Erg is a crucial regulator of endocardial-mesenchymal transformation during cardiac valve morphogenesis

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SUMMARY
During murine embryogenesis, the Ets factor Erg is highly expressed in endothelial cells of the developing vasculature and in articular chondrocytes of developing bone. We identified seven isoforms for the mouse Erg gene. Four share a common translational start site encoded by exon 3 (Ex3) and are enriched in chondrocytes. The other three have a separate translational start site encoded by Ex4 and are enriched in endothelial cells. Homozygous Ergex3/ΔEx3 knockout mice are viable, fertile and do not display any overt phenotype. By contrast, homozygous Ergex3ΔEx4 knockout mice are embryonic lethal, which is associated with a marked reduction in endocardial-mesenchymal transformation (EnMT) during cardiac valve morphogenesis. We show that Erg is required for the maintenance of the core EnMT regulatory factors that include Snail1 and Snail2 by binding to their promoter and intronic regions.

KEY WORDS: Ets factor, Erg isoforms, Valve morphogenesis, EMT, ENMT, Snail1 (Snai1), Snail2 (Snai2)

INTRODUCTION
During mouse development, the Ets factor Erg is sequentially expressed in endothelial cells (ECs) of the yolk sac blood islands [embryonic day (E) 7.5], ECs of the developing vasculature (E8.5), and then in chondrocytes of the developing cartilage (E10.5) (Vlaeminck-Guillem et al., 2000). Overexpression of Erg in zebrafish promotes the proliferation of mesodermal angioblasts (Ellett et al., 2009). Knockdown of Erg in murine embryonic stem (ES) cells significantly reduces their differentiation into ECs (Nikolova-Krstevski et al., 2009). Whereas Erg expression gradually decreases in the developing vasculature of zebrafish, Erg remains highly expressed in ECs of most adult tissues in mouse and human (Balzinger et al., 1999; Vlaeminck-Guillem et al., 2000; Hewett et al., 2001; Ellett et al., 2009; Yuan et al., 2009). Erg can function as a transcriptional activator of several EC-restricted genes, including angiopoietin 2, endoglin, VWF and VE-cadherin (VE-cad; cadherin 5) (Birdsey et al., 2008; Yuan et al., 2012; Schwachtgen et al., 1997; Pimanda et al., 2006; Hasegawa et al., 2004). Erg also acts as a transcriptional repressor of pro-inflammatory genes in ECs; for example, Erg represses the chemokine interleukin 8 to promote endothelial quiescence and inhibit neutrophil attachment in response to pro-inflammatory cytokines such as tumor necrosis factor (Yuan et al., 2009).

During mouse and chicken bone development, Erg is selectively expressed in articular chondrocytes (Iwamoto et al., 2000; Iwamoto et al., 2001; Iwamoto et al., 2007). Erg is induced by the bone morphogenetic protein Gdf5 and is highly expressed in regions of the articular cartilage that express lubricin (Prg4) (Iwamoto et al., 2000). Interestingly, overexpression of Erg in developing chick limbs effectively blocks maturation of endochondral ossification by maintaining the entire limb chondrocyte population in an immature state (Iwamoto et al., 2000).

Erg is also transiently expressed during the early stages of T and B cell differentiation but is silenced permanently after T and B cell lineage commitment (Anderson et al., 1999). Two recent studies support an important regulatory role for Erg during murine hematopoiesis (Loughran et al., 2008; Taoudi et al., 2011). In these studies, mice were characterized containing a single point mutation (Mld2) in the DNA-binding domain of Erg that significantly inhibits Erg transactivation. This mutation is associated with multiple defects in definitive hematopoiesis and a failure to sustain self-renewal of hematopoietic stem cells (HSCs), ultimately leading to exhaustion of HSCs and death at E11.5. Interestingly, no defects in cardiac development or vasculogenesis were observed.

Formation of the murine cardiovascular system begins prior to heart tube formation at E7 and involves a series of precisely orchestrated events involving morphogenesis and differentiation. In the heart, the atrioventricular canal (AVC) and outflow tract (OFT) that eventually give rise to the atrioventricular valves and pulmonic and aortic valves, respectively, are characterized by localized swellings called cardiac cushions that are evident by E9.5 (Butcher and Markwald, 2007). These cushions provide a valve-like function, allowing unidirectional blood flow. The cardiac cushion is composed of myocardial-derived extracellular matrix, which is primarily populated by mesenchymal cells as a result of endocardial-mesenchymal transformation (EnMT) of the endocardial cells in the AVC and OFT (DeLaughter et al., 2011). Maturation of the valve is a multistep process involving activation, proliferation, EnMT of the endocardial ECs and, finally, maturation of the cellularized cushions into functional valve leaflets by E13.5. Remodeling of the valve leaflets continues into the postnatal period.

