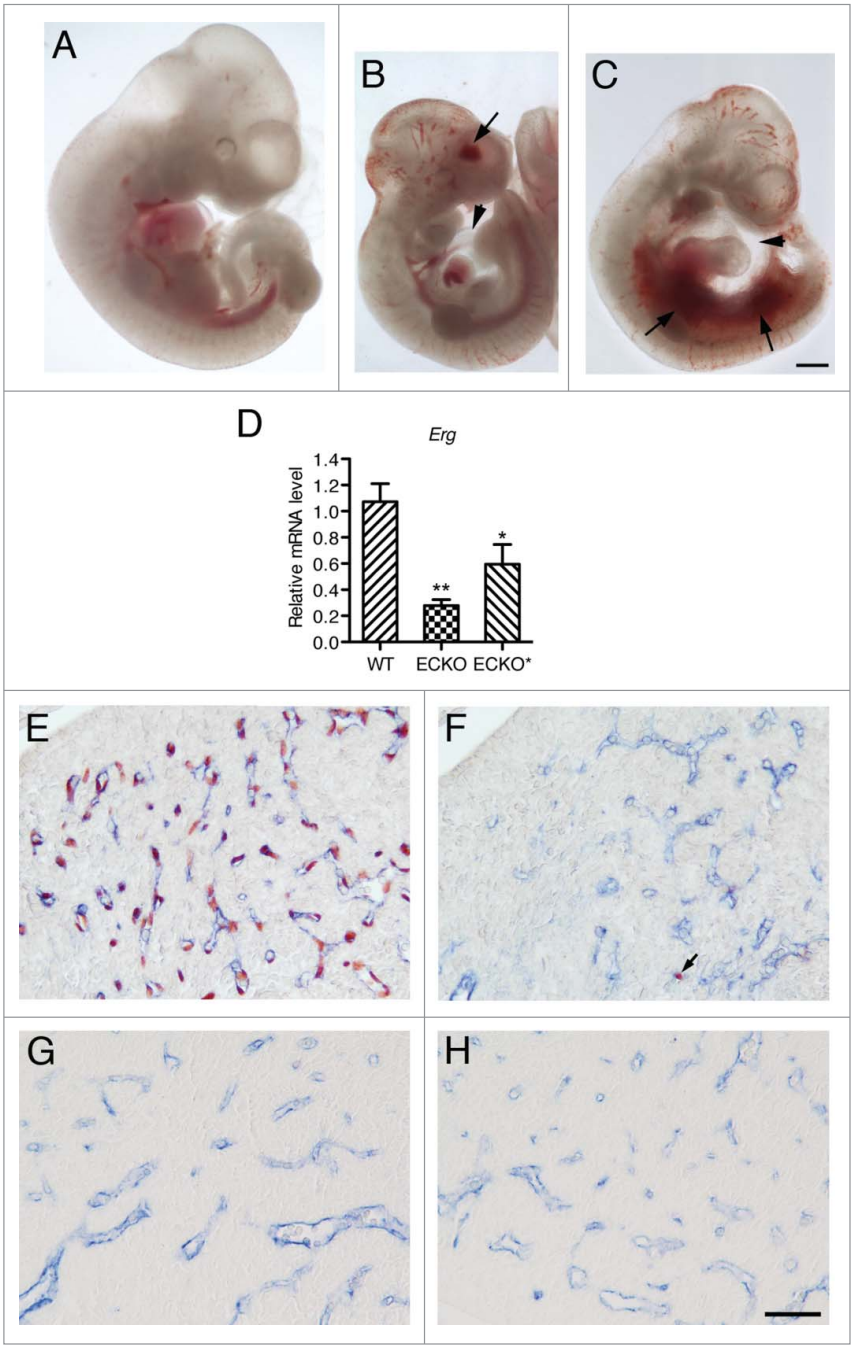
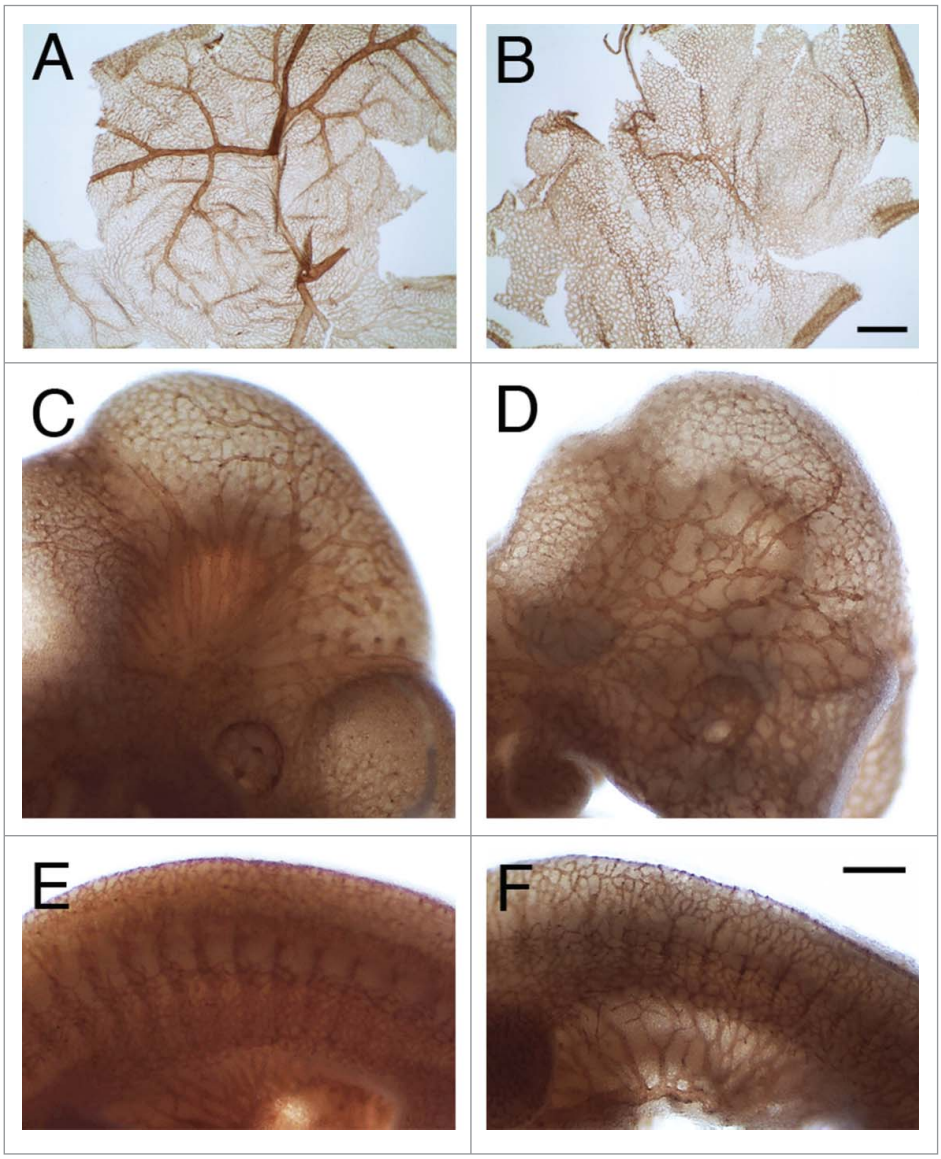