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This transition is accompanied by upregulation of the Snail family of zinc-finger transcription factors, which are subsequently responsible for the downregulation of EC-specific genes such as VE-cad (Timmerman et al., 2004) that lead to the induction of EnMT. Many cell types in the developing embryo that undergo EnMT have been reported to express Ets genes (Remy and Baltzinger, 2000). However, no report to date has suggested a role for any Ets family genes in EnMT during valve morphogenesis.

The primary purpose of this study was to evaluate the role of different Erg isoforms during embryonic development. We identified four Erg isoforms that are highly expressed in articular chondrocytes and share a commonly spliced exon 3, and three Erg isoforms that share a commonly spliced exon 4 and are highly enriched in ECs. Exons 3 and 4 encode separate translational start sites. We report that mice with targeted disruption of exon 3 (ErgEx3/H9004) are viable, fertile and do not display any overt phenotype. By contrast, targeted disruption of exon 4 (ErgEx4/H11032) is embryonic lethal and is associated with defects in cardiac and vascular development. The cardiac defects are primarily related to a failure of the endocardial ECs to undergo EnMT. We have identified Erg as a crucial regulator of the zinc-finger transcriptional repressors Snail1 and Snail2, which are required to repress VE-cad during EnMT.

MATERIALS AND METHODS
Generation of ErgEx3 and ErgEx4 knockout mice and genotyping
To generate the ErgEx3 knockout construct, 2.4 kb of the 5' and 3' flanking regions of exon 3 were amplified by PCR (for primers see supplementary material Table S1) and cloned on either side of the lacZ-Neo cassette and were then inserted into the pM253 vector (kindly provided by Dr W. C. Aird, Boston, MA, USA). The linearized construct was targeted into the Erg locus of W4/129S6 ES cells. The targeted ES clones were digested with NheI and screened by Southern blotting for homologous recombination events (primers used to prepare probes are listed in supplementary material Table S1).

Similarly, 3.8 kb 5' and 2.5 kb 3' arms of homology spanning exon 4 of the mouse Erg locus were PCR amplified from genomic DNA isolated from the W4/129S6 ES cell line. The fragments were cloned on either side of the lacZ-Neo cassette and were inserted into the pM253 vector backbone digested with KpnI and XhoI. The plasmid was linearized with KpnI before gene targeting. Two hundred micrograms of linearized and purified plasmid was transfected into W4/129S6 ES cells at 3 µg/ml. All PCR reactions were performed in triplicate. Relative expression was calculated using Gapdh as an endogenous internal control. Primers are listed in supplementary material Table S1.

Ex vivo AVC and OFT explant culture assay
Ex vivo explant assays for EnMT were performed as described previously (Camenisch et al., 2002a). Briefly, 1.5% rat tail collagen type microscropy, β-galactosidase-stained embryos were fixed overnight in 4% paraformaldehyde (PFA), embedded in paraffin, sectioned (5 µm) and counterstained with Eosin and photographed. For immunofluorescent staining, embryos were fixed overnight in 4% PFA, rinsed, cryoprotected overnight in 20% sucrose, embedded in OCT (Tissue-Tek) and sectioned (6 µm). Sections were permeabilized with 0.5% Triton X-100 for 5 minutes and blocked in 5% goat serum for 30 minutes. The sections were then incubated with the primary antibody for 1 hour followed by secondary antibody for 45 minutes. Nuclei were stained with 5 ng/ml DAPI, the sections were mounted using Vectastain (Vector Labs) antifade mounting medium and photographed using a Zeiss AxioVision microscope. Antibodies are listed in supplementary material Table S1.

Fig. 1. Genomic structure and expression of mouse Erg isoforms.
(A) Genomic organization of Erg and representation of the different isoforms that result from the presence of two independent translation start sites within exons 3 and 4. The seven isoforms differ in the variable region and in the 5' UTR. F1 and R1 denote forward and reverse qPCR primers used to identify Erg isoforms 1-4, whereas primers F2 and R2 were used to identify Erg isoforms 5-7. The pointed (PNT), variable and Ets domains are indicated. (B-E) Real-time PCR analysis of differential Erg isoform expression in various mouse cell lines. Isoforms 1-4 are predominantly expressed in non-endothelial cells (ECs) and in articular cartilage (B), whereas isoforms 5-7 are predominantly expressed in ECs (C). Similarly, adult organs enriched in blood vessels, such as heart and lung, predominantly express isoforms 5-7 (E), whereas organs such as the trachea with large amounts of chondrocytes express isoforms 1-4 (D). Error bars indicate s.d. of at least three independent qPCR reactions.
I (BD Biosciences) was prepared by diluting 3% collagen in cell culture grade water. Equal parts of 2.2% sodium bicarbonate solution and 10× DMEM medium were mixed and added to the collagen solution at 33.3%. The resulting solution was mixed immediately and dispensed into 4-well plates (Nunc) and allowed to solidify for 1 hour inside a 37°C, 5% CO2 incubator. The collagen pads were then conditioned for 4 hours with 1× DMEM medium containing 10% fetal bovine serum (FBS). AVC and OFT tissues were dissected from E10.5 embryos and placed on drained collagen pads with the endocardium facing down on the collagen. The explants were allowed to attach for 24 hours at 37°C and 5% CO2 in a humidified incubator. Then 1× DMEM with 10% FBS was added to the collagen pads and incubated for an additional 24 hours. Transforming endocardial cells were identified as spindle-shaped cells that either migrated away from the explants or invaded the gel. The collagen pads and explants were fixed overnight with 4% PFA, rinsed several times with PBS and then stained for smooth muscle α-actin (Sigma) and counterstained with anti-mouse Alexa 488 secondary antibody (Invitrogen).