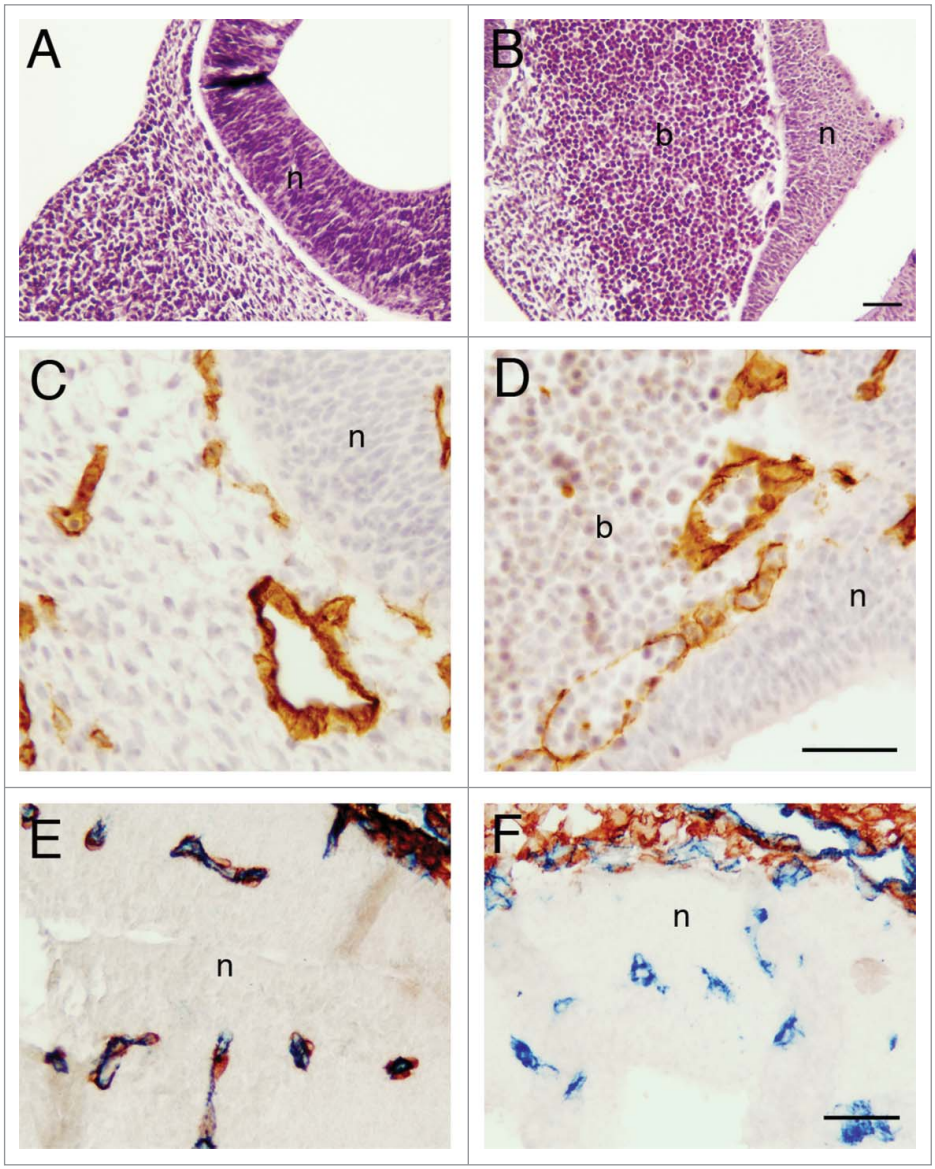