**Chromatin immunoprecipitation (ChIP) assays**

ChIP was performed as described (Carey et al., 2009). Briefly, ~50 hearts were isolated from E10.5 Erg+/+ and Erg ΔEx3:ΔEx4/+ mouse embryos, cross-linked for 15 minutes with 1% formaldehyde and lysed. Chromatin was sheared to obtain DNA fragments of 200-500 bp. The chromatin slurry was incubated with anti-mouse Erg (SC354, Santa Cruz). Anti-rabbit IgG was used as a control. The immunoprecipitated DNA fragments were recovered and subjected to qPCR (for primers see supplementary material Table S1).

**Fig. 2. Targeting of Erg ΔEx3/+ and Erg ΔEx4/+ loci.** (A) The wild-type murine Erg locus, the targeting construct, and targeted locus with exon 3 replaced by the lacZ-Neo reporter cassette. The numbered boxes represent Erg exons. Bold lines indicate the arms of homology. The positions of Southern blotting probes are indicated. (B) Southern analysis to identify Erg ΔEx3/+ ES cell clones using 5′ and 3′ probes that distinguish NheI fragments in the wild-type and targeted alleles. (C) Erg wild-type locus, the targeting construct, and targeted locus with exon 4 replaced by the lacZ-Neo reporter cassette. (D) Southern analysis to identify Erg ΔEx4/+ ES cell clones using 5′ and 3′ probes that distinguish BssSI and NheI fragments in the wild-type and targeted allele, respectively.
RESULTS

Genomic structure of Erg

The mouse Erg gene contains 13 exons that span a region of ~225 kb on chromosome 16qC4 (Fig. 1A). By carefully analyzing the UCSC and Ensembl genome databases, we identified seven known transcript variants of mouse Erg. Isoforms 1-4 contain the same translational start site within exon 3. Isoforms 1 and 2 differ only in the 5’ UTR. By contrast, isoforms 3 and 4 have a truncated variable region, lacking either exon 9 or 10, respectively. Isoforms 5-7 have a separate translational start site within exon 4. Isoforms 5-7 differ by the presence or absence of exons 9 and 10 in the variable region. All isoforms contain the Ets DNA-binding domain and the pointed domain (PNT) known to be crucial for protein-protein interactions (Fig. 1A).

Differential expression of Erg isoforms in chondrocytes and ECs

Previous studies have demonstrated differential expression of Erg in chondrocytes of developing bone and in ECs during vascular development (Iwamoto et al., 2001; Nikolova-Krstevski et al., 2009; Yuan et al., 2009). Isoform-specific primers were designed to evaluate the expression of Erg in different cell types in mouse tissues and cultured cells (Fig. 1B-E). Isoforms 1-4 exhibit high levels of expression in articular chondrocytes such as AtdcD13 and minimal expression in all other cell types tested. By contrast, isoforms 5-7 are highly expressed in mouse ECs including MS1, bEND and PY41, but not in other cell types, and in organs with abundant ECs such as the lung and heart.

Generation of Erg exon 3 and exon 4 knockout mice

To further define the role of exon 3- and exon 4-specific isoforms during development, we generated ErgΔEx3/ΔEx3 and ErgΔEx4/ΔEx4 knockout mice using standard gene targeting strategies. In brief, we designed a targeting vector to replace either the ATG of exon 3 or exon 4 with a lacZ-Neo cassette (Fig. 2A,C). Targeting of these exons blocks protein expression of Erg isoforms 1-4 and 5-7, respectively, which is replaced by expression of lacZ. Mouse ES cells were transfected with the linearized targeting vectors and neomycin-resistant ES cell clones were picked and screened. Successful homologous recombination was verified by PCR and Southern blot analysis (Fig. 2B,D). ErgΔEx3/+ and ErgΔEx4/+ ES cells were used to produce ErgΔEx3/+ and ErgΔEx4/+ heterozygous knockout mice, respectively. lacZ staining was first observed in the ErgΔEx3/+ mice at E9.5 and in ErgΔEx4/+ mice at E7.5 (Fig. 3A).

*ErgΔEx3/ΔEx3* mice do not display any overt phenotype

lacZ staining in ErgΔEx3/+ and ErgΔEx3/ΔEx3 was first observed at E9.5 in the limb bud. At E10.5, expression was seen in the spine and ear.
regions. By E12.5, lacZ expression was detected in all bony joints, including the skull, spine and limbs (Fig. 3A). To further define the cellular distribution of the lacZ staining in the joints, 10 μm sections of the knee joints from postnatal day (P) 4 pups were counterstained with eosin (Fig. 4). We observed strong lacZ staining only in the articular cartilage in the Erg<sup>Ex3/+</sup> and Erg<sup>Ex4/+</sup> P4 mice. This is consistent with the Erg expression pattern previously observed in the mouse by in situ hybridization (Iwamoto et al., 2007). No appreciable morphological changes were observed in the joints of Erg<sup>Ex3/Ex3</sup> versus control P7 mice. Radiographs of Erg<sup>Ex3/+</sup> and Erg<sup>Ex3/Ex3</sup> mice revealed no differences in joint bone structure at 4 or up to 14 months of age (data not shown). Despite the wide expression pattern of Erg isoforms I-4 in the developing cartilage and joints, the homozygous null animals were viable, fertile and did not display any overt phenotype.

**Erg<sup>Ex3/Ex4</sup> mice are embryonic lethal at E10.5-11.5**

lacZ expression in Erg<sup>Ex3/Ex4</sup> was seen throughout the entire vasculature of the embryo proper by E8.5-10.5 (3A-G) with particular accentuation of expression in the endocardium of the AVC. This pattern of lacZ expression was similar to the endothelial expression pattern that we reported previously by Erg immunohistochemistry in the developing mouse embryo (Nikolova-Krstevski et al., 2009). The homozygous Erg<sup>Ex3/Ex4</sup> embryos appeared smaller than heterozygous or Erg<sup>Ex3/+</sup> embryos by E9.5 (Fig. 5A,B). By E10.5, the embryos also displayed a significant reduction in size and exhibited hemorrhages in the head, trunk, or both regions (Fig. 5D; see Fig. 7A-D). These abnormalities were more striking in surviving embryos at E11.5, with marked reductions in the size and vascularization of Erg<sup>Ex3/Ex4</sup> embryos (Fig. 5F). No live Erg<sup>Ex3/Ex4</sup> embryos were observed beyond E11.5 (Table 1).

**Erg<sup>Ex3/Ex4</sup> embryos exhibit abnormal vascular remodeling**

Vascular defects in the Erg<sup>Ex3/Ex4</sup> embryos were observed as early as E10.5. The large arborized vessels that are characteristic of the remodeled vascular plexus were completely absent in the yolk sac of Erg<sup>Ex3/Ex4</sup> embryos (Fig. 6B). Whole-mount CD31 (Pecam1) staining of the Erg<sup>Ex3/Ex4</sup> yolk sac revealed that the primary capillary plexuses were present but that these capillaries failed to fuse into the larger vessels seen in the controls (Fig. 6B), indicating a vascular remodeling defect.

Vascularization of the central nervous system begins at ~E10.0, when angiogenic vessels from the perineural vascular plexus invade the embryonic neuroectoderm (Marin-Padilla, 1985; Risau and Wolburg, 1990). In E10.5 Erg<sup>Ex3/Ex4</sup> embryos brains we failed to detect a normal alignment and invasion of vessels from the perineural vascular plexus into the developing ganglionic eminence, as seen in the controls (Fig. 6C,D, arrowheads). We consistently observed disrupted vessels by CD31 staining (Fig. 7E-J) and hemorrhages (Fig. 7A-D) in the brain of Erg<sup>Ex3/Ex4</sup> embryos.

We also observed defective vasculature in the labyrinth region of the placenta in Erg<sup>Ex3/Ex4</sup> embryos. Whereas the controls displayed normal placental villi and capillaries, in the Erg<sup>Ex3/Ex4</sup>...
placentas the capillaries were not clearly discernible by CD31 staining (Fig. 7F). Instead, the placental capillaries appeared collapsed, with a drastic reduction in fetal blood (Fig. 8F, arrows), possibly a consequence of abnormal cardiac function and circulatory collapse. Taken together, these results indicate that the vasculature in both the embryo and extra-embryonic tissues of \(Erg^{\Delta Ex4}/H9004\) has vascular remodeling defects.