FIGURE 2. *Erg*^{ECKO} embryos display vascular modeling and remodeling defects. Blood vessels were visualized by whole-mount PECAM1 staining (brown) in the yolk sacs (**A–B**) and embryonic head (**C–D**) and trunk (**E–F**) regions of E10.5 WT (**A, C, E**) and *Erg*^{ECKO} embryos (**B, D, F**). Scale bar in (**A–B**): 500 μm; (**C–F**): 250 μm.



tools were used to identify genes that were up- or down-regulated in the mutant *Erg*^{ECKO} embryo by at least 2-fold and make predictions on possible biological pathways and processes affected by those changes. The data showed that genes related to carbohydrate (especially glucose) and heme metabolism were strongly up-regulated in mutant embryo, together with several genes that were associated with vascular endothelium including *Vegfa*, *Edn1*, *Epo*, *Flt1* and *Adm*. The up-regulation of such genes could have been a secondary effect from tissue hypoxia as a consequence of vascular leakage and malfunction. Thus we turned our attention to genes that were downregulated in the mutant. Two previously reported ERG target genes,

FIGURE 3. *Erg*^{ECKO} embryos show compromised vascular integrity. Sections of E10.5 WT (**A, C, E**) and *Erg*^{ECKO} embryos (**B, D, F**) were stained with hematoxylin and eosin (**A–B**), anti-PECAM1 (brown, **C–D**), or simultaneously with anti-PECAM1 (blue) and anti-PDGFRB (red) (**E–F**). Pictures shown are from the head region. Note the pool of nucleated blood cells next to the neural tube in the mutant embryo (**B**) and outside of blood vessels (stained positive for PECAM1, D). Microvessels in the neural tube of control embryo contain both endothelial cells (PECAM1 positive) and mural cells (PDGFRB positive) (**E**), while those in the mutant embryo lack mural cells (**F**). Note the positive PDGFRB staining of mesenchymal cells next to the neural tube in (**F**). n: neural tube, b: hemorrhaging blood. Scale bars: 50 μm.



RhoJ and *Cldn5*,[10,14] were in fact down-regulated in the mutant embryo affirming the validity of our microarray analysis. Genes involved in cell cycle regulation and complement/coagulation pathways were strongly downregulated in the mutant as revealed by GSEA and GATHER analysis. In addition, we compared down-regulated genes (>1.4 fold) to mutant

genes listed in the Mammalian Phenotype Browser (http://www.informatics.jax.org/searches/MP_form.shtml) in the Mouse Genome Informatics (MGI) database that are associated with phenotypes such as "abnormal vascular development" (MP: 0000259) or "embryonic growth retardation" (MP: 0003984). As a result of this approach we identified a small number of common genes downregulated between 1.4 and 2.5 fold (**Table 2**). Interestingly, 3 genes (*Krit1*, *Ccm2*, and *Pdcd10*) previously found to be responsible for a familial form of Cerebral Carvenous Malformation (CCM)[20] were all down-regulated in the mutant embryo. We used quantitative RT-PCR to verify the down-regulation of *Krit1* and *Ccm2* in Erg^{ECKO} embryos (**Fig. 4**).

DISCUSSION

The results of our study provide clear evidence that ERG is an essential regulator of endothelial cell development and vascular integrity. Our conditional gene ablation approach exploited *Tie2* promoter-driven *Cre* to target and ablate *Erg* specifically in the endothelial lineage and thus, the phenotypes we observed are attributable to defects in that cell lineage. We find that vascular development and associated processes including vascular integrity were all markedly affected and deranged in the mutants, likely representing the cause of embryonic lethality by mid-gestation. These phenotypes were highly consistent, and resemble those recently observed in embryos lacking endothelial-enriched *Erg* isoforms in all cells.[16] The similarity of phenotypes obtained by these 2 different genetic strategies further strengthens the notion that ERG is important for the development of blood vessels and exerts a cell autonomous and indispensable function in that process.

The vascular defects we observe in the *Erg* mutants are severe and include a lack of hierarchical vessel organization, deranged vascular branching and remodeling, leakage of blood cells into the surrounding environment, and cardiac effusion. These encompassing defects indicate that ERG has multiple roles in vessel development and homeostasis. As pointed out above, ERG could not only direct and sustain endothelial cell commitment and differentiation, but could also regulate behavior, function and survival of the cells. These possibilities are sustained by the multiple changes in gene

Table 2. Genes down-regulated in Erg^{ECKO} embryo with known function in embryogenesis or vascular development[a]