**Endocardial Erg is necessary for endocardial cushion development**

At E10.5, many \(Erg^{\Delta Ex4}/H9004\) embryos exhibited pericardial effusions (Fig. 7K,L), a classic sign of cardiac dysfunction. \(\beta\)-galactosidase-stained sections of control and \(Erg^{\Delta Ex4}/H9004\) embryos that had reached similar developmental stage revealed trabeculae that were well connected to the myocardium of the ventricular wall, and the \(\beta\)-galactosidase-stained ECs lined the trabeculae in both the \(Erg^{\Delta}/H9004\) and \(Erg^{\Delta Ex4}/H9004\) heart (Fig. 9A,B). We observed regional swellings of the extracellular matrix in the AV and OFT (Fig. 9C-F) of the heart in both control and mutant embryos; however, invasion of the mesenchyme was dramatically decreased in the mutants (Fig. 9D,F). The endocardial cells maintained their adherens junctions and failed to invade the cardiac extracellular matrix, as observed by VE-cad staining of the AV and OFT (Fig. 10A,B). By contrast, VE-cad in other tissues, such as the developing lung (Fig. 10C-F), showed a significant reduction in the knockouts compared with wild-type controls.

We next analyzed the transformation of endocardial cells to mesenchymal cells by performing AV and OFT explant cultures, using a collagen gel culture system (Runyan and Markwald, 1983).

### Table 1. Genotype analysis of progeny from \(Erg^{\Delta Ex4}\) heterozygous intercrosses

<table>
<thead>
<tr>
<th>Age</th>
<th>(Erg^{\Delta Ex4})</th>
<th>(Erg^{\Delta Ex4}/\Delta Ex4)</th>
<th>(Erg^{\Delta Ex4}/H9004)</th>
</tr>
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<tbody>
<tr>
<td>P0</td>
<td>158</td>
<td>290</td>
<td>0</td>
</tr>
<tr>
<td>E8.5</td>
<td>7</td>
<td>16</td>
<td>5</td>
</tr>
<tr>
<td>E9.5</td>
<td>16</td>
<td>28</td>
<td>10</td>
</tr>
<tr>
<td>E10.5</td>
<td>96</td>
<td>154</td>
<td>80 (57*)</td>
</tr>
<tr>
<td>E11.5</td>
<td>31</td>
<td>55</td>
<td>37*</td>
</tr>
<tr>
<td>E12.5</td>
<td>18</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>E13.5</td>
<td>10</td>
<td>18</td>
<td>0</td>
</tr>
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Postnatal mice were scored at P0. No live embryos were observed beyond E11.5. Asterisks indicate dead embryos.
Fig. 8. Placental vascular defects in Erg$^{\Delta Ex4/\Delta Ex4}$ embryos at E10. (A,B) The dashed lines in Erg$^{+/-}$ (A) and Erg$^{\Delta Ex4/\Delta Ex4}$ (B) indicate the extent of decidual invasion in each placental section. (C,D) Magnification of the boxed areas in A and B. Well-defined vasculature is observed in Erg$^{+/-}$ placentas (C), whereas the mutants, although they have abundant maternal blood sinuses, lack well-defined fetal-derived capillaries (D). (E,F) Higher magnification of C and D reveal abundant nucleated fetal blood in the controls (arrows, E), whereas the mutants show a drastic reduction in the number of fetal blood cells and exhibit collapsed capillaries (arrows, F). D, maternal decidua; L, labyrinth; E, embryo; MB, maternal blood; EB, embryonic blood.

from E9.5 and E10.5 embryos, respectively. Endocardial cells from Erg$^{+/-}$ AVC and OFT explants were motile and invaded the collagen matrix, whereas the cells from Erg$^{\Delta Ex4/\Delta Ex4}$ explants showed a marked reduction in migration and invasion (Fig. 11A). We further characterized EnMT in the Erg$^{\Delta Ex4/\Delta Ex4}$ and Erg$^{+/-}$ AVC and OFT explant cultures by immunofluorescence analysis. The explants were stained for the mesenchymal marker smooth muscle α-actin (α-SMA) and stained cells that had migrated away from the explant into the matrix were counted and quantified (Fig. 11B,C). α-SMA-stained cells were found in abundance in Erg$^{+/-}$ explants, whereas very few were observed in the explants from Erg$^{\Delta Ex4/\Delta Ex4}$ embryos (Fig. 11C).

Genes involved in EnMT are differentially regulated in Erg$^{\Delta Ex4/\Delta Ex4}$ hearts

In an attempt to identify regulators and downstream targets of Erg in endocardial cushion formation, we collected RNA from Erg$^{+/-}$ and Erg$^{\Delta Ex4/\Delta Ex4}$ hearts at E10.5 and isolated RNA for qRT-PCR analysis of several genes involved in EnMT in the developing heart (Fig. 12A; supplementary material Table S1). Of the genes analyzed, Snail1 (also known as Snail or Snai1) and Snail2 (Slug or Snai2) were significantly downregulated in Erg$^{\Delta Ex4/\Delta Ex4}$ heart. Snail1 and Snail2 encode zinc-finger transcriptional repressors that have been reported to induce EnMT during embryonic development. Deficiency of Snail2 is partially rescued by the expression of Snail1 (Niessen et al., 2008). Accordingly, deficiency of both Snail1 and Snail2 leads to lethality at E10.5 from EnMT defects (Niessen and Karsan, 2008). This helped us identify Snail1 and Snail2 as potential downstream targets of Erg. Snail2 has been reported to be a direct target of Notch during EnMT and cardiac cushion cellularization (Timmerman et al., 2004; Grego-Bessa et al., 2007; Niessen et al., 2008). Interestingly, there were no significant changes in the expression of Notch1/4, Bmp2/4 or Tgfb1/2, which are the main pathways involved during valve morphogenesis. Consistently, hearts stained with Snail1 plus Snail2 antibody revealed a drastic reduction in the number of stained cells in the AVC region (Fig. 12C) and OFT (data not shown) of Erg$^{\Delta Ex4/\Delta Ex4}$ embryos compared with Erg$^{+/-}$ controls (Fig. 12B). These data suggest that Erg acts downstream of crucial regulators such as Notch, BMP and Tgfb and upstream of Snail1 and Snail2.

Erg binds to highly conserved Ets binding sites in the Snail1 proximal promoter and Snail2 intronic region

To examine whether Snail1 and Snail2 are direct targets of Erg, we first analyzed the proximal promoter and intronic regions of Snail1 and Snail2 for highly conserved Ets binding sites. We identified one highly conserved Ets binding site at –650 in the...
proximal promoter of *Snail1* (Fig. 13A). Similarly, the intronic region of *Snail2* at +157 contained a highly conserved Ets binding domain (Fig. 13B). To evaluate whether Erg binds to these regions in vivo, we conducted ChIP assays from mouse embryo hearts at E10.5 using an Erg antibody. Hearts from *Erg<sup>ΔEx4/ΔEx4</sup>* embryos were employed as negative controls. From the genomic DNA fragments immunoprecipitated with Erg antibody, amplification of the regions spanning the Ets binding sites were analyzed by qPCR (Fig. 13C,D; supplementary material Table S1). A significant amplification of the region

![Fig. 10. Endocardial cells maintain their adherens junctions in *Erg<sup>ΔEx4/ΔEx4</sup>* embryos.](image)

(A,B) Heart sections from E10.5 *Erg<sup>+/+</sup>* and *Erg<sup>ΔEx4/ΔEx4</sup>* mouse embryos stained for VE-cadherin reveal that the endocardial cells maintain their adherens junctions and fail to invade the cardiac jelly in the mutants. Arrowheads, VE-cadherin staining. (C-F) Lung bud sections of E10.5 *Erg<sup>+/+</sup>* and *Erg<sup>ΔEx4/ΔEx4</sup>* embryos reveal a reduction in VE-cadherin staining in the mutant. A, atrium; AVC, atrioventricular canal.

![Fig. 11. Deletion of endothelial enriched Erg in endocardial cells prevents EnMT in vitro.](image)

(A) In vitro collagen gel assays performed with AVC and OFT explants isolated from *Erg<sup>+/+</sup>* (i,iii) or *Erg<sup>ΔEx4/ΔEx4</sup>* (ii,iv) E10.5 hearts. The number of invading and migrating cells formed in *Erg<sup>+/+</sup>* explants was substantially higher than in *Erg<sup>ΔEx4/ΔEx4</sup>* explants. (B) *Erg<sup>+/+</sup>* (i,iii) and *Erg<sup>ΔEx4/ΔEx4</sup>* (ii,iv) explant cultures stained with an antibody (green) against the mesenchymal marker α-SMA. Nuclei were visualized by staining with DAPI (blue). (C) The numbers of mesenchymal cells formed in *Erg<sup>+/+</sup>* (n=12) and *Erg<sup>ΔEx4/ΔEx4</sup>* (n=7) explants were quantified. The number of migrating and invading mesenchymal cells was 16.66±2% in the AVC and 13.15±2% in the OFT regions in *Erg<sup>ΔEx4/ΔEx4</sup>* compared with the *Erg<sup>+/+</sup>* embryos. *P<0.005. Error bars indicate s.d.
spanning the Ets binding sites was observed in the Erg+/- heart samples compared with the IgG or negative controls. It is imperative to note that the input was diluted 1:200 because whole embryo hearts were used for the assay and it is likely that Erg may only regulate Snail1 and Snail2 in a subset of cells, and therefore the signal might not be highly enriched over the input. Overall, these results indicate that Erg binds specifically to regulatory regions in the Snail1 and Snail2 genes in E10.5 heart.