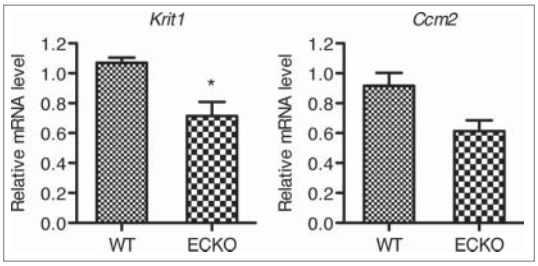
Gene symbol	Fold change	In MGI	Gene symbol	Fold change	In MGI
Agrt2	−1.53	V[b]	Ccne1	−2.50	Ec
Arntl	−1.64	V	Ccne2	−1.55	E
Casp8	−1.93	V	Cops8	−1.52	E
Ccm2	−1.69	E, V	Fadd	−2.22	E
Cx3cr1	−2.04	V	Fen1	−1.51	E
E2f7	−1.58	V	Gjb2	−1.50	E
Krit1	−1.60	E, V	Gnpnat1	−1.87	E
Mecom	−1.41	V	Hira	−1.62	E, V
Mpi	−1.71	E, V	Hus1	−1.42	E, V
Nrarp	−1.87	V	Mmp14	−1.45	E, V
Paxip1	−1.52	E, V	Mtfmt	−1.51	E
Pdcd10	−1.55	V	Pmm2	−1.59	E
Plg	−1.88	V	Rbbp6	−1.55	E
Vash1	−1.50	V	Rbm12	−2.06	E
Vash2	−1.86	V	Xbp1	−1.66	E

[a]This list contains genes that were downregulated in Erg^{ECKO} by >1.4 fold as revealed by microarray analysis. Mutations in these genes are listed in the MGI database as being associated with "abnormal vascular development" or "embryonic growth retardation" phenotypes.

[b]Gene mutations associated with "abnormal vascular development" in the MGI database are indicated here as V.

[c]Gene mutations associated with "embryonic growth retardation" in the MGI database are indicated here as E.

FIGURE 4. Gene expression changes in Erg^{ECKO} embryos. Expression of *Krit1* and *Ccm2* in E10.5 embryos was measured by qRT-PCR. Student's t-test was performed to compare the expression levels between WT and Erg^{ECKO} groups. *: $P < 0.05$. N = 3 per group.



expression we observe in the Erg^{ECKO} embryo by microarray analysis. Thus, the upregulation of genes involved in sugar metabolism and vascular induction such as *Vegfa* could be an attempt by the mutant embryos to sustain overall cell survival, counter anoxia and boost angiogenesis. In a similar fashion, the several genes downregulated in the Erg^{ECKO} embryo, including such known *Erg* targets as *RhoJ* and *Cldn5*,[10,14] indicates that there are no effective compensating mechanisms to restore normal gene expression patterns and developmental processes in the absence of ERG. In this regard, it will be interesting to assess whether pathologies such as CCM may involve deranged *Erg* expression as a contributor to its pathogenesis.

Due to alternative splicing, *Erg* is transcribed into at least 7 mRNA isoforms.[16,21] Previous in vitro studies did not uncover major functional differences among Erg isoforms when over-expressed in chondrocytes.[21] Recently Vijayaraj et al. reported that *Erg* transcripts expressed by mouse endothelial cells contain exon 4 as translational start site, while those expressed by chondrocytes contain exon 3 as translational start site.[16] In the *Erg* floxed mice we describe here, the loxP sites were placed flanking exon 6 that encodes a portion of the Pointed domain, is not subjected to alternative splicing and when ablated, results in a stop codon in exon 7. Thus, our strategy eliminated all *Erg* isoforms following effective Cre recombination. In Vijayaraj et al.'s study, *Erg* mutant embryos displayed defects in endocardial cushion formation due to reduced endothelial mesenchymal transition, and *Snail1* and *Snail2* were identified as *Erg* downstream targets.[16] In our microarray and qRT-PCR analyses, however, we observed no significant changes in expression in those genes, probably because we used whole embryos for RNA preparation while only embryonic hearts were used in that study. However, we did observe decreases in several potential *Erg* target genes that are known to be required for embryo survival or vascular development (**Table 2**). Because these changes were significant but not dramatic, they suggest the interesting possibility that ERG contributes to vascular development and integrity by regulating multiple genes in a synergistic and/or coordinated manner.