DISCUSSION

Ets transcription factors regulate the expression of several genes that are important for signaling pathways involved in cell differentiation during embryonic development. They influence cell specification and coordinate changes in the expression of adhesion molecules and the degradation of the extracellular matrix, thereby regulating cell motility, epithelial-mesenchymal transition (EMT) interactions, morphogenesis during embryonic development, as well as other physiological and pathological processes (Maroulakou et al., 1994; Raghow, 1994; Werb, 1997; Lukashev and Werb, 1998; Werb and Chin, 1998). During embryonic development, EMT is crucial for appropriate differentiation and is a mechanism for dispersing cells (Kalluri, 2009). The EMT program allows cells to separate from neighboring cells, become motile and migrate to other locations (Ben-Porath et al., 2008). For example, in the developing embryo the neural crest develops from a small portion of the dorsal neural tube (Saint-Germain et al., 2004; McKeown et al., 2005). The neural crest cells then undergo EMT, migrate and differentiate into bone, smooth muscle cells, peripheral neurons, glia and melanocytes. Similarly, in the heart, the endocardial ECs lining the AVC undergo EMT to form the earliest progenitors of the tricuspid and mitral valves. Likewise, the ECs in the OFT undergo EMT to form the precursors of the aortic and pulmonary valves (Eisenberg and Markwald, 1995).

Among the Ets factors known to regulate EMT, Ets1 expression in the chick embryo is induced in cranial neural folds and in migrating neural crest cells (Tahtakran and Selleck, 2003). Although Ets1 does not directly induce EMT, it cooperates with Snail2 to initiate EMT (Theveneau et al., 2007). The neural crest cells then undergo EMT, migrate and differentiate into bone, smooth muscle cells, peripheral neurons, glia and melanocytes. Similarly, in the heart, the endocardial ECs lining the AVC undergo EMT to form the earliest progenitors of the tricuspid and mitral valves. Likewise, the ECs in the OFT undergo EMT to form the precursors of the aortic and pulmonary valves (Eisenberg and Markwald, 1995).

Fig. 12. ErgAE4/AE4 embryos show changes in genes regulating EnMT. (A) Differential expression of genes that regulate EnMT in ErgAE4/AE4 compared with Erg+/+ E10.5 heart samples. For each gene evaluated, control expression was normalized to 1 in order to compare the relative change in expression in the knockout versus control. Error bars indicate s.d. of at least six independent qPCR reactions per genotype. (B, C) Snail1 plus Snail2 staining of sections of Erg+/+ and ErgAE4/AE4 AVC regions at E10.5, showing a marked reduction in Snail1/2-positive cells in the mutant. Dashed lines outline the endocardial cells. (D, E) Representative serial sections of B and C, respectively, stained with IgG controls. A, atrium; AVC, atrioventricular canal.
vascularization by regulating the expression of MMPs in ECs, thereby activating EMT (Fafeur et al., 1997). Similarly, the PEA3 subfamily of the Ets family directly targets MMPs in tumor settings, thereby promoting EMT (Waslylyk et al., 1993; Higashino et al., 1995). By contrast, the prostate-derived Ets factor (PDEF) suppresses EMT by negatively regulating the expression of Snail2 (Findlay et al., 2011). Along similar lines, Erg is also involved in epithelial-mesenchymal interactions through the regulation of extracellular matrix proteins, including stromelysin 1 and collagenase 1 (Buttice et al., 1996).

Despite evidence supporting a role for Ets factors in EMT, any involvement in regulating valve morphogenesis in the developing heart has not been previously reported. Transcripts for several Ets factors, including Erg and Ets1, have been recently identified in the E8.5 mouse heart (Schachterle et al., 2012). By contrast, the prostate-derived Ets factor (PDEF) suppresses EMT by negatively regulating the expression of Snail2 (Findlay et al., 2011). Along similar lines, Erg is also involved in epithelial-mesenchymal interactions through the regulation of extracellular matrix proteins, including stromelysin 1 and collagenase 1 (Buttice et al., 1996).

In the current study, we have identified an important regulatory role for the EC-enriched Ets factor Erg in the regulation of murine vascular development. Our data indicate that Erg is expressed throughout the entire vasculature during early development from E7.5. Previous studies, including those from our own laboratory, have shown an important role for Erg in the regulation of a number of EC-enriched genes, including VE-cad, claudin 5, VWF, endoglin and angiopoietin 2 (Birdsey et al., 2008; Yuan et al., 2012; Schwachtgen et al., 1997; Pimanda et al., 2006; Hasegawa et al., 2004). In the current study we report a novel and essential role for Erg during valve morphogenesis in the developing mouse heart. Deletion of the EC-enriched isoforms leads to lethality between E10.5 and E11.5, with reduced EnMT during valve morphogenesis. We show for the first time the involvement of an Ets factor in fate determination of endocardial cells during the initiation of cardiac cushion cellularization. We further demonstrate that Erg binds directly to the proximal promoter of Snail1 at the –650 bp region and to the intronic region of Snail2 at +157, thereby regulating the expression of Snail1 and Snail2.

Endocardial cushion formation and EnMT are regulated by a number of pathways, either synergistically or via inhibitory
interactions (DeLaughter et al., 2011). Studies in chicken first suggested that Tgfβ2 and Tgfβ3 are the major myocardial EnMT inducers. Given that mice deficient for either of these factors do not exhibit dramatic EnMT defects in vivo (Dickson et al., 1995; Kaartinen et al., 1995; Sanford et al., 1997; Bartram et al., 2001; Stenvers et al., 2003), it was later suggested that other factors, such as Bmp2, that function upstream of Tgfβ2 are crucial inducers of EnMT and cushion formation in the AVC (Ma et al., 2005). Within the endocardial cells destined to undergo EnMT, Notch signaling is a crucial regulator of this process. Mice with targeted disruption of Notch1 die in utero with severe defects in valve morphogenesis as the AVC fails to undergo cellularization (Timmerman et al., 2004). Loss of expression of Notch targets such as Snail1 and Snail2 lead to failure of cells to undergo EnMT. Snail1 and Snail2 are transcriptional repressors of VE-cad, thereby inducing EnMT by downregulating endocardial cell adhesion. Notch1 mutants also display reduced Tgfβ2 expression in the AVC and OFT regions (Timmerman et al., 2004), suggesting that Notch signaling exerts a non-cell-autonomous effect in myocardial Tgfβ2 signaling. In our studies, we did not observe a significant change in Notch1, placing Erg downstream of Notch1. The most significant change was observed in the levels of Snail1 and Snail2, both of which are Notch1 targets required to repress the expression of VE-cad in the endocardium. These results appear paradoxical given that Erg is known to activate VE-cad expression in ECs (Birdsey et al., 2008), and, in support, we too see an overall reduction of VE-cad in other tissues, including the lung bud (Fig. 10C-F). However, this phenomenon is not previously unreported for Erg. Higher levels of androgen receptor (AR) activity have been shown to increase Erg protein through Tmprss2 regulatory regions, which in turn downregulates AR and Erg, and vice versa in prostate cancers (Yu et al., 2010). It is similarly possible that Erg and the Snail1/2 transcription factors function in a negative autoregulatory loop so that Erg and VE-cad are suppressed by Snail1/2 once they are induced by Erg.

Cardiac valve development is also tightly regulated by the differential expression of several transcription factors. During the early stages of heart development, the cardiac chambers are formed when the linear heart tube loops rightward, balloons outward and the endocardial cushions appear at the level of the AVC and OFT. The newly formed endocardial cushions are marked by the expression of selected transcription factors, including the T-box factor Tbx2 (Habets et al., 2002). Interestingly, we observed a marked downregulation of Tbx2 in our Erg mutant hearts. EnMT involves overlapping steps of repopulation, differentiation and migration of ECs into the cardiac jelly (Armstrong and Bischoff, 2004). Hence, during the first step, endocardial cells that are fated to transdifferentiate and are destined to undergo EnMT delaminate. During this process, expression of the Snail1/2 transcription factors leads to marked repression of VE-cad, allowing the endocardial cells to separate from each other. The Erg knockout embryos show substantially decreased levels of Snail1/2 and a sustained expression of VE-cad, indicating a failure of the endocardial cells to undergo transdifferentiation. The T-box factor Tbx20 has recently been shown to be involved in early AVC development. EnMT is markedly perturbed in mice lacking Tbx20 (Cai et al., 2011). In addition to Tbx2, we also observed significant reductions in Tbx20 in Erg knockout mice. Similarly, Msx1 and Msx2, which belong to the Nk family of homeobox genes, have also been shown to play an overlapping regulatory role in BMP-induced cardiac EnMT (Chen et al., 2008). We also observed a significant reduction in the expression level of Msx1 in the Erg-deficient embryonic hearts.

During the second stage of EnMT, the endocardial cells repopulate and replace the cells undergoing transdifferentiation, a process that is controlled by Vegf signaling through Nfatc1 (Wu et al., 2011). Our mutant embryos did not show a significant change in Nfatc1 expression, which is consistent with its recently described role in the inhibition of EnMT during valve development (Wu et al., 2011). During the third stage of EnMT, the endocardial cells destined to undergo EnMT are then subjected to hyaluronic acid signaling and signaling through ErbB2/3 heterodimers that promote cell migration into the cardiac jelly (Camenisich et al., 2002b). Consistently, the Erg knockout embryos showed decreased Erbb2. It is also possible that Ets factors can interact cooperatively with other factors to regulate EnMT in the heart. Recent evidence shows that the Ets factor ETV5 regulates the EnMT process in endometrial carcinomas through crosstalk with lipoma-preferred partner protein as a principal event in initiating EC invasion (Colas et al., 2012).

In conclusion, the current study identifies a previously unknown function of Erg during valve morphogenesis and clearly delineates functional variation in isoform-specific activity. These findings also suggest that isoform-specific Erg mutations might underlie some forms of human congenital cardiac valve defects.

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