Structurally, ERG is closely related to the ETS family member FLI1, and the 2 factors can presumably recognize the same DNA sequences.[22] *Fli1* expression patterns are much broader than those of *Erg*, and expression is appreciable in endothelial cells, haematopoietic cells, fibroblasts and other cells.[4,6,23,24] It remains unclear whether FLI1 and ERG have redundant function. *Erg* and *Fli1* double heterozygous mice ($Erg^{mld2/+}$; $Fli1^{+/-}$) have a more severe phenotype than single mutants, suggesting that they have overlapping but also distinct functions in regulating homeostasis of megakaryocytes and haematopoietic stem cells.[25] *Fli1*-null embryos die by E11.5-E12.5 due to defects in vascular development and megakaryopoiesis.[26,27] Failure by *Fli1*- or *Erg*-null embryos to survive past mid-gestation suggests that these factors cannot compensate for each other during development, although it is still not known whether they regulate the same set of genes. An example of closely related ETS family members with redundant functions is the pair ETS1/ETS2. While *Ets1* or *Ets2* single null mutant mice develop a normal vasculature, double mutant mice have severe angiogenesis defects and die by E11.5–15.5.[28] Whether *Erg/Fli1* double null mutants would have an even more severe defect in angiogenesis/vasculogenesis than single null mutants shown here and previously awaits further investigation.

MATERIALS AND METHODS

Mice

Erg floxed mice were generated previously.[17] *Tie2-Cre* mice were purchased from the Jackson Laboratory (B6.Cg-Tg(Tek-cre) 12Flv/J). Genotyping primers used were: Erg^{loxP} allele was detected with primers F: 5′-CAC ATT CGT GCA TGT TTC TGT GGA-3′ and R: 5′-CAC CCA TCC TCC TAG AGC TTC CAA-3′; Erg^{-} allele was detected with the same forward primer used for Erg^{loxP} allele and the reverse primer 5′-CAG GGA ACA GTG GAG CTT ACG-3′; *Tie2-Cre* transgene was detected with primers F: 5′-GAA CCT GAT GGA CAT GTT CAG G-3′ and R: 5′-AGT GCG TTC GAA CGC TAG AGC CTG T-3′.

Embryo analysis

For timed mating, female $Erg^{loxP/loxP}$ or $Erg^{loxP/-}$ mice were crossed to male *Tie2-Cre;* $Erg^{loxP/+}$ mice, and the day the vaginal plug was found was designated E0.5. At pre-designated time points, pregnant females were sacrificed and individual embryos were removed from the uterus and kept in phosphate-buffered saline (PBS). Live embryos were examined and photographed in PBS under a stereo microscope (Leica).

Whole-mount immunocytochemistry was carried out as described with slight modifications.[29] Briefly, E10.5 embryos and visceral yolk sacs were fixed in 4% paraformaldehyde for 2 hr at 4°C, then dehydrated in methanol. Embryos were treated with 6% H_2O_2 in methanol to quench endogenous peroxidase and then rehydrated. Anti-PECAM1 antibody (BD Biosciences) was used at 1:400, followed by horseradish peroxidase conjugated-anti-rat antibody (Jackson ImmunoResearch) at 1:200. Signal was detected with the liquid DAB+ kit (Dako) for 10 min. Embryos were photographed in PBS, while yolk sacs were spread out onto glass slides and mount with Aqua-Mount (Thermo Scientific) without coverslips.

Histology and immunohistochemistry

Embryos were fixed in 10% formalin for 2 hours (E10.5) or overnight (E11.5 and older), and then embedded in paraffin and cut into 5 μm sections. Sections were stained with hematoxylin and eosin for histological analysis.

For immunohistochemistry, deparaffinized and rehydrated sections were heated in citric acid based Antigen Unmasking Solution (Vector Labs) before blocking and incubation with antibodies. Anti-mouse CD31 (PECAM1) antibody (Dianova) was used at 1:200, followed by biotinylated rabbit-anti-rat antibody (Vector Labs) at 1:200. Signal detection was done using VECTASTAIN Elite ABC kit (Vector Labs) followed by liquid DAB+ substrate (Dako). Sections were counterstained with Hematoxylin 7211, dehydrated, cleared, and mounted with Cytoseal XYL (Thermo Scientific).

For simultaneous detection of PDGFRB and PECAM1 by immunohistochemistry, a 2-colored staining scheme was employed. Following antigen retrieval, sections were blocked with BLOXALL (Vector Labs) and 5% horse serum sequentially. Sections were then incubated with the anti-PDGFRB antibody (Cell Signaling, 1:75) at room temperature for 1 hour, followed by ImmPRESS anti-rabbit (peroxidase) (Vector Labs), and the first color was developed with the ImmPACT AMEC substrate (Vector Labs). After a brief wash, sections were incubated sequentially with the anti-CD31 antibody (Dianova), unconjgated rabbit-anti-rat (Vector Labs), and ImmPRESS-AP anti-rabbit (Vector Labs). The second color was developed with the VECTOR Blue substrate. Sections were washed with water and mounted with Aqua-mount.

Simultaneous detection of ERG and PECAM1 by immunohistochemistry was similarly performed, except that for ERG staining, a method of staining mouse sections with mouse antibodies was used.[30] After antigen retrieval and blocking of endogenous enzymes, sections were blocked with 0.1 mg/ml unconjugated donkey-anti-mouse Fab fragment (Jackson ImmunoResearch), and then incubated with mouse-anti-ERG antibody (Biocare Medical) at 1:100, followed by biotinylated donkey-anti-

mouse Fab fragment (Jackson ImmunoResearch) at 1:400, then the 2-color staining procedure was followed from the first color development (AMEC) step. Purified mouse IgG from unimmunized animals (Jackson ImmunoResearch) was used in the place of anti-ERG as a negative control.

RNA extraction, quantitative RT-PCR and microarray

RNAlater (Qiagen) stabilized embryos were homogenized with TissueLyser II (Qiagen) at 20 Hz for 1 min, and total RNA was isolated with the RNeasy Mini Kit (Qiagen). 1 μg of total RNA was reverse transcribed with random hexamers using the Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science). Diluted cDNA was mixed with SYBR Green PCR Master Mix (Applied Biosystems) to quantitatively measure gene expression using a StepOnePlus Real-Time PCR system (Applied Biosystems). The primers used are: for *Krit1*, F: 5′-CGA TAA AGG ACT TTC AGT CAA CC-3′ and R: 5′-TAT GCG AGT GGC CTC AAC TT-3′; for *Ccm2*: F: 5′-GTC ATC ATG GCC ACA GAG AG-3′ and R: 5′-TGG ACT CCG TGT AAA CAA TCT G-3′. The *B2m* gene (F: 5′-TCG CTC GGT GAC CCT AGT CTT T-3′ and R: 5′-ATG TTC GGC TTC CCA TTC TCC-3′) was used as internal control.

For microarray analysis, residue DNA was removed from total RNA samples by on-column DNase digestion using the RNeasy mini kit, and purified RNA was then submitted to the Boston University Microarray Resource Core. GeneChip Mouse Gene 1.0 ST Array (Affymetrix) was used for generating the gene expression profiles. Raw Affymetrix CEL files were normalized to produce gene-level expression values using the implementation of the Robust Multiarray Average (RMA) in the affy package included within in the Bioconductor software suite (version 2.10.0) and an Entrez Gene-specific probeset mapping from BrainArray (version 14.0.0). All microarray analyses were performed using the R environment for statistical computing (version 2.12.0). GSEA[31,32] and GATHER[33] (http://gather.genome.duke.edu) were used to analyze the microarray data.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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SUPPLEMENTAL MATERIAL

Supplemental data for this article can be accessed on the publisher's website.

